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Biophysical Properties of the Zebrafish Posterior Lateral Line *in vivo*

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Abstract

The zebrafish have specialised sensory hair cells along the posterior lateral line (pLL) that are morphologically and functionally similar to those in mammalian inner ears. The zebrafish uses the superficial hair cells of the pLL to detect water velocity but has become an important animal model in which to explore hair cell function and defects *in vivo*. However, there are many fundamental questions about the pLL that have not yet been explored.

The aim of this study was to provide a detailed account of the biophysical properties of the zebrafish pLL at rest, during development and maturation. This involved developing a new method to record from the pLL ganglion with electrophysiology. I identified that the pLL afferent fibres display increasingly higher frequencies of spontaneous activity during development in 2-18 dpf zebrafish. I hypothesised that the increased presence of higher frequency firing is likely to be due to the increased number of hair cells that the afferent fibres innervate and the refinement of synaptic connections into a more adult-like configuration.

I also identified the profile of spontaneous activity during regeneration of hair cells of the pLL after $CuSO_4$ exposure. After exposure at 3 or 12 dpf, hair cells - and thus spontaneous activity - were present within 24 hours post treatment (hpt). It took longer to regenerate the same number of hair cells as age-matched controls in older zebrafish and consequently, spontaneous activity also took longer to reach to similar frequencies to controls.

As hair cell regeneration is not possible in most mammalian species, it is important to understand the mechanisms and effects of hair cells loss on the afferent fibres, and also how spontaneous activity is shaped by hair cell regeneration in order to apply findings to further research and therapeutics.

Contents

Figures

Tables

Abbreviations

AEPs – auditory evoked potentials

aLL – anterior lateral line

AMPA - alphaamino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

CV – Coefficient of Variation

dpf – days post fertilisation

E3 – Embryo medium 3

GECI – genetically encoded calcium indicators

HC - Hair cell

hpf – hours post fertilisation

hpt – hours post treatment

IHC – inner hair cell

MET - mechanotransduction

mpf – months post fertilisation

MS-222 - tricaine methanesulfonate

OHC – outer hair cell

pLL - posterior lateral line

pLLg – posterior lateral line ganglion

prim1 – primordium 1

wpf – weeks post fertilisation

wph – weeks post hatching

ZF – Zebrafish

α-Btx – α – bungarotoxin

Chapter 1 General Introduction

1.1 Hair cells

Hair cells are specialised sensory cells of lower vertebrates and mammals in the vestibular, auditory and lateral line system. Hair cell biophysical properties vary depending on location in the organ they reside and the physical stimulus which they are exposed to in order to reliably transmit the biological signal to the brain with high fidelity and precision.

The hair cells of the auditory and vestibular system are vulnerable to damage from environmental insults and genetic predispositions. To understand disorders that effect our hearing and vestibular system, we must advance our knowledge of the general function of the hair cells in order to prevent damage and restore function. Fortunately, we can turn to lower vertebrate and invertebrate models to investigate hair cells *in vitro* and *in vivo* as the auditory organs of many vertebrate and invertebrates share genetic and physiological similarities. For example, the Johnston's organ is the hearing organ of the Drosophila melanogaster that contains Usher proteins that are associated with congenital deafness in humans (Li *et al.*, 2018).

This chapter will summarise the key structural features of the hair cell, how a stimulus is transmitted by the hair cell to higher order structures and their function within different sense organs. I will focus on the hair cells of the zebrafish (*Danio rerio*), whilst referencing additional sense organs with hair cells from other animals where necessary.

1.1.1 The general structure of hair cells

Hair cells can be functionally divided into two distinct parts; the apical portion which detects and transduces the sensory stimulus, and the basal portion that transmits the signal to the brain.

Finger-like projections called stereocilia extend from the apical surface of the hair cell (Figure 1.1A). The stereocilia have a core of actin filaments; therefore, the stereocilia do not bend but pivot at the base - where they meet the hair cell body - during stimulation (Crawford & Fettiplace, 1985; Howard & Ashmore, 1986). The stereocilia emerge from the hair cell body early in development and grow in rows to a restricted height (Kindt *et al.*,

2012). Once developed, the stereocilia are highly organised with optimum arrangement and length for their position and function that varies between sensory organs (Tilney et al., 1986). The stereocilia are connected together by many different types of links that ensure they maintain their projection and that deflection of the stereocilia occurs in unison (Pickles et al., 1984). The links of most significance are the tip links that gate the mechanoelectrical transducer (MET) channel (Figure 1.1B), located at the top of the stereocilia, but not in the tallest row (Pickles et al., 1984; Beurg et al., 2009). The hair cell bundles are asymmetric and polarised; therefore, the tip links are aligned with the hair bundle's axis of mechanotransduction (Pickles et al., 1984). The tip links comprise of protocadherin15 (Seiler et al., 2005) and cadherin23 (Siemens et al., 2004) which are implicated in human deafness (Kazmierczak et al., 2007). Disorders in either protocadherin15 or cadherin23 cause splaying of stereocilia, abnormal directional sensitivity and reduced mechanotransduction (Schwander *et al.*, 2009; Alagramam *et al.*, 2011). Also present apically in the hair cell is a singular

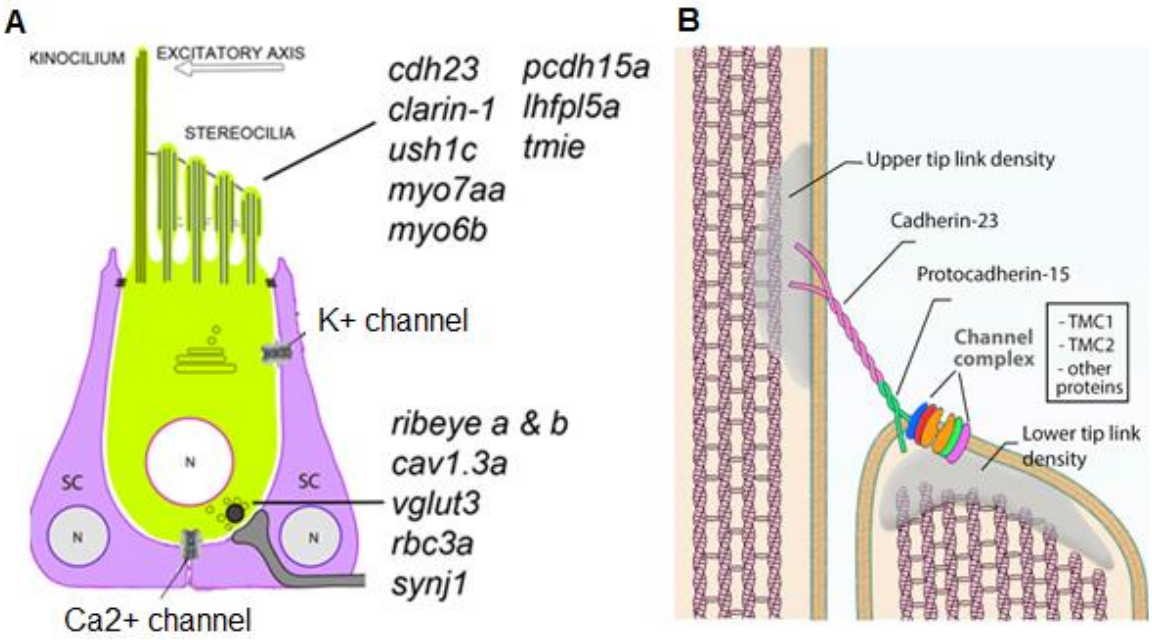

Figure 1.1 The hair cell

A, Schematic of a hair cell. Featuring the stereocilia hair bundle with kinocilia, potassium channels (K^+) for depolarisation and repolarisation of the cell, calcium channels (Ca^{2+}) and synaptic machinery with ribbons for neurotransmitter release to the afferent fibre and important genes for hair cell function. Image adapted from Nicolson (2017). **B,** Schematic of MET channel (channel complex) that is gated by the tip link (cadherin 23 & protocadherin 15) and is attached to the corresponding stereocilia. Image obtained from Schwander *et al.* (2010)**.**

kinocilium, located towards the longest stereocilia (Bashtanov et al., 2004). The kinocilium is present throughout life in the zebrafish lateral line (Kindt et al., 2012) and in the mammalian vestibular system (Denman-Johnson & Forge, 1999), or just during development in the mouse cochlea (Furness et al., 1989). The kinocilium is a true cilia with a microtubular core (Kikuchi *et al.*, 1989). Whereas it is not thought that the kinocilium functions as an aid to mechanosensation in vestibular hair cells of mammals (Hudspeth & Jacobs, 1979), in zebrafish nascent hair cells the kinocilium is important for mechanosensation. Zebrafish mutants that display stunted kinocilia cannot perform mechanotransduction despite normal stereocilia morphology (Kindt et al., 2012). In zebrafish the kinocilium appears prior to stereocilia during development, but does not assume its polar arrangement until later on (Denman-Johnson & Forge, 1999).

When the stereocilia are deflected towards the tallest row due to a stimulus, tension is applied to the tip links and the open probability of the MET channel increases (Hudspeth & Corey, 1977; Peng et al., 2016; Kazmierczak et al., 2007), that allows an influx of ions through the pore (Corey & Hudspeth, 1983). There are on average 1.5 MET channels per stereocilia (Ricci et al., 2003) which each have a pore size of approximately 1.25 nm in diameter (Farris et al., 2004). The MET channel is large enough to allow entry of many ions and compounds into the hair cells of the cochlea from the external endolymph (Johnstone et al., 1989). The influx of positively charged ions through the MET channel (K^+ is usually abundant in the surrounding endolymph of the cochlea hair cells) causes alteration in the membrane potential. If the change in membrane potential exceeds the threshold to cause opening of voltage-sensitive calcium channels near the ribbon synapses, neurotransmitter is released (Lewis & Hudspeth, 1983; Brandt et al., 2003; Sidi et al., 2004). Consequently, once the hair bundle is matured (to the correct orientation, length and with all appropriate tip links) if the hair cell stereocilia are stimulated in the opposing direction (towards the shortest row), there is a reduction in the MET open probability compared to at rest and hyperpolarisation of the cell occurs (Shotwell *et al.*, 1981; Kindt *et al.*, 2012).

In the basal region of the hair cell, neurotransmitter release occurs. This involves specialised structures to ensure the hair cell can convey the sustained changes to the membrane potential during stimulation. The ribbon synapses are an important feature of the

hair cell and are present in the auditory and vestibular system of vertebrates, as well as the visual system and in electroreceptors (Sejnowski & Yodlowski, 1982; Lysakowski & Goldberg, 1997; Lenzi et al., 1999; Khimich et al., 2005). Ribbon synapses are involved in tethering greater amounts of vesicles near the pre-synaptic membrane. As a larger quantity of vesicles are associated to the active zone, fast and graded vesicle release can be achieved, which is important for hair cell function (Khimich et al., 2005). Ribbon synapses are comprised of many protein components, which have not all been identified. Of note is the protein ribeye which is abundantly found in the ribbon synapse (Schmitz et al., 2000; Zenisek et al., 2004). Variation is seen between species and location in the quantity of vesicles tethered by the ribbon, which (from species investigated) ranges from 20 vesicles in type 2 vestibular hair cells (Lysakowski & Goldberg, 1997) to >400 vesicles in frog saccular hair cells (Lenzi et al., 1999). Ribbon synapse quantity, size and shape also vary across species (Khimich et al., 2005) and even within individual hair cells (Merchan-Perez & Liberman, 1996) which is likely to reflect the different exocytosis needs of each system.

The influx of K^+ from the opening of the MET channel leads to an influx of Ca^{2+} through the opening of voltage-gated Ca^{2+} channels; thus, exocytosis at the ribbon synapse of hair cells is regulated by Ca^{2+} (Beutner *et al.*, 2001). The L-type voltage-gated Ca^{2+} channel, $Ca_v1.3$, is colocalised at the pre-synaptic membrane of hair cells to ribbon synapses (Zenisek *et al.*, 2003). $Ca_v1.3$ channels are fast acting for rapid release of neurotransmitter and thus fast signalling (Mennerick & Matthews, 1998; Johnson & Marcotti, 2008). Aside from signalling, this channel has also been shown to be important in spontaneous activity in the inner hair cells of the immature mouse cochlea pre-hearing (Zampini *et al.*, 2010). The primary neurotransmitter released by hair cells is glutamate, which activates alphaamino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors post-synaptically in the afferent fibre (Glowatzki & Fuchs, 2000). An action potential is triggered in the neuron that will consequently send a message to higher order contacts such as the brain (Matsubara *et al.*, 1996). Hair cell innervation by afferent fibres varies between, and within, organs. For example, the vestibular hair cells are classified into two types based on the afferent innervation pattern: innervation is either in the classical bouton shape, or in a calyx which has a higher surface area contact to the hair cell. Either innervation type has implications for action potential propagation (Desai *et al.*, 2005; Eatock & Songer, 2011). Calyxes in the

chinchilla vestibular system have been shown to have irregular spontaneous firing compared to afferents with boutons (Baird *et al.*, 1988). The afferent fibre can be depolarised by one vesicle, although the vast amount of vesicles that the ribbon synapse can dock to the hair cell membrane suggests that this configuration is suitable for high frequency firing (Glowatzki & Fuchs, 2002).

1.1.2 The function of hair cells in various systems

Hair cells differ morphologically depending on the organ in which they reside (Xue & Peterson, 2006), and even between other hair cells in the same organs (Olt et al., 2014) due to factors previously mentioned, such as kinocilium length and innervation from afferents. The inner ears of mammals have auditory organs that contain hair cells in a bony capsule called the cochlea. Two types of hair cells reside here, the inner hair cell for auditory detection, and the outer hair cell for sound amplification. Both types of hair cell are arranged into rows and perceive sound of varying frequencies depending on their position along the cochlea (Fettiplace & Crawford, 1980; Ashmore, 1987). Hair cells are also present in the semi-circular canals and otolith organs of the inner ear that function as the vestibular system for mammals to detect rotational and acceleration movement. Other species also have hair cells located in auditory and vestibular organs; however, aquatic life such as amphibians (before metamorphosis) and teleost fish have hair cells superficially located along their body for detection of water movement.

In the adult teleost fish, hair cells are present in distinct areas:

- The saccule; located caudomedially in the inner ear for hearing (Higgs *et al.*, 2002). Hearing is possible in zebrafish as young as 5 dpf (Mo *et al.*, 2010), but during juvenile stages Weberian ossicles develop that amplify sound that is delivered to the saccule from compression of the swim bladder (Fritzsch & Straka, 2014).

- The utricle; located rostrally in the inner ear and is thought to be used for vestibular movements (Riley & Moorman, 2000; Whitfield *et al.*, 2002), as well as the hair cells that reside in the cristae of the semi-circular canals (Waterman & Bell, 1984; Fritzsch *et al.*, 2002).

- The lagena; located in the inner ear which appears to function as a vestibular and auditory organ (Whitfield, 2002; Olt *et al.*, 2014).

- The lateral line has hair cells clustered together in superficial neuromasts for detection of water velocity (Metcalfe *et al.*, 1985). The lateral line can be split into two distinct areas based on location. The anterior lateral line (aLL) is located on the head of the fish, and the posterior lateral line (pLL) is located along the flank. During postembryonic development, neuromasts also become located in canals for acceleration detection rather than velocity like the superficial neuromasts (Webb & Shirey, 2003; Wada *et al.*, 2014).

1.2 The Zebrafish Lateral line

Fish and amphibians possess hair cells clustered together in neuromasts along their lateral lines that are responsible for detecting water velocity (Dijkgraaf, 1963; Chou *et al.*, 2017). Research using fish and amphibians has mostly focused on the pLL as it develops in a controlled and predictable manner that is easily visualised with basic microscopy equipment.

1.2.1 Structure of the posterior lateral line in zebrafish

The detection of water movement by the zebrafish pLL is necessary for behaviours such as non-visual feeding (Sampson *et al.*, 2013) and rheotaxis (Suli *et al.*, 2012). Due to the function of the pLL hair cells, the structure of the pLL varies from other mammalian structures that contain hair cells. The hair cells of lateral lines are clustered together in rosette-like structures called neuromasts (Figure 1.2) which are surrounded by supporting cells that are encircled by mantle cells (Lopez-Schier *et al.*, 2004). Hair cells are

mechanosensory; therefore, they transduce a mechanical stimuli (water movement) into a meaningful electrical signal that the brain can process (Wang, 2017). On the apical surface of the hair cell are three or four rows of stereocilia, arranged in a stair case like structure, with a long kinocilium that is around five times as high as the tallest stereocilia (Kindt *et al.*, 2012). To protect the hair bundles from damage, each neuromast has a gelatinous cupula that encases the cilia. The cupula also helps to coordinate uniform stimulation of the hair cells of a neuromast and also provides some rigidity against deflection, so there is a limit to the structure's sensitivity (McHenry & van Netten, 2007). The basal region of the hair cell contains 2-5 ribbon synapses in early-larval zebrafish (3-5 dpf) (Obholzer *et al.*, 2008), and two ribbons in the same active zone are sometimes observed (Sheets *et al.*, 2012). The ribbon synapses become larger as they mature which can be disrupted if *Ribeye* is knocked out as ribbons are absent or greatly reduced in size (Sheets *et al.*, 2011).

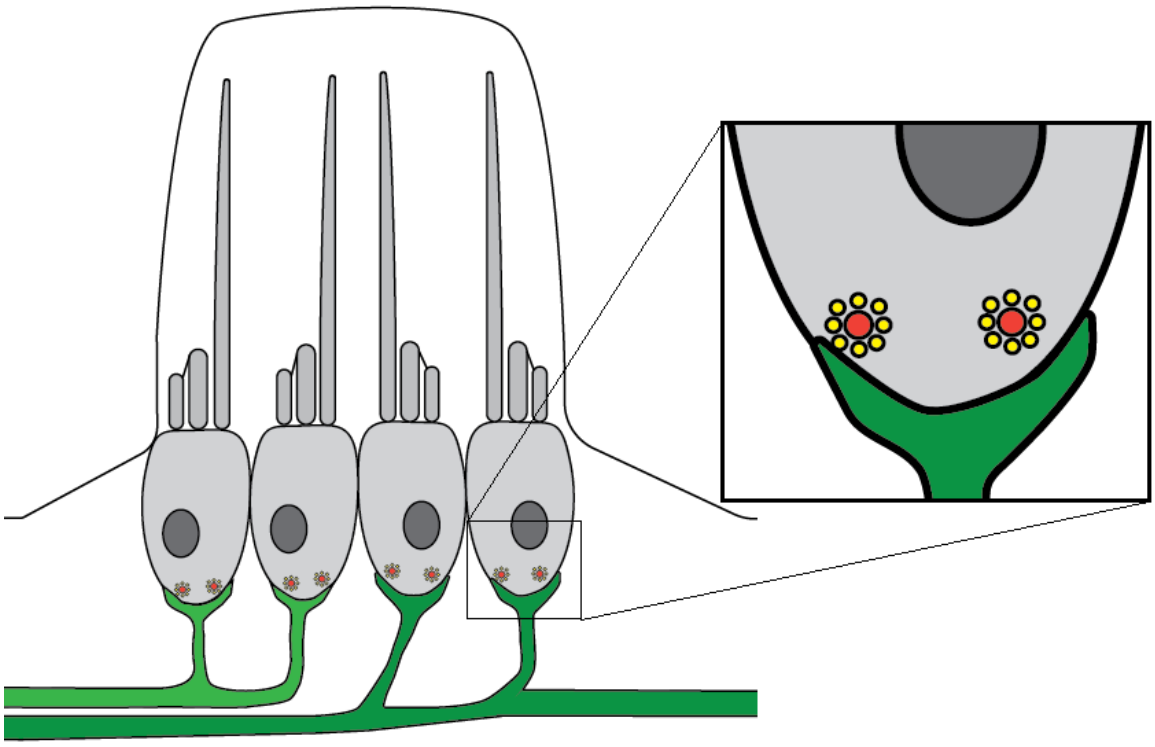

Figure 1.2 Structure of the lateral line neuromast

Schematic of a cross section of a superficial neuromast of the zebrafish posterior lateral line and a close up of the ribbon synapse. Depicting: hair cells (grey) of opposing polarities, afferent fibres (green), and ribbon synapses (red and yellow).

At 5 dpf, two or more afferent fibres form synapses with hair cells of one neuromast in the zebrafish pLL. The afferent fibres send the information of stimuli detection to higher order contacts such as the hindbrain (Pujol-Marti *et al.*, 2012). The somas of the afferent fibres are located in a ganglion found anterior to the inner ear. In 4-7 dpf zebrafish, there are almost double the amount of neuromasts than afferent fibres as some afferent fibres will innervate multiple neuromasts (Faucherre *et al.*, 2009). This innervation pattern is unlike many other hair cell containing organs. Each inner hair cell of the mammalian cochlea is innervated by multiple individual afferents (Liberman, 1982), rather than in the zebrafish where an afferent contacts multiple hair cells. The innervation of the pLL will have implications for the zebrafish's receptive field as the afferent fibre will receive a summation of signals from multiple hair cells, rather than in the cochlea where and afferent fibre will receive input from one active zone of a hair cell.

Efferent fibres act to modulate hair cell response to mechanical stimulation (Dawkins *et al.*, 2005). Efferent fibres are also present in the zebrafish pLL, like in the mammalian cochlea (Guinan *et al.*, 1983). At larval stages efferent projections appear to emerge from the diencephalon of the brain and the hindbrain (Metcalfe *et al.*, 1985) and around 9 efferent fibres contact the hair cells of the pLL neuromasts and appear to exist at similar levels in the adult (Metcalfe *et al.*, 1985; Toro *et al.*, 2015). Although the organisation has not yet been determined to the same degree as the afferent fibres in the pLL (Haehnel-Taguchi *et al.*, 2018), dopaminergic efferents are known to be present as dopamine antagonists regulate activity of the hair cells (Toro *et al.*, 2015). The efferent system has not been as extensively studied like the afferent system. Cholinergic efferents are commonly found in species such as the African clawed frog (*Xenopus laevis)* lateral line (Dawkins *et al.*, 2005) and mouse cochlea (Glowatzki & Fuchs, 2000). Cholinergic efferents have been found in other teleost fish such as the plainfin midshipman (*Porichthys notatus)* (Brantley & Bass, 1988) but have not been well described in the zebrafish pLL (Bricaud *et al.*, 2001).

1.2.2 Development of the posterior lateral line

Zebrafish development has been highly documented as visualisation of the embryo is easily achieved using a stereomicroscope without causing harm to the fish. There are seven stages of embryogenesis that encompasses the transition from the zygote at the 1 cell stage, to pharyngula stage and hatching from the chorion at 2-3 dpf (Kimmel *et al.*, 1995). The chorion is a tough shell of glycoprotein to protect the embryo during early development from physical and chemical insults from the external environment, which contains a jelly-like perivitelline fluid (Cotelli *et al.*, 1988; Ninness *et al.*, 2006; Mu *et al.*, 2017).

Kimmel *et al.* (1995) produced a paper that documented the development of the zebrafish in a succinct manner in order to align the developmental stages and class them accordingly. The paper has since been frequently used as a standard for staging embryos.

Once fertilised, from 0-2.25 hours post fertilisation (hpf), the embryo undergoes many cell divisions known as the cleavage stage. For the consequent 3 hours, the blastula period occurs where the cells are more motile. During gastrulation - occurring until 10 hpf - the anterior-posterior and dorsal-ventral axes are established and then the segmentation period occurs for the next 14 hours. The segmentation period involves the formation of organ-like structures and development of the somites (segments) that give rise to mesoderm - such as the myotome (muscles) and sclerotome (vertebrate cartilage). By 16 hpf, an epithelial thickening called the otic placode is present which, within a further three hours, hollows to give rise to the otic vesicle. Between the otic vesicle and the first somite, there is another placode that will give rise to the primordium (prim1) of the pLL, after the neural tube has formed throughout the length of the fish. The primordium migrates posteriorly down the central flank of the fish and by 22 hpf is located near the third somite (prim-3 stage)(Kimmel *et al.*, 1995).

The next 24 hours are termed the pharyngula stage, where most of the cells of the pLL are deposited (Kimmel *et al.*, 1995). The prim1 deposits cells that become the hair cells and supporting cells of the neuromasts, and growth cones of the pLL afferent nerve migrate with the primordium. After the initial first growth of afferent axons occurs (leaders), other axons

follow (followers) (Metcalfe *et al.*, 1985; Sato *et al.*, 2010). By 40 hpf, the primordium has completed migration and has reached the posterior end of each side of the fish (Kimmel *et al.*, 1995). At this stage, there are around eight neuromasts (L1-8) and 20 afferent fibres on both sides of the zebrafish (Sarrazin *et al.*, 2010). The zebrafish is now capable of small bouts of swimming but does not usually hatch from the chorion until 48-72 hpf (Kimmel *et al.*, 1995).

By the long-pec stage (48 hpf) the hair cells of the aLL and pLL, as well as the otic vesicle, have differentiated and the kinocilia can be seen at the apical ends of the cells. By 60 hpf, the zebrafish will start to rest dorsal-up, unlike previous ages where they rest on their side (Kimmel *et al.*, 1995). The pLL ganglion (pLLg) grows in size during the first six days of development due to increased quantity of afferent fibre somas (Sato *et al.*, 2010) and growth in diameter of pre-existing ones (Pujol-Marti *et al.*, 2012).

Two dopaminergic efferent fibres (one on each side) follow the tracks of the afferent fibres but are not observed to do so until 2 dpf where efferents have extended to the first neuromast (L1). Efferent outgrowth continues until 5 dpf. The efferents do not appear to contact the hair cells directly, as the terminals are located near supporting cells, and form not as extensively as the afferents do (Toro *et al.*, 2015).

1.2.3 Post-embryonic development of the posterior lateral line

By 3 dpf, the embryo has now reached the larval stage, and most morphogenesis has taken place. However, more development needs to occur before the zebrafish pLL is mature like in the juvenile fish, meaning that it is functioning optimally with the correct configuration of hair cells and afferent synapses. Knowledge on the post-embryonic development of the pLL isn't as extensive as it is during larval development as tissue density increases and obscures optical clarity; however, some studies have identified key aspects of post-embryonic development of the zebrafish pLL.

Three additional lines of neuromasts develop on the posterior flank of the zebrafish by late larval/juvenile stages that exist in association with the pLL (ventral, dorso-lateral and dorsal) (Figure 1.3) (Ledent, 2002). Due to the increased size of the fish, neuromasts continue to increase in number (~60 at 25 dpf on the flank of the fish) as cells bud off from existing neuromasts throughout adulthood to form new neuromasts that cluster together in stitches (Nunez *et al.*, 2009).

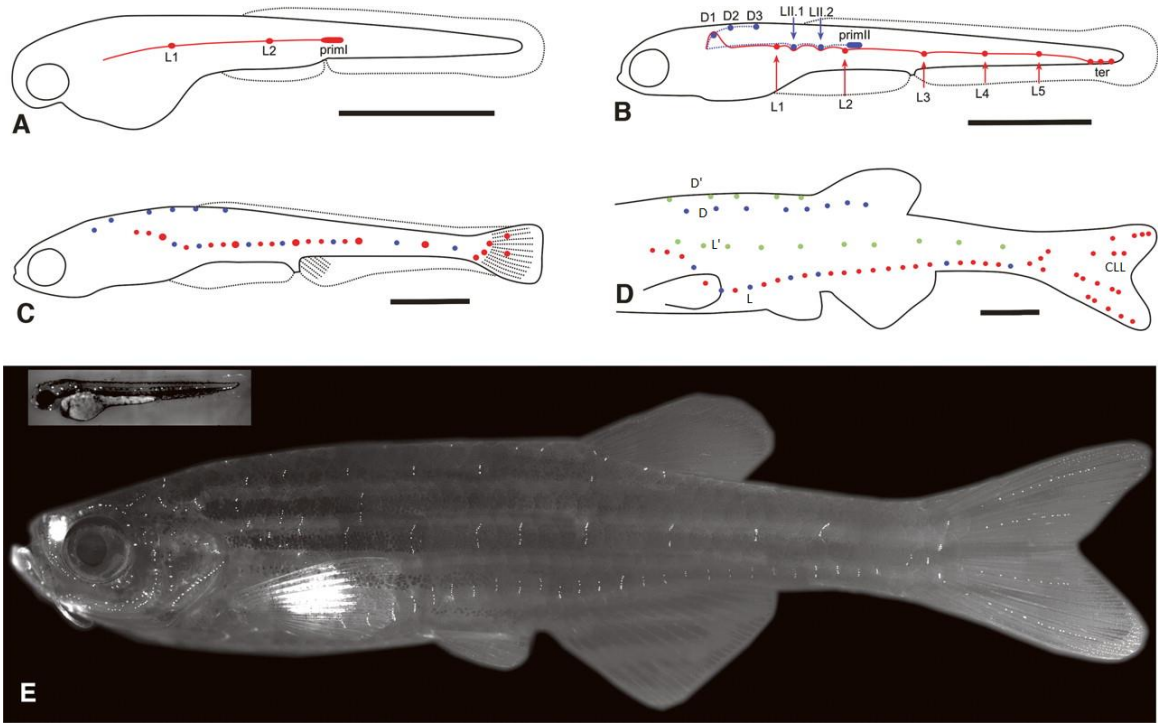

Figure 1.3 Development of the four lateral lines, from embryonic to juvenile stages

A, At 32 hpf, prim1 has started to migrate and cells that will become two neuromasts have been deposited. **B**, At 48 hpf prim1 has finished migrating, the dorsal primordium (primD) and primII has begun migration. **C**, By 3–4 weeks, prim1, primD and primII has finished migration and intercalary neuromasts form. **D**, At the late-larval stage, neuromasts migrate and dorsal and lateral lines are complete. **E**, Adult zebrafish with hair cells labelled with a hair cell specific dye, compared to embryonic zebrafish in inset. Stitches are now present. D, D', L, and L', the four lines of the juvenile pLL system; CLL, caudal fin lateral line. Scale bars: 1 mm. Adapted from Ghysen & Dambly-Chaudière, 2004.

The growth of additional lateral lines begins relatively early on in development. At 40 hpf, another primordium is present which then splits into two. One portion of the split (primII) follows the same path as prim1, the other migrates more dorsally (primD). By 6 dpf, primII has deposited a further 3-4 neuromasts (LII.1-4) and has migrated fully by

approximately 2 weeks post fertilisation (wpf) (Sapede *et al.*, 2002). Twelve intercalary neuromasts also slowly develop between the neuromasts of prim1 (Nunez *et al.*, 2009). Delays in outgrowth of the various lateral lines and positioning of the neuromasts seem to depend, in part, due to neuromasts having an inhibitory effect on another forming at the same location; however, the molecular mechanisms of this effect and general patterning of the lateral lines at post-embryonic stages is not known (Nunez *et al.*, 2009).

The neuromasts of the original pLL will migrate ventrally from their original central position during post-embryonic growth (Figure 1.3D). Each neuromast does not migrate a uniform distance; however, the afferent fibre axons that contact each neuromast reliably follow them to their new location, leaving space for a new lateral line to form in its original location (Ledent, 2002). It is not known what the molecular mechanisms of how the hair cells know where to migrate to, nor how the afferent fibres so reliably follow them are.

Although it is easy to identify superficial structures like the neuromasts of the pLL, the development of the corresponding afferent fibres is not as well documented. It has been suggested that the number of afferent fibres in the pLLg increases after embryonic growth, but whether the new afferents originate from another primordium is unknown (Sapede *et al.*, 2002). In adulthood when stitches of neuromasts form, the new neuromasts are innervated by branches of afferents from the pLL nerve that forms contacts with the original neuromast of the stitch. The nerve arborizes to follow the budding neuromast prior to hair cells differentiating (Wada *et al.*, 2010; Wada *et al.*, 2013). The afferent fibre that innervates a stitch will receive a summation of information from the hair cells of the neuromasts. As discussed previously, multiple neuromasts are more efficient than one large neuromast, to maintain sensitivity of the hair bundle and stiffness of the cupula.

1.3 Function of the Lateral line in zebrafish

The development of the pLL allows optimum organisation in order to fulfil its function as a sense organ for water velocity.

1.3.1 Function of the hair cells

Hair cells are designed to carry out specific functions based on what sensory organ in which they reside. In the mammalian cochlea, hair cells are capable of fast and sustained release of neurotransmitter in response to continuous sound stimulation. As previously discussed, ribbon synapses are specialised pre-synaptic structures in hair cells that allow larger tethering of vesicles to the membrane for graded release that is necessary for constant sound stimulation (Glowatzki & Fuchs, 2002). The pLL lacks structural features that are integral for the cochlea's sensitivity, such as the basilar membrane and the endocochlear potential (Marcotti, 2012; Goodyear *et al.*, 2017). However, the hair cells of the pLL contain ribbon synapses like those found in the cochlea, to allow for sustained detection of water velocity of varying strengths. The presynaptic ribbons have also been found to juxtapose the synapse of each hair cell in the pLL near calcium channels ($Ca_v1.3$) (Sheets *et al.*, 2012). The close association of ribbon synapses to $Ca_v1.3$ channels is integral for successful release of neurotransmitter from the hair cell, as found in other species such as the gerbil and mouse cochlea, which is refined during development (Johnson & Marcotti, 2008; Wong *et al.*, 2014).

Many functional studies on the zebrafish pLL take place at larval stages due to the rapid development of the fish and regulations that prevent use in more developed fish (>5.2 dpf - UK Home Office). Rodent hair cells take approximately 3 weeks to differentiate *in utero* (Marcotti, 2012), whereas by 2 dpf the hair cells of the zebrafish pLL are suggested to be functional as they are associated to afferent fibres (Nagiel *et al.*, 2008). Further studies revealed that mechanosensation is present before the hair bundles have fully developed in the zebrafish pLL which was proven by stimulation of neuromasts at 2 dpf in zebrafish with calcium reporters in the hair cells (Kindt *et al.*, 2012). The speed of development of the lateral line suggests a dependence on the system in order for the fish to function and survive as soon as they hatch.

Investigations into the pre-synaptic mechanisms of the zebrafish hair cell have taken place. In zebrafish the ribbon synapse gene, *Ribeye*, is present in two isoforms (*a+b*) (Zenisek *et al.*, 2004) and assists in stabilising the contact of the active zone to the afferent fibre, and also clustering voltage gated calcium channels near the active zone in the zebrafish pLL

(Sheets *et al.*, 2011); therefore, the pLL is always prepared for a continuous detection of stimuli. When expression of *ribeye a+b* is disrupted in the hair cells of the pLL, "ghost" ribbons form which lack electron density and are smaller than usual (Lv *et al.*, 2016).

Directional sensitivity is important for hair cells of all species to convey stimulus of a certain direction. The hair cells of a pLL neuromast are organised into two groups that have opposing polarities (Figure 1.4), an organisation that occurs even in absence of afferent and efferent innervation. The directional polarity ensures that stimuli can be detected by opposite directions as afferent fibres only make contacts with hair cells of the same polarity (Nagiel *et al.*, 2008; Obholzer *et al.*, 2008) and stimulation in the opposite direction causes inhibition from the other half of the hair cells in the neuromast (Lopez-Schier *et al.*, 2004). The afferents are such loyal selectors of polarity that even after hair cell loss, they will re-establish contacts with hair cells of the same polarity (Faucherre *et al.*, 2009). Neuromasts deposited from the first primordium display anterior-posterior or dorsal-ventral patterning (Lopez-Schier *et al.*, 2004; Mirkovic *et al.*, 2012; Chou *et al.*, 2017). To ensure that there is no cross talk of hair cells of different polarities, an afferent fibre will not contact neuromasts of opposing polarity axes (Nagiel *et al.*, 2008).

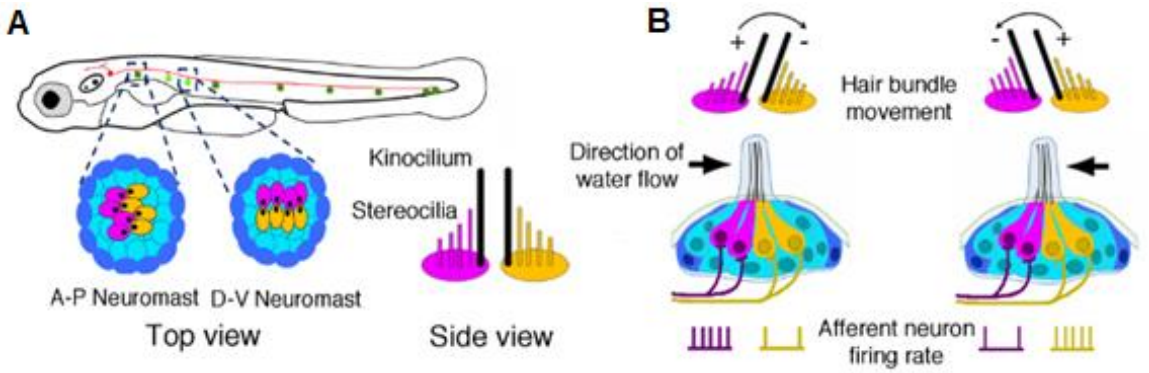

Figure 1.4 Opposing polarities of the hair cells of the pLL neuromasts

A, Schematic of the larval zebrafish, featuring the pLL (red) and neuromasts (green). Two neuromasts with differently orientated hair cells (yellow and magenta). **B**, Schematic of the stimulation of neuromast hair bundles with directional sensitivity to water flow. Figure modified from Ji *et al.* (2018).

1.3.2 Function and organisation of the afferent fibres

The afferent fibres form broad contacts with the afferent fibres of the pLL, which are considered to be slightly calyx-like (Nicolson, 2015). The afferent fibres main purpose is to convey the signal of stimulation from the hair cells to higher order contacts, in order for the zebrafish to initiate an appropriate behavioural response, such as a startle response to escape the stimulus source (Faucherre *et al.*, 2009).

The afferent fibres of the pLL converge along the flank of the fish to form the pLL nerve. The somas of the afferent fibres are found in the pLLg, just posterior to the inner ear, on both sides of the fish. There are approximately 22 afferent fibres (on each side) in the pLLg at 5 dpf (Liao, 2010) that project connections to the hindbrain and other higher order contacts such as the mauthner cell to elicit behaviours such as an escape response (Korn & Faber, 1975; Haehnel *et al.*, 2012). In the anterior, posterior and dorsal lateral line, the number of afferent fibres is relatively stable between 3-15 dpf; however, the number of afferent fibres a neuromast is innervated by does fluctuate depending on its location and therefore is not uniform in the anterior, posterior and dorsal lateral lines. For example in the aLL, the number of afferent fibres that form contacts with the hair cells of the SO1 neuromast (located more anteriorly on the head) decreases with age, whereas the SO3 neuromast (located more posteriorly) increases in innervation during the same time frame (Haehnel *et al.*, 2012). It is unknown whether there is a functional consequence to particular neuromasts having more afferent innervations than others. Some researchers even suggest that some hair cells in a neuromast may be “synaptically silent”, signifying that not all hair cells will release neurotransmitter due to stimuli, but are readily available to be recruited after damage to form synapses with afferent fibres (Zhang *et al.*, 2018).

The sensory receptive field of the neuromasts are mapped in the brain by the positioning of the corresponding afferent neuron terminals. This is somatotopy, which provides the brain of the fish with a representation of the lateral line, in order to perform the correct behaviours after a neuromast of a certain position is stimulated. The projections in the hindbrain are organised so that the neuron corresponding to a more anterior neuromast is located ventrolaterally to the corresponding neurons of the more posteriorly located

neuromasts (Alexandre & Ghysen, 1999). Somatotopy is established before the afferent fibres make contacts with the hair cells, therefore is not activity dependent (Gompel *et al.*, 2001).

1.4 Spontaneous activity

Spontaneous activity is defined here as spontaneous action potentials in the neuron in absence of external stimulation, as defined in previous literature from the field (Trapani & Nicolson, 2011).

1.4.1 The function of spontaneous activity

Spontaneous activity is important for many sensory systems during development for functions such as strengthening dendrite contacts to postsynaptic targets and processing information correctly (Kennedy, 2012; Kutsarova *et al.*, 2016), and can also be thought to modulate sensitivity as seen in vestibular synapses (Peusner *et al.*, 2012). The mechanisms that cause spontaneous activity vary greatly across different systems, from gap junction propagation to homeostatic regulation (Blankenship & Feller, 2010) but is not as widely investigated in all species and systems.

It is not practical to investigate the function of spontaneous activity in mammals *in vivo* as hair cells and afferent fibres are encased in bone. Zebrafish provide an opportunity for investigation of spontaneous activity in an intact and alive animal. Some i*n vitro* work has elucidated some interesting insights into spontaneous activity. There has been evidence to suggest that spontaneous activity in the immature system is a sign of synaptic competition (Buffelli et al., 2003) to organise a developing neural circuit (Petersson et al., 2003). For example, whilst a young rat is asleep, spontaneous muscle twitches modify the sensorimotor neural patterning that leads to more refined and corrected muscle movements (Petersson et al., 2003). Spontaneous activity is also found in the visual system as retinal waves for activity-dependent refinement of neural patterning prior to the onset of vision. Retinal waves are the

propagation of bursts of action potentials among neighbouring retinal ganglion neurons. The consequence of the spontaneous retinal waves is to ensure that higher-order projections are organised correctly before the onset of vision (Ackman *et al.*, 2012).

Manipulation of spontaneous activity has mainly been achieved using gene editing, and rarely the outputs have been measured *in vivo*. However, in the afferent neurons of the mouse olfactory system, spontaneous activity has been investigated and it was shown to have an important role in establishing and maintaining the somatotopic organisation of neural connections. If spontaneous and evoked activity is inhibited in a subsection of olfactory neurons, the corresponding axons can establish connections in the glomeruli, but cannot maintain these contacts (Yu *et al.*, 2004). Alternatively, if the general functioning of an olfactory neuron is disrupted - for example when overexpressing a K^+ channel (hyperpolarising inward rectifying channel, Kir2.1) - formation of the olfactory map is delayed and disrupted (Yu *et al.*, 2004). It is unclear whether the results of these experiments are due to reduced spontaneous activity or reduced total activity (including evoked). However, the results suggest that spontaneous activity in the mouse olfactory system is key for development and maintenance of the olfactory somatotopic organisation (Yu *et al.*, 2004). This finding has not been confirmed *in vivo* but spontaneous activity that is present in other sensory systems has been investigated and has found to have varying functions. Such as the retinal ganglion cells of rats have not only been found to have spontaneous activity for somatotopic organisation, but also to enhance trophic factors that cause axonal growth (Goldberg *et al.*, 2002) and is therefore essential for neural maturation. However, little research has focused on spontaneous activity during development in the zebrafish lateral line. Spontaneous activity is present in the neurons of the lagenar macula in birds, which has a functional resemblance to the mammalian vestibular system (Galicia *et al.*, 2010). In early embryonic stages, the frequency of this activity is low, and becomes higher and more regular as development progresses (Galicia *et al.*, 2010) as seen in other vestibular systems. These hair cells have a regenerative capacity (further discussed in section 1.7) (Bermingham-McDonogh & Reh, 2011), which spontaneous activity is thought to play a significant role in refining the connections between newly regenerated hair cells and existing afferent fibres.

Spontaneous activity is not just a property associated with neurons. In the auditory system of murine animals, spontaneous activity is present in inner hair cells (Marcotti *et al.*, 2003). However, spontaneous activity has only been observed embryonically until the onset of hearing in acutely dissected cochlea (Johnson *et al.*, 2012). Due to the limitations of the preparations, it is unknown whether spontaneous activity in the inner hair cells is present *in vivo* and how it would mature in the adult. However, it is proposed that there is spontaneous activity that influences the refinement of the cochlear development pre-hearing (Blankenship & Feller, 2010). Understanding spontaneous activity *in vivo* may help us to identify key important characteristics for the maintenance, maturation and repair of hearing in humans. Using functional MRI, patients with presbycusis (age-related hearing loss) have been shown to have disrupted spontaneous neural activity in areas including the superior temporal gyrus and parahippocampal gyrus (Chen et al., 2018) for unknown reasons.

1.4.2 Spontaneous activity in the zebrafish posterior lateral line

Spontaneous activity is present in the pLL afferent fibres of zebrafish. Spontaneous activity has been characterised in larval zebrafish (3-7 dpf) through the combination of genetic manipulation studies and electrophysiology *in vivo* (Trapani & Nicolson, 2010; Liao & Haehnel, 2012; Haehnel-Taguchi *et al.*, 2014). Through extracellular loose-patch recordings of the pLLg, it is possible to visualise spontaneous and stimulated activity. It is possible to distinguish the difference between spontaneous and evoked activity, as spontaneous activity has a more random firing pattern that is of a lower frequency compared to evoked activity caused by stimulation (Liao, 2010).

Trapani and Nicolson (2011) hypothesised that the $Ca_V1.3$ voltage gated calcium channel in the hair cells of the pLL was involved in spontaneous release of neurotransmitter (Trapani & Nicolson, 2011). The membrane potential can fluctuate at rest so it is within the activation range for opening the $Ca_V1.3$ channel, as seen previously in the frog saccular hair cells (Jorgensen & Kroese, 2005). The MET channel has a low open probability at rest which contributes to the resting potential of the hair cells. However, when mechanotransduction was blocked in a zebrafish strain with ineffective tip links caused by a mutation in *cadherin23*

(cdh23), spontaneous activity was still present, but the rate of firing was reduced (Figure 1.5). In a zebrafish strain which lacked $Ca_V1.3$ channels, evoked and spontaneous activity was not present (Trapani & Nicolson, 2011), as exocytosis of neurotransmitter is calcium dependent. The absence of $Ca_V1.3$ channels leads to changes in ribbon synapse morphology and retraction of post-synaptic densities occurs, which leads to non-functional synapses (Sheets *et al.*, 2012). The spontaneous firing rate is also reduced in zebrafish with vesicular glutamate transporter 3 (*vglut3*) mutations which cause the hair cells of these fish to have altered neurotransmitter release, similar to those with $Ca_V1.3$ mutations (Trapani & Nicolson, 2011) due to fewer vesicles associated with the ribbon synapse (Figure 1.5) (Obholzer *et al.*, 2008). As it was clear that the spontaneous activity in the afferent fibres not intrinsic, Trapani and Nicolson (2011) then sought to identify what aspect of the hair cell would cause the exocytosis of neurotransmitter. The hyperpolarisation-activating current (I_h) was blocked in the hair cells of the pLL with the antagonists ZD7288 and DK-AH 269, spontaneous activity

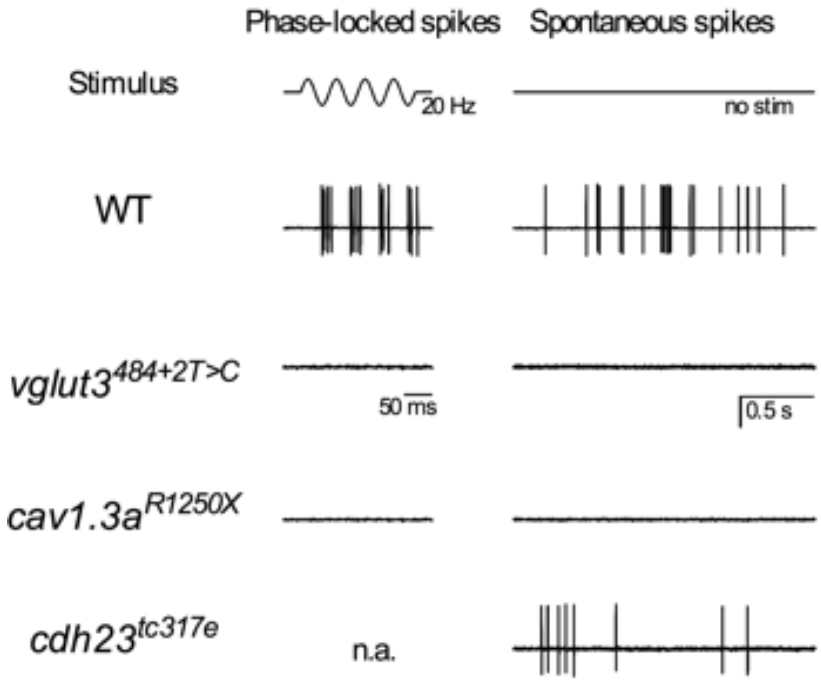

Figure 1.5 Spontaneous activity in mutant lines of zebrafish

Example extracellular loose-patch traces from the pLLg after stimulation (phase-locked spikes, left), or at rest (spontaneous spikes, right) from wildtype, vglut3, $Ca_V1.3$ and cdh23 mutant zebrafish. Figure adapted from (Trapani & Nicolson, 2011).

was only reduced in frequency until the contribution of the MET current (I_{MET}) was also blocked (with dihydrostreptomycin), which completely eliminated spontaneous activity. These findings indicated that the depolarising currents due to I_h and I_{MET} contribute to spontaneous activity in the pLL (Trapani & Nicolson, 2011). A similar finding has been found in the vestibular ganglion neurons of the mouse, as mechanotransduction and hyperpolarising-activating currents contribute to spontaneous activity (Horwitz *et al.*, 2014).

There are two outgrowths of afferent fibres that innervate the pLL in early development. The initial outgrowth projects down to innervate neuromasts in the tail, whereas the neurons of the second outgrowth are suggested to only contact one neuromast each located more anteriorly. The population of afferent fibres that are first to develop have larger soma diameter which is distinguishable from the later born afferent fibres that have smaller somas (Liao & Haehnel, 2012). If the rate of spontaneous activity is different between the two populations of afferent fibres, the spontaneous activity may be modulating how readily the afferent fibre can respond to an evoked stimulus. However, it is unknown what would occur over time after development to spontaneous activity.

Using whole cell patch-clamp electrophysiology, it has been proposed that early born afferent fibres have a lower spontaneous firing rate and a lower input resistance, implying that it takes a larger stimulus to occur in order for a threshold to be reached for evoked activity to be triggered. In contrast, later born afferent fibres have a higher input resistance and a higher spontaneous rate (Liao & Haehnel, 2012; Pujol-Marti *et al.*, 2012). The function of this variation is suggested to exist so that early born afferent fibres are able to detect strong stimuli before further development and refinement of the pLL nerve occurs. Therefore, spontaneous rate differences are involved in initial gross escape behaviour and finer local stimuli responses later on in development.

Another factor which influences escape behaviour refinement is afferent innervation of neuromasts. During embryonic development, afferent fibres initially innervate only one neuromast, but during larval development afferent fibres will branch to also innervate neuromasts located close by, even if they are already innervated by another fibre (Nagiel *et*

al., 2008; Faucherre *et al.*, 2009). This enlarges the perceptive field of an afferent fibre so it is able to detect stimuli from a wider range. However, the increase in hair cells that one afferent fibre innervates is likely to have consequences to the spontaneous activity as one afferent fibre will receive more input and thus the frequency of spontaneous activity may increase. Do later born afferent fibres have a higher spontaneous rate (Liao & Haehnel, 2012) due to increased coordinated hair cell output?

Although the early-larval stages of the pLL have been investigated, it is not fully known how the properties of spontaneous activity may change as the zebrafish develops and matures into an adult.

1.5 Maturation and aging of hair cells

After larval development, some older hair cells will die but new hair cells will also arise in neuromasts of the pLL from mitosis of existing hair cells (Lopez-Schier & Hudspeth, 2006). These cells must rearrange their orientation to match the polarity of other hair cells in the neuromast and become the correct shape. This happens throughout the zebrafish's lifetime; therefore, even in adulthood there is a constant turnover of hair cells (Williams & Holder, 2000; Cruz *et al.*, 2015). The afferent fibre must keep up with the innervation of new hair cells and to retract their processes from dying ones. How the spontaneous activity is affected by the constant turnover of hair cells is not known. Hair cells in the zebrafish inner ear continue to increase in number after development, without overcrowding, until 10 months of age. The increase in quantity of hair cells has no impact on hearing sensitivity (Higgs *et al.*, 2002) which remains constant after 2 wpf despite the further increase in sensory cells (Lu & DeSmidt, 2013). The zebrafish inner ear, like the pLL, also has a constant turnover of hair cells throughout life (Higgs *et al.*, 2002). Therefore, as the hearing sensitivity does not change, it is possible that the addition and constant turnover of hair cells in the pLL does not significantly impact the zebrafish pLL function or spontaneous activity during maturation and aging.

Maturation and aging is known to have impact on the functioning of hair cells, especially in the mammalian cochlea (Perez & Bao, 2011). In zebrafish hair cells, maturation

and aging is not as well investigated. However, it has been found that during development from larval to juvenile stages, the biophysical properties of the hair cells alter as the zebrafish pLL matures. Whole-cell patch clamp electrophysiology identified that a higher proportion of mature hair cells are present in the juvenile zebrafish based on the channels present in the hair cells compared to at larval ages. The immature hair cells, like in those of the larval zebrafish, are found in the periphery of the neuromast past larval stages (Olt *et al.*, 2014), where new hair cells develop due to differentiation from surrounding supporting cells (Williams & Holder, 2000). Functionally, this gives the zebrafish a benefit as if cells are lost during damage, new cells will always develop to help maintain pLL related behaviours at all ages of the zebrafish. However, it is not known if there is a time scale of the life cycle of hair cells in the pLL and what the biophysical properties are in hair cells of the adult zebrafish.

In the auditory saccule of the zebrafish, the hearing sensitivity can be measured by electrodes with microphonics or Auditory Evoked Potentials (AEPs). During the first week of development (3-7 dpf) zebrafish lose some hearing sensitivity as seen in an increased shift in hearing thresholds (Yao *et al.*, 2016). However, at this time point, the zebrafish are still capable of detecting sounds below the level which would trigger a startle response (Bhandiwad *et al.*, 2013). From 40 dpf to 20 months post fertilisation (mpf) zebrafish display a degree of developmental enhancements and age-related declines in hearing sensitivity as seen in AEPs. Total hair cell number increases almost 8-fold from 30 dpf to 14 mpf, and then declines. The ribbon synapse protein, Ribeye b, also reduces in number in older age which is consistent with the AEP threshold decline and reduction in hair bundle morphology (Wang *et al.*, 2015).

As the biophysical properties of the hair cell change, the afferent fibres mature and sensitivity of the hair cells of the saccule declines with age, it is clear that the small-time window in which zebrafish are unprotected (<5.2 dpf), and thus frequently used for research, represents a very early developmental stage which may not be representative of the mature, adult system.

1.6 Zebrafish genetics

The zebrafish is an animal model that is widely used for many aspects of scientific research and has become an increasingly popular tool for investigating hearing loss and deafness (Whitfield, 2002). The zebrafish is easy to breed, maintain and it is possible to manipulate their genes to model human auditory and vestibular disorders.

Zebrafish have many advantageous characteristics for hearing research compared to mammalian animal models. The zebrafish pLL has sensory hair cells that are morphologically and functionally similar to those of the mammalian inner ear (Coffin *et al.*, 2010). The superficial nature of the hair cells of the pLL means that these cells are easy to access with an electrode and are easy to image, especially at embryonic and larval ages when the zebrafish is relatively pigment free and transparent.

Zebrafish and humans share similar genes that are involved in hearing loss (Whitfield, 2002). Many auditory and vestibular genes have been identified through large mutagenesis screens of zebrafish which are confirmed by behavioural assessments such as startle reflexes and swimming behaviour (Granato *et al.*, 1996; Nicolson *et al.*, 1998). Genetic manipulation of these genes in the zebrafish allows us to identify deficits and the mechanisms of action involved in hearing loss in humans, which we cannot map *in vivo* as easily in other animal models (Erickson *et al.*, 2017). Hair cell genetic defects in auditory and vestibular disorders are mainly classified as issues in either mechanotransduction or synaptic transmission. However, despite the frequent use of zebrafish in hearing and deafness research - such as identifying otoprotectants (compounds that may protect against hair cell damage) and mutagenesis studies to identify genes of interest - there is still a lot we do not know about the physiology of the pLL (Pujol-Marti *et al.*, 2012).

1.7 Regeneration of hair cells

Many people worldwide suffer from vestibular and hearing defects due to hair cell damage and loss. The treatment for hearing and vestibular issues is mainly palliative when

hair cells are damaged as humans are not capable of hair cell regeneration and there is no cure. Murine models are used frequently for hearing loss and deafness research; however, they are also not capable of regenerating hair cells. Therefore, it is important to investigate regenerative capacities of auditory and vestibular systems in other animal models in order to identify methods of regeneration that may be initiated in the human inner ear.

1.7.1 Regenerative capacities of different species

The African clawed frog, teleost fish and avian species are capable of regeneration of many different tissues, including hair cell regeneration following damage due to acoustic trauma, ototoxic exposure or aging (Corwin, 1981, 1985; Cruz *et al.*, 1987; Corwin & Cotanche, 1988; Harris *et al.*, 2003).

There are varying degrees of regeneration in different species. The avian vestibular system constantly renews hair cells due to apoptosis of existing hair cells (Jorgensen & Mathiesen, 1988) but an increase of hair cell regeneration can be seen after vast hair cell damage (Weisleder & Rubel, 1993) and re-innervation is rapid (Bermingham-McDonogh & Rubel, 2003). Supporting cells are the origin of new hair cells through two different mechanisms. Supporting cells can mitotically divide and the daughter cells will either become another supporting cell and a hair cell, or two hair cells. Alternatively, supporting cells can differentiate into hair cells without mitosis, this is termed transdifferentiation (Figure 1.6B) (Warchol & Corwin, 1996; Stone & Cotanche, 2007; Atkinson *et al.*, 2015).

The mammalian inner ear does not have a comparable regenerative capacity to birds. In both the vestibular and auditory system of mammals cells do not re-enter the cell cycle upon damage post-embryonically (Cotanche, 1987). This leads to very little recovery of cell numbers after insult and therefore permanent hearing loss and vestibular disorders (Bermingham-McDonogh & Reh, 2011). The avian cochlea, chicken utricle and non-

vertebrate models have become key into identifying mechanisms and signalling pathways important for the regeneration process.

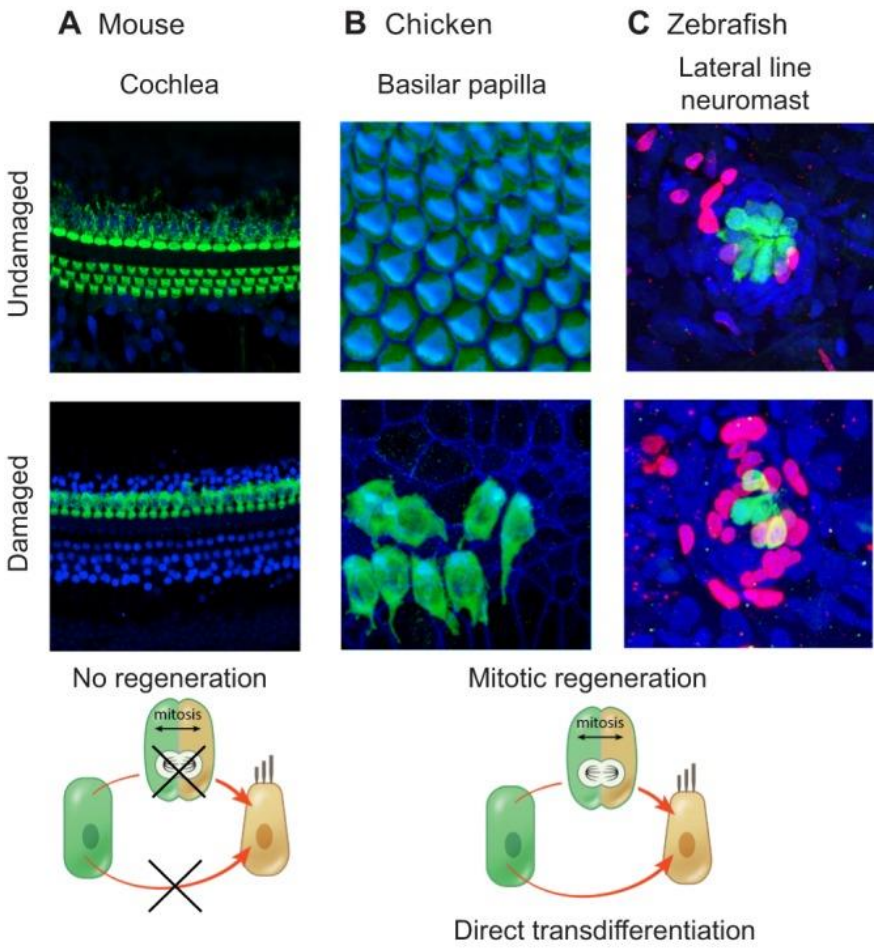

Figure 1.6 Hair cell regeneration in different species

Regenerative capacities of the adult mouse cochlea **(A),** the chicken basilar papilla **(B)** and the zebrafish lateral line system **(C).** Sensory hair cells before (myosin VIIa = green) (top) and after (middle) hair cell damage. No hair cell regeneration is present in the adult mouse cochlea, but some is present in the chicken basilar papilla and pLL neuromast. BrdU used in the zebrafish to label proliferating cells. Bottom panel is a schematic of hair cell regeneration. Adapted from Atkinson *et al.* (2015).

1.7.2 Regeneration in the zebrafish pLL

Despite having many similar hearing and deafness related genes to mammals, the zebrafish lateral line hair cells are able to regenerate after damage unlike in humans (Nicolson, 2005). The hair cells regenerate rapidly in order for the zebrafish to continue to carry out behaviours that are largely reliant on the pLL, such as rheotaxis and the startle

response (Wang *et al.*, 2017). The regenerative capacity of the hair cells is nearly inexhaustible throughout the zebrafish's life, which requires a continuous maintenance of the precursor pool to ensure a steady supply of new cells when needed (Cruz *et al.*, 2015). The mechanism of regeneration in the zebrafish pLL contrasts to regeneration in avian and amphibian hearing organs, where hair cells can regenerate through transdifferentiation (supporting cells become hair cells without division)(Adler & Raphael, 1996; Baird *et al.*, 1996; Baird *et al.*, 2000). In the pLL, during regeneration precursors divide and give rise to two daughter cells that will become hair cells. As the neuromast comprises of hair cells of two polarities, these hair cell precursors must then rearrange to orientate next to existing hair cells of the same polarity (Wibowo *et al.*, 2011). The zebrafish allows us to work with an *in vivo* animal model that regenerates hair cells quickly; therefore, is chosen more favourably over avian and amphibian systems to explore regeneration of hair cells as cell fate mapping is easily visualised.

Zebrafish are small and easy to breed, therefore are suitable for high-throughput screening to detect various behavioural and morphological defects (Bang *et al.*, 2002; Ou *et al.*, 2010; Baxendale & Whitfield, 2016). There are many ototoxic compounds (such as neomycin (Harris *et al.*, 2003), cisplatin (Ton & Parng, 2005; Ou *et al.*, 2007) and copper (Hernandez *et al.*, 2006; Linbo *et al.*, 2006)) that have been shown to be ototoxic to both humans and zebrafish. As the pLL is externally located, it is in a good position in which to assess damage and protection, allowing for research in zebrafish to be conducted in order to find preventative measures for hearing loss. Previously approved drugs are screened to see if they can be co-administered to prevent the effects of the ototoxic compounds on the hair cells, reducing the time taken to generate new drugs and test in higher vertebrate species such as mice (Coffin *et al.*, 2010; Ou *et al.*, 2010; Stawicki *et al.*, 2015). These studies look at the protection of the hair cells based on if they are present or not after exposure, not what happens to the physiology of the cell, but give a good overview of protective capabilities of certain compounds.

Due to the transparency and optical clarity of larval zebrafish, regeneration of the pLL hair cells is well documented using microscopy. Certain compounds such as YO-PRO1 and DASPEI (Linbo *et al.*, 2006) label hair cells by selectively entering and accumulating in hair

cells through the MET channel. Although hair cell labelling is good for quantification purposes, this method does not identify if the hair cell is functional, merely if the hair cell is present and MET channel is open. Zebrafish strains that express a hair cell-specific marker can be more useful if the protein it is tagged to is expressed early enough in the hair cell development; therefore, hair cells are able to be seen prior to having functional MET channels. However, this also provides a similar issue of only giving evidence for the presence of hair cells, not hair cell quality or synaptic viability (Kindt & Sheets, 2018).

One ototoxic compound used in zebrafish hair cell regeneration research is copper sulphate ($CuSO_4$). $CuSO_4$ causes the hair cells of the pLL to degenerate and after only two hours of exposure, the hair cells of the pLL are completely absent. Regeneration of hair cells in larval zebrafish occurs within 72 hours, as the number of hair cells has reached similar levels as in control zebrafish (Mackenzie & Raible, 2012). This is best achieved at low doses of around 10 µM $CuSO_4$. It has been shown that $CuSO_4$ can irreversibly damage the pLL after just 50 µM, whereas the aLL is more resilient and can withstand up to 400 µM before it can no longer regenerate the hair cells (Hernandez *et al.*, 2006). This is evident throughout the zebrafish lifespan. It is not known how, or to what extent ototoxic compounds such as $CuSO_4$ affect the spontaneous activity during regeneration in larval or older zebrafish, when the pLL is more mature and there are more hair cells to regenerate after damage.

1.8 Aims and hypothesis

There is currently relatively little understanding of how the biophysical properties of the zebrafish pLL develops. I have identified what current knowledge has found using electrophysiology.

There are three main aims to this study. The first aim is to identify when spontaneous activity first appears and whether it is present both during development and maturation. The second aim is to identify through extracellular loose-patch electrophysiology if spontaneous activity recorded from the afferent fibres of the pLL alters during development and refinement of the pLL, such as increased frequency of firing. The third aim is to identify the effect of regeneration of hair cells on spontaneous activity. Hypotheses have been developed in order to address these aims as follows:

1) It is hypothesised that spontaneous activity will be present from when the hair cells are "functional" at 2 dpf. This will identify when the hair cell forms a synapse with the afferent fibre.
2) It is hypothesised that spontaneous activity properties - such as frequency - will alter with development, past previously reported ages due to the increased number of hair cells and the refinement of afferent connections.
3) It is hypothesised that copper will abolish spontaneous activity in the pLL until hair cells regenerate.
4) It is hypothesised that compared to early-larval zebrafish, spontaneous activity in older zebrafish will take longer to recover to similar frequencies as age-matched controls after copper treatment due to the larger number of hair cells per neuromast in older fish.

These hypotheses will be tested using extracellular electrophysiology and confocal microscopy with transgenic zebrafish.

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Chapter 2 General Methods

2.1 Ethics Statement

All zebrafish studies were licensed by the UK Home Office under the Animals (Scientific Procedures) Act 1986 and were approved by the University of Sheffield Ethical Review Committee. Maximum effort was used to employ the three Rs, Replacement, Reduction and Refinement.

A personal licence was obtained for all work on zebrafish >5.2 dpf and individual study plans were written prior to breeding parent zebrafish. Special care was taken when using zebrafish >5.2 dpf due to the Animals (Scientific Procedures) Act 1986 and, therefore, the protocol was altered to accommodate these regulations. Zebrafish are reared in the aquarium until needed by the experimenter due to Section 17 of the Animals (Scientific Procedures) act 1986, it is not possible to use neuromuscular blocking agents in regulated procedures. Thus, zebrafish >5.2 dpf were not paralysed with α-bungarotoxin as they were in <5.2 dpf zebrafish to paralyse the fish and consequently stop movement of the fish causing interference to the microelectrode recording. As a result of the lack of paralysis by neuromuscular blocking agents, pins were not used to restrain zebrafish >5.2 dpf as it would cause too much harm and distress to an older zebrafish compared to the paralysed and anaesthetised larvae. Low-melting point Agarose was used to restrain zebrafish >5.2 dpf instead after consultation with the Home Office.

2.2 Animals and Husbandry

Fish were maintained in the University of Sheffield aquarium facility on a 14-10 hour light-dark cycle at 28°C. Adult fish were fed artemia twice daily, 5.2 dpf – 10 dpf were fed powdered food and raised in a light/dark incubator due to the facility's regulations.

Table 1 Zebrafish strains

Strain	Origin
AB	The University of Sheffield Zebrafish Facility, Sheffield, UK
Tg(NeuroD:EGFP)	Prof. Teresa Nicolson, Oregon, USA
Tg(Myo6b:R-GECO)	Prof. Teresa Nicolson, Oregon, USA

2.2.1 Zebrafish husbandry <5.2 dpf

For larval (<5.2 dpf) experiments zebrafish were raised in petri dishes in Embryo Medium (E3) with methylene blue to prevent fungal diseases in an incubator (SLS, Hessle, UK) at 28°C. Fish were culled using bleach after experiments/before they reached the age of 5.2 dpf as permitted by the Home Office.

Table 2 Composition of Embryo Medium (E3)

Compound	Final concentration (mM)
NaCl	4.9
KCl	0.174
$CaCl_2.2H_2O$	0.432
$MgSO_4.7H_2O$	0.332

2.2.2 Zebrafish husbandry >5.2 dpf

For experiments using zebrafish under 10 dpf, zebrafish were raised as mentioned above (5.2 dpf) and then transferred in a petri-dish with E3 medium in to a light/dark incubator in the University of Sheffield Zebrafish Facility as zebrafish obtain circadian rhythms at this age, and thus development is inhibited by a reduced or absent light/dark cycle (Villamizar *et al.*, 2014). Embryo medium was changed daily and fish were fed powdered food until 10 dpf.

Zebrafish older than 10 dpf were raised in the zebrafish facility in system tanks from 5.2 dpf until needed for optimum feeding and light/dark cycles controlled by the facility.

For experiments involving treating zebrafish with copper sulphate, the zebrafish were placed in flow out tanks, so that the tank water was not recycled and therefore disposed of to avoid contamination of copper into the rest of the aquarium.

2.2.3 Zebrafish euthanasia

Tricaine methanesulfonate (MS-222) was obtained in a 0.4% stock solution from the University of Sheffield Aquarium Facility. The MS-222 was dissolved in water and buffered with Tris to pH 7. Zebrafish that were restrained in agarose >5.2 dpf were culled by immersion in 0.4% MS-222 until secondary endpoints were met or immersed in MS-222 until unresponsive and then decapitated as per Home Office regulations.

2.3 Zebrafish tissue preparation

All experiments were conducted *in vivo,* which is discussed in further detail in chapters 3 and 4.

2.3.1 Paralysis by α-bungarotoxin

Larval zebrafish were paralysed by injection into the heart cavity of α-bungarotoxin (α-Btx; Tocris Bioscience, Bristol, UK) to reduce interference to the recordings by movement of the zebrafish as previously described by Trapani & Nicolson (2010). A 2.5 µl of 500 µM α-Btx stock solution was kept frozen (-20°C) until day of use, thawed and 7.5 µl phenol red (0.5 % in DPBS, Sigma-Aldrich Co. Ltd, Gillingham, UK) added for visibility. Injection pipettes were made from pulled borosilicate glass capillaries (O.D. 1 mm; I.D. 0.58 mm, Harvard Apparatus Kent, UK) using a Narishige puller (Model PP-830; Narishige Japan). Injection pipettes were then back filled and inserted into an Eppendorf Femtojet injection system (Eppendorf, Stevenage, UK). Using a micro-manipulator, the injection pipette was used to penetrate the heart cavity where approximately 150 µM α-Btx was injected. Successful injection was confirmed by inflation of heart cavity and presence of red phenol red (as seen in Figure 2.1).

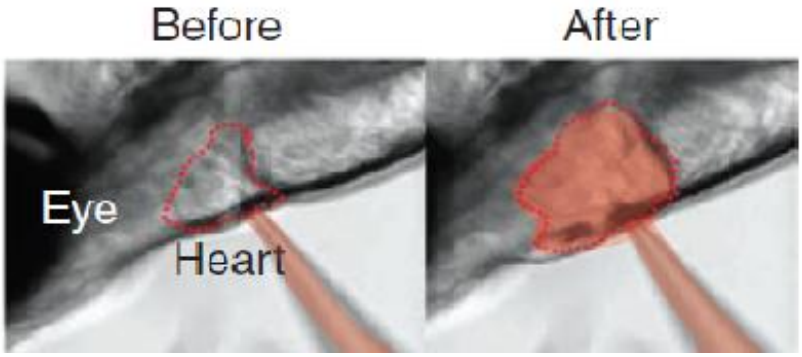

Figure 2.1 α-bungarotoxin injection

Image of zebrafish heart cavity before (left) and after (right) microinjection **of** α-bungarotoxin and phenol red and visualisation of a successful injection. Image adapted from Trapani & Nicolson (2010).

2.3.2 Restraint in agarose

For electrophysiology with zebrafish > 5.2 dpf, zebrafish were restrained using 2% low melting-point (LMP) agarose. Agarose was dissolved in a microwave to 2% in E3. On days of experiments, agarose was melted in the microwave and left to cool to avoid heat shock to the zebrafish, then maintained at ~37°C in a water bath for addition of the zebrafish.

Figure 2.2 Zebrafish restrained in agarose

Photo image of zebrafish (~12 dpf) restrained in 2% LMP-agarose on a coverslip in a Perspex chamber. Image obtained prior to addition of extracellular solution.

Zebrafish were gradually brought up to ~37°C to avoid heat shock by individually placing in a 50 ml falcon tube with 15 ml of room temperature E3 in a water bath. After the E3 had reached ~37°C, zebrafish were then transferred using a glass pipette/ pasteur pipette (depending on zebrafish size), into the liquid 2% LMP agarose, and then immediately pipetted onto a cover slip. Fish were orientated before the agarose set so they would lie on their left lateral side. Once the agarose was set, the area near the posterior lateral line ganglion (just posterior of the ear) was gently cleared of agarose to allow for microelectrode recordings. A drop of liquid agarose was added to the Perspex recording chamber to allow for the zebrafish in agarose to adhere to the chamber. The zebrafish was transferred into the chamber and orientated so it lay on its lateral side once more (figure 2.2).

2.4 Experimental solutions

2.4.1 Afferent Fibre Extracellular Solution (AFS)

For extracellular loose patch recordings, afferent fibre solution was used to bathe the fish, and was constantly perfused into the chamber by a peristaltic pump. The AFS was also used to fill the electrodes for extracellular recordings (Trapani & Nicolson, 2011). The osmolarity was approximately 290 mOsm with an adjusted pH of approximately 7.8. The AFS was made in 2 litre batches and stored in the fridge.

Table 3 Composition of extracellular solution for afferent fibre recordings <5.2 dpf

Chemical	Final mM
NaCl	140
$CaCl_2$	2
KCl	2
$MgCl_2$	1
HEPES	10

2.4.2 Afferent Fibre Extracellular Solution with bicarbonate

For recordings obtained from zebrafish >5.2 dpf, an adapted extracellular solution was used. Due to previous reports that HEPES in the AFS precipitates during oxygenation (95% oxygen, 5% carbon dioxide), bicarbonate ($NaHCO_3$) was used as a replacement buffer. The extracellular solution with bicarbonate was made in 1 litre batches and frozen in 100 ml glass bottles. The osmolarity and pH was adjusted to be the same as the AFS. The solution was oxygenated as per request from the Home Office to ensure the fish could respire whilst restrained in agarose.

Table 4 Composition of extracellular solution >5.2 dpf zebrafish recordings

Chemical	Final mM
NaCl	140
$CaCl_2$	2
KCl	2
$MgCl_2$	1
$NaHCO_3$	25

2.5 Electrophysiology

2.5.1 Glass microelectrode preparation

For extracellular recordings, borosilicate glass with filaments (inside diameter 0.86 mm, outside diameter 1.5 mm. Sutter instrument, Novato, USA) were sterilised in the oven at 400°C for 4 hours. The glass is then pulled using a Narishige puller (Model PP-830; Narishige Japan) with a tip resistance of ~13 MΩ. The filament ensures filling the pipettes with solution without bubbles.

2.5.2 Experimental set-up

For extracellular recordings, <5.2 dpf zebrafish were anaesthetised in 0.005% MS-222 in afferent fibre solution and transferred onto a sylgard-coated cover slip in a Perspex recording chamber. Zebrafish were then pinned to the sylgard-coated cover slip by two pieces of Tungsten wire (0.025 mm. Advent, Eynsham, UK). The tungsten wire pins were inserted posteriorly through the fish tail, and anteriorly through the jaw region to restrain zebrafish (figure 2.3).

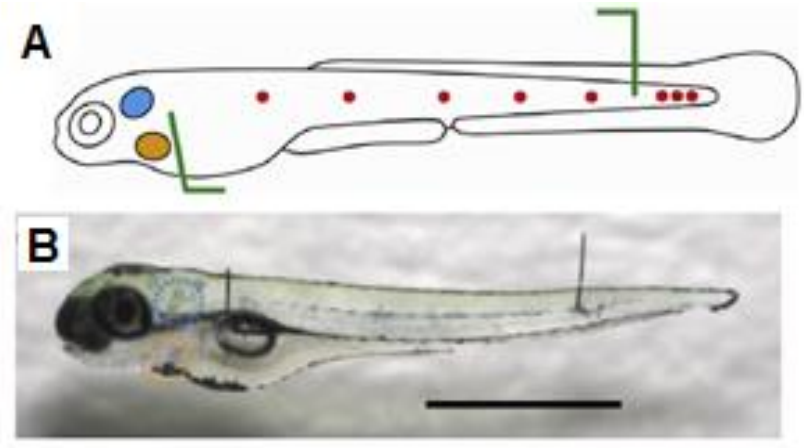

Figure 2.3 Diagram of the zebrafish preparation (<5.2 dpf)

A, schematic of larval zebrafish preparation with pins for restraint. **B**, DIC image of restrained larval zebrafish. Blue highlights the otocyst. Scale bar = 1 mm. Figures adapted from Olt *et al.* (2016).

The chamber was then placed under an upright microscope (Olympus BX51WI, Olympus, Japan) and the preparation perfused with AFS by a peristatic pump (Cole-Palmer, IL, USA) at ~10 ml/min. All experiments were conducted at room temperature (~21°C).

2.5.3 Extracellular recordings of the posterior lateral line ganglion

A lens with x60 magnification was used on an upright microscope for identification of the posterior lateral line ganglion. The arc lamp was used to identify GFP expressing afferent fibres and therefore the ganglion in the zebrafish strain Tg(NeuroD:EGFP).

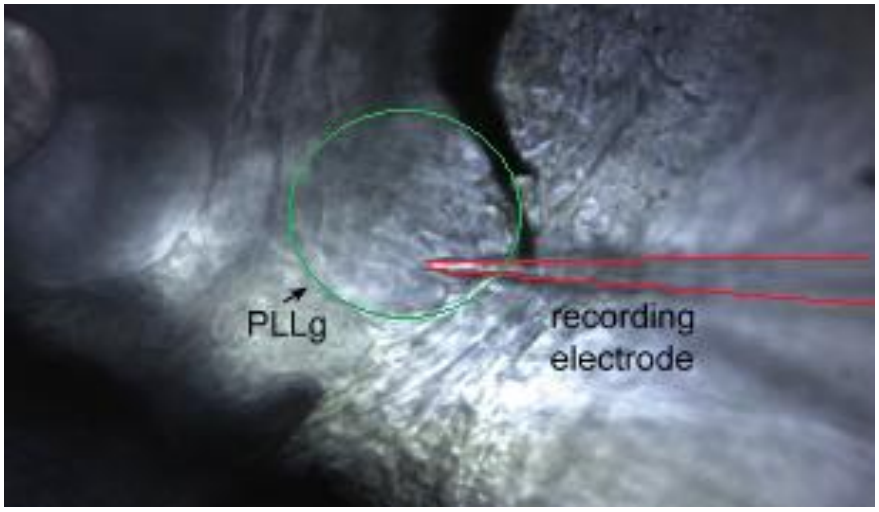

Figure 2.4 Posterior lateral line ganglion and recording electrode

DIC image of the pLLg at x60 magnification. Recording electrode highlighted in red and pLLg highlighted in green. Figures adapted from Olt *et al.* (2016).

For extracellular recordings, the electrode was filled with AFS and inserted into an electrode holder of the patch clamp amplifier headstage, which has a tube attachement to allow for positive and negative pressure to be applied to the electrode by a 60 ml syringe. The recording electrode was put into position to record from the pLL ganglion through the use of a micromanipulator (PatchStar, Scientifica, UK) (figure2.4). A reference electrode was also filled with AFS and placed in the bath in the chamber.

In voltage clamp, the pipette offset is compensated for prior to advancing into the ganglion using the automatic control of the Multiclamp 700B (Axon Instruments, Molecular Devices, USA). Once the pipette offset was corrected, the pipette was advanced into the ganglion through gradual forward and down motions until the pipette had penetrated the

skin layers efficiently. To ensure entry into the ganglion, slight pressure was applied to the pipette to inflate the ganglion. If this is not achieved, then the pipette was blocked or not effectively entered the ganglion. The resistance remained the same throughout this procedure. Once an afferent fibre soma was identified, pressure was relieved, and then negative pressure was applied until the resistance reached between 35-120 MΩ and confirmation of spikes was obtained using Clampex 10.6 software (Molecular Devices, USA). This provided a fairly stable seal; therefore, the activity can be recorded for many minutes.

The headstage pre-amplifies signals from the recording electrode in voltage clamp, which is then sent to the amplifier Multiclamp 700B (Axon Instruments, Molecular Devices, USA), filtered by a Bessel low pass filter set at 2.5 kHz, then passed through the Digidata 1440A to turn the signal from an analogue to a digital output (Axon CNS, Molecular Devices, USA) before being sent to the computer.

In the computer, the signal is finally captured by Clampex 10.6 (Molecular Devices) and then analysed offline using minianalysis and OriginPro software (Origin Lab, USA). Minianalysis was used to identify the time between spikes and therefore the inter-spike interval (ISI) was calculated in OriginPro, as well as the coefficient of variation (CV).

2.6 Confocal microscopy

To identify the number of hair cells regenerating over time, the zebrafish strains Tg(NeuroD:EGFP) and Tg(myo6b:R-GECO) were crossed for visualisation of the afferent fibres (green) and hair cells (red) respectively. Zebrafish were culled either by overdose of anaesthetic (MS-222) or decapitation before fixing in 4% PFA as stated previously.

Zebrafish were mounted 2-3 to a glass slide using Vectashield (Vector Laboratories, Burlingame, USA) for confocal microscopy and sealed with a cover slip and nail varnish.

Slides were imaged using a Nikon A1 confocal microscope in the University of Sheffield Wolfson Light Microscopy Facility. Images and z-stacks were obtained using a x60 magnification oil immersion lens. Images were processed using ImageJ software.

2.7 Statistics

Tests for normality were conducted using Origin (Shapiro Wilk test). Kruskall-Wallis tests were also conducted on Origin to identify significant differences between groups that were not normally distributed.

If normally distributed, a one-way analysis of variance (ANOVA) was used to determine statistically significant differences between the means of two or more independent groups. Statistical comparisons in experiments with two potentially interacting factors (e.g., treatment condition and hours post treatment) were calculated using a two-way ANOVA with a Bonferroni post hoc test. Comparisons of two groups were conducted using a student's t-test using Prism and Origin.

Pearson's correlation coefficient (if normally distributed) and Spearman correlation coefficient (if not normally distributed) was calculated in Origin.

F-tests were calculated on Origin.

Mean values are displayed with ± SEM.

Significant values are indicated in figures by:

P-Value	Symbol
>0.05	n.s.
<0.05	*
<0.01	**
<0.001	***

2.8 References

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Chapter 3 Developing Biophysical Properties of the Zebrafish Posterior Lateral Line *in vivo*

3.1 Introduction

Zebrafish have become an increasingly popular animal model in the field of hearing loss and deafness research due to the superficial nature of the hair cells of the lateral line that are morphologically and functionally similar to hair cells of the inner ear (Coffin *et al.*, 2010). Zebrafish as an animal model have led to important advancements into the field, such as studying progression of congenital deafness diseases (Whitfield, 2002). Due to beneficial factors such as the optical clarity of larval zebrafish as well as restrictive welfare laws, most research is conducted under 5.2 dpf in the UK and 7 dpf in the USA. This encompasses is a very narrow time window of development for the zebrafish and doesn't incorporate a time when hair cells are considered mature and the system is refined.

Previous work has identified some key features of spontaneous activity in the pLL of larval zebrafish (Trapani & Nicolson, 2011; Liao & Haehnel, 2012). As discussed in chapter 1, spontaneous activity in the pLL is defined as activity in the afferent fibre in absence of external stimulation. The origin of the spontaneous activity has been identified to be due to stochastic exocytosis of glutamate from the hair cell (without mechanotransduction) that depolarises the afferent fibre (Dijkgraaf, 1963; Trapani & Nicolson, 2011). As the afferent fibre contacts numerous hair cells of the same polarity (Nagiel *et al.*, 2008), it is thought that the spontaneous activity in the afferent fibre is a cumulative signal from many hair cells (Song *et al.*, 2018).

The presence of spontaneous activity in the afferent fibres of the pLL suggests a functioning lateral line as a sense organ, as the afferent fibre is innervating the hair cells. Evidence that spontaneous activity is present in larval zebrafish (Trapani & Nicolson, 2011; Levi *et al.*, 2015) is correlated with behavioural findings that identify that the pLL is responsive to stimuli. The zebrafish relies on the visual system and lateral line for rheotaxis, a behaviour used by fish to orientate themselves to face oncoming water current (Olszewski *et al.*, 2012; Suli *et al.*, 2012; Olive *et al.*, 2016). By 5 dpf zebrafish have appropriate rheotactic orientation and startle response (escape behaviour due to lateral line stimulation)(McHenry

et al., 2009; Suli *et al.*, 2012). This suggests that the pLL circuitry is already able to provide orientation information and drive escape behaviour, for other functions such as predator avoidance, in an early stage of development. Adult zebrafish also display rheotaxis (Montgomery *et al.*, 1997) that is receptive to low flow rates, like in the larval zebrafish (Suli *et al.*, 2012). However, it is speculated that the larval zebrafish relies more on visual input for rheotaxis than in the adult, suggesting that the pLL and visual system are still not fully integrated in larval stages to fine tune rheotaxis behaviour (Olive *et al.*, 2016). The development of the pLL is also evident in zebrafish swimming behaviour. Although many factors add to the development of swimming behaviour, such as the myelination of the Mauthner cells (neurons that have input from the pLL for escape behaviour (Korn & Faber, 1975)) at 2 dpf (Triller *et al.*, 1997), the pLL is likely to play a role in ensuring swimming behaviour is precise. At 2 dpf the zebrafish is mostly inactive, whereas by 4 dpf swimming behaviour has increased and become more purposeful (Muller & van Leeuwen, 2004), which suggests further refinement of behaviour and the pLL.

Development of the larval zebrafish is well documented; however, if we are interested in the development of the pLL physiology at rest, we must first characterise spontaneous activity in larval stages at rest. Spontaneous activity has previously been recorded by electrophysiology in the zebrafish pLL ganglion (pLLg) before the age of 7 dpf (Obholzer *et al.*, 2008; Trapani *et al.*, 2009; Trapani & Nicolson, 2011; Levi *et al.*, 2015; Olt *et al.*, 2016a; Sheets *et al.*, 2017). The mean frequency (Hz) of spontaneous activity in early-larval zebrafish varies somewhat between different groups. Levi *et al.* (2015) found that in zebrafish aged 4-6 dpf the average spontaneous spike rate was 8.6 Hz. Whereas Sheets *et al.* (2017) and Olt *et al.* (2016a) observe the mean frequency of spontaneous activity to be around 6.8 Hz and 3.57 Hz respectively. Inconsistencies found in mean spontaneous activity spike frequencies can be due to many methodological considerations including the composition of the extracellular solution, area targeted by the electrode in the pLLg and age and strain of zebrafish. Although it is important to know the presence of spontaneous activity at a time when the zebrafish is most frequently used for hearing loss research (3-5.2 dpf in the UK and <7 dpf in the US), we must assess it at other ages in order to determine if biophysical changes in the pLL occur and if they relate to factors such as behavioural changes, for example for faster or more sensitive

escape reactions. It is not known when the onset of spontaneous activity occurs or how long it persists for. Neurons of the pLL do not start to differentiate until 18 hpf and by early-larval stages there are 21 differentiated neurons per ganglion (Sato *et al.*, 2006; Pujol-Marti *et al.*, 2010). Therefore, spontaneous activity is not expected to be present until after neurons have differentiated and have innervated hair cells.

It is also not known how long spontaneous activity in the zebrafish pLL persists for and how it may alter during development. The influence of increasing hair cells on spontaneous activity in development has been investigated using the African clawed frog. The African clawed frog possesses a lateral line system, which has also been extensively studied due to the superficial location of the sensory epithelia. The African clawed frog has neuromasts, like the zebrafish, that are innervated by two or more afferent fibres (Davis, 1965) and the afferent fibres that innervate the neuromasts also have spontaneous activity (Harris & Milne, 1966). It has been found that the number of hair cells an afferent fibre innervates increases during development in the African clawed frog, which leads to an increase in overall activity in the fibre. However, it is likely that the increase in activity is independent of spontaneous activity and is more likely to reflect the change in threshold needed to elicit an action potential in the afferent fibre in the presence of a stimulus, rendering the system more sensitive to weaker stimuli (Kroese *et al.*, 1978; Kroese *et al.*, 1980). It is not known if the zebrafish spontaneous firing rate increases in development in order to detect weaker stimuli or if there is a limit to the sensitivity of the hair cells of the neuromast. A superficial neuromast has a restriction to the number of hair cells it can have before becoming too large. A larger quantity of hair cells in a neuromast causes more hair bundles to be present in the cupula; therefore, sensitivity is reduced due to the large diameter and increased flexural stiffness of the cupula (McHenry & van Netten, 2007). This theory explains why neuromasts of zebrafish do not continue to increase in size, but new neuromasts are created with a limited amount of hair cells (Faucherre *et al.*, 2009). If an afferent fibre receives increased input from additional hair cells in different neuromasts during development, then the spontaneous firing rate may increase, like in the African clawed frog. Afferent fibres that have a higher rate of spontaneous activity may be more sensitive to stimuli, as has been found in the cichlids fish (*Sarotherodon niloticus*) lateral line (Münz, 1985), as smaller stimulations are

more likely to cause an action potential. In zebrafish, the average amount of hair cells per neuromast doubles between 5.2-18 dpf (Figure 3.1) (Olt *et al.*, 2014). Therefore, it is expected that the increase in hair cells per neuromast during development would cause an increase in neurotransmitter release that one afferent fibre would receive, and thus a higher rate of spontaneous firing. If this is the case, one would expect that the pLL would not be deemed mature until the number of hair cells remains stable later in development and spontaneous firing also becomes stable. However, there is a constant turnover of hair cells in neuromasts throughout life (Williams & Holder, 2000; Cruz *et al.*, 2015). As this is a continuous and slow occurrence, changes to the spontaneous firing rate is likely to be minimal in adulthood, especially after the zebrafish starts to produce of scales - as neuromasts are no longer capable of budding to form more neuromasts at this stage (Nunez *et al.*, 2009).

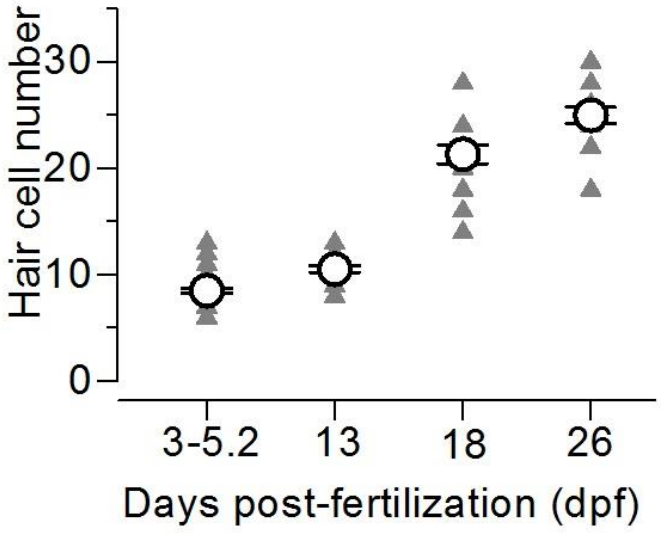

Figure 3.1 Hair cell number per neuromast increases with development

Hair cell quantification per neuromast (grey triangles) across zebrafish larval development. N = 66, 20, 24 and 20. Image obtained from Olt *et al.* (2014).

In certain animals - such as cats, mice and rats - the onset of hearing is not until after birth, but the auditory nerve fibres display spontaneous activity pre-hearing that is inversely correlated with the acoustic range of the fibre (high spontaneous rate fibres are more

sensitive to stimuli and have a narrower dynamic range) (Liberman, 1978). Through extracellular loose-patch recordings from afferents of the rat cochlea, it was identified that the spontaneous rate changes over time to become more mature and is related to pre-synaptic properties. Different auditory nerve fibres that contact the same hair cell can display varying spontaneous rates to each other, indicating that at rest the pre-synaptic release sites differ even in individual cells (Wu *et al.*, 2016). As the hair cells in the zebrafish pLL mature and the pre-synaptic properties are refined, the spontaneous firing rate may reflect these changes.

Through whole-cell patch clamp electrophysiology, it was found that during development from larval to juvenile stages, the basolateral membrane properties of hair cells in the zebrafish pLL changes over time, based on location of the hair cell in the neuromast. Mature hair cells appear to be located more centrally in a neuromast and need less calcium after stimulation to trigger neurotransmitter release; therefore, exocytosis is more efficient. Olt *et al.* (2014) postulate that the mature biophysical properties present in the hair cells in juvenile zebrafish are reminiscent of lower vertebrate vestibular and auditory systems due to the current profiles that the hair cells of both systems share (Olt *et al.*, 2014). Therefore, if we are to use the zebrafish pLL to investigate auditory research, it is important to consider the age of the animal to be in line with the hypothesis that is being addressed. Hair cells located centrally in the neuromast appear mature from juvenile ages (> 30 dpf) and thus may obtain ion channels and pre-synaptic machinery configurations that are more representative of the human inner ear at this stage (Olt *et al.*, 2014). Developmental refinement has also been seen in auditory organs of other species. In mice inner hair cells of the cochlea, the synaptic contact with the spiral ganglion neuron is refined during development, including tighter coupling between the calcium influx and exocytosis (Wong *et al.*, 2014).

As a result of hair cell maturation in the pLL, it is assumed that the neurotransmitter output is more refined. Therefore, spontaneous activity in the afferent fibre may be different when it receives input from immature hair cells at larval stages than when it innervates mature hair cells. Spontaneous activity alteration is seen in the hair cells of the mouse cochlea as the hair cells mature (Wong *et al.*, 2014). However, we do not know to what

extent the pre-and post-synaptic densities mature and when the pLL is functionally mature. We would like to identify if the spontaneous activity pattern reflects the hair cell accumulation and maturation, and if it continues to develop with the maturing system.

Spontaneous activity has been observed in different animals but alterations to spontaneous activity during development is not as well documented. Manley *et al.* (1991) recorded spontaneous activity in the auditory nerve of chicks at 2 and 21 days post hatching (dph). Despite the growth and development of the system, the spontaneous firing rate remained the same from 2 to 21 dph (Manley *et al.*, 1991), suggesting that mature, adult-like spontaneous firing rates are present from 2 dph. However, another group discovered that the spontaneous firing rate in adult chickens (16-24 weeks) was four-fold that of the chick (Salvi *et al.*, 1992), suggesting further refinement occurs after 21 dph. In other avian species such as the barn owl and the pigeon, spontaneous activity in the auditory nerve has been seen to closely correlate with the characteristic frequency (the frequency of action potentials that a neuron responds to a stimulus at) (Gummer, 1991; Koppl, 1997), which draws a comparison between activity and function. Not all avian species have similar auditory nerve activity. Spontaneous rate in the auditory nerve of pigeons reaches a stable rate by 4 weeks of age, that persists into adulthood (1-4 years)(Richter *et al.*, 1996). It is assumed that in the zebrafish pLL afferent fibres that spontaneous firing rate alters with development, and therefore it will continue to do so with further development that may be more evident the older the zebrafish becomes.

It is speculated that during development, the proportion of hair cells that one afferent fibre will innervate will increase (Nunez *et al.*, 2009), which would increase input that the afferent fibre receives and thus the spontaneous firing rate would increase in frequency - as afferent fibres do not proliferate as greatly as hair cells. More hair cells in a neuromast leads to more active zones that release neurotransmitter to one afferent fibre. Previous work has sought to identify what mutations in the hair cell active zone are essential for hair cell function and thus spontaneous activity. An overexpression of *Ribeye-b*, causes an increase in ribbon density size in the pre-synapse of hair cells in the pLL (Sheets *et al.*, 2017). An increase in ribbon size could lead to stronger exocytosis as a larger amount of vesicles are associated

to membrane (Usukura & Yamada, 1987). However, the spontaneous firing rate in the afferent fibres was lower in larval zebrafish with an over-expression of *Ribeye-b* (Sheets *et al.*, 2017). Thus, the size of the ribbon density does not increase the spontaneous firing rate, possibly as increasing the size does not increase the number of vesicles that are docked to the membrane, or due to many compensatory factors such as post-synaptic site size. This demonstrates that an alteration in pre-synaptic properties can alter spontaneous firing rates in the normal developing fish during development.

3.2 Hypotheses and aims

The aims of this chapter were to:

1. Characterise spontaneous activity in early-larval zebrafish (<5.2 dpf) and to identify the onset of spontaneous activity through extracellular loose patch recordings from the pLLg using previously established methods.
2. To establish a suitable protocol to restrain zebrafish (5.2-18 dpf) in order to obtain extracellular recordings from the pLLg whilst following UK Home Office Guidelines for zebrafish >5.2 dpf.
3. To identify whether the pattern of spontaneous activity changes past the age of protection (5.2 dpf), in line with additional changes during development and maturation of the system.

I hypothesised that spontaneous activity would be present from the onset of hair cells differentiating and receiving innervation from the afferent fibres. I also hypothesised that the mean firing frequency of spontaneous activity would increase with development due to maturation and refinement of the pLL, as the afferent fibres are receiving higher input from increased number of hair cells.

These hypotheses were tested using previously established methods of extracellular loose-patch electrophysiology in the posterior lateral line ganglion of early-larval (<5.2 dpf) zebrafish and was adapted for use with zebrafish past the age of protection (>5.2 dpf).

3.3 Methods

Briefly detailed below is the methodology for experiments in early-larval and older zebrafish. For full details, see chapter 2.

3.3.1 <5.2 dpf zebrafish

Zebrafish were anaesthetised using MS-222 and restrained using pins inserted into a sylgard coated coverslip and immersed in solution. Zebrafish were paralysed with an α-bungarotoxin injection into the heart cavity and then transferred to the microscope for recording acquisition. A recording electrode was used to penetrate the pLLg and record from the afferent fibres. A continuous protocol was used to obtain spontaneous activity for a duration of approximately 5-10 minutes.

3.3.2 >5.2 dpf zebrafish

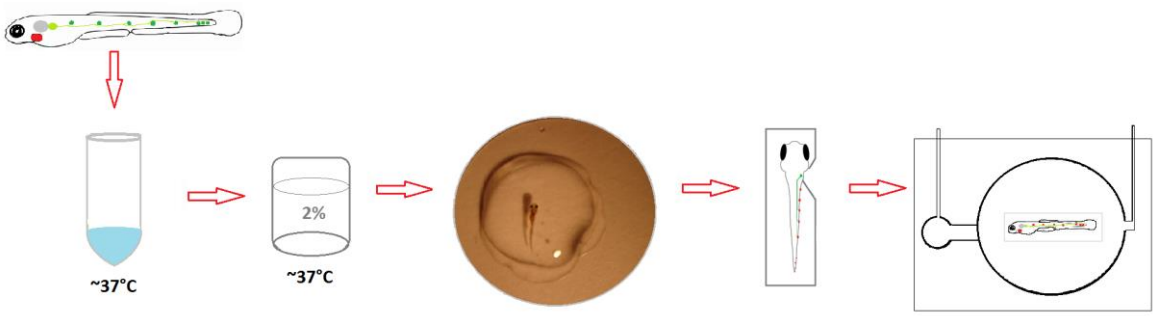

Figure 3.2 Diagram of preparation for extracellular recordings in >5.2 dpf zebrafish.

Zebrafish are slowly heated to liquid 2% LMP agarose temperature, added to agarose then transferred onto a coverslip to dry. The area by the ganglion is freed from agarose and then the fish is transferred into a recording chamber for electrophysiology.

Due to UK Home Office regulations, there are restrictions on zebrafish use over 5.2 dpf in the UK and therefore a personal licence is needed. A new method of restraint was

employed to abide by the Home Office regulations and ensure the zebrafish was not able to move, whilst not feeling discomfort or suffocation during the experiment.

Zebrafish were embedded in agarose from 5.2 dpf (Figure 3.2), through mid-larval ages until recordings were deemed too technically challenging with our method of restraint and recording acquisition.

Full details of the preparation are listed in the chapter 2. Briefly, I will explain the rationale behind the process. Agarose was used in replacement for the "pinning" method used for zebrafish <5.2 dpf as it was not possible to use the pins in the older zebrafish without MS-222 anaesthesia and the paralytic α-bungarotoxin. As the low melting point (LMP) agarose is maintained as a liquid in a water bath at ~36˚C, the zebrafish must be brought up to temperature in E3 in a waterbath before being submerged in liquid agarose to prevent heat shock. The zebrafish is then pipetted onto a glass coverslip and arranged using pipette tips so that the fish is either laying on its right lateral side or dorsal side up if necessary (this requires removal of agarose above the ganglion and then flipping the fish to lie on its lateral side). Once the agarose is solidified (approximately after 5 minutes at room temperature), the agarose is gently removed with a pipette tip above the ganglion (posterior to the otocyst). The zebrafish is then transferred to a chamber and is stuck down with more agarose at the sides to adhere the fish to the chamber and prevent movement during recordings.

LMP agarose appeared to be a good substitute method for restraining the zebrafish as breathing occurs through diffusion of oxygen across the skin at the age range the zebrafish were used (<21 dpf)(Olt *et al.*, 2016a). Therefore, perfusing the extracellular solution with oxygen was enough to keep the fish breathing with minimal stress (assessed by heart beats). A concentration of 2% LMP agarose was used in E3 as it restrained the zebrafish without impacting the overall health of the fish. A lower concentration of agarose did not restrain the fish appropriately.

Zebrafish are reliable photoperiodic breeders. Marbling/pair mating are set up the afternoon before breeding is desired by the experimenter; therefore, upon the initiation of the light cycle in the morning, zebrafish are likely to breed within the hour. Therefore, it is possible to estimate the hours post fertilisation (zebrafish from the University of Sheffield Zebrafish Facility are fertilised at ~9am as the light cycle begins at 8am) and days post fertilisation is accurately known.

Although zebrafish are not completely restrained from movement in agarose, a stable recording can be maintained as long as minimal agarose is removed from the area above the ganglion; heart beat and fin movement can interfere with the stable electrode. Due to licencing laws, the use of paralytics was prohibited and was therefore omitted from the >5.2 dpf preparation to prevent movement.

3.4 Results

3.4.1 Spontaneous activity is present from 2 dpf

Previous literature has identified that spontaneous activity is present at 3-7 dpf (Trapani *et al.*, 2009; Sheets *et al.*, 2011; Olt *et al.*, 2016b). However, it is not known at what age spontaneous activity is initially present from. Therefore, I obtained extracellular loose-patch recordings from the pLLg from zebrafish under 3 dpf. The pLL is not suggested to be "functional" until the last cells have been deposited along the fish by the first primordium at around 48 hpf (Kimmel *et al.*, 1995). Prior to this time, spontaneous activity is unlikely to occur (Figure 3.3A+B) and may only be present from differentiated hair cells that were first deposited by the first primordium (Kindt *et al.*, 2012). Attempts were made to obtain recordings of spontaneous activity from 24 hpf; however, it was clear that the otocyst and ganglion had not developed sufficiently and so spontaneous activity was not present (data not shown). Spontaneous activity was obtainable by extracellular loose-patch recordings from ~48 hpf (Figure 3.3C) as this is also a time when the somas of the ganglion are large enough to be seen using a GFP marker (Figure 3.3A+B).

Using zebrafish that have naturally hatched, extracellular loose-patch recordings are relatively simple to obtain at 2 dpf due to the high optical clarity of the cells of the ganglion. The frequency of spontaneous activity at 2 dpf is relatively low (0.44 – 2.61 Hz, mean = 1.88 ± 0.31) (Figure 3.3C) compared to what was previously reported in the literature in zebrafish age 3-7 dpf (Trapani & Nicolson, 2010; Olt *et al.*, 2016a). I suspect that the low frequency of spontaneous firing is due to the early stage of development of the pLL at 2 dpf; therefore, not all hair cells have formed functional synapses with the afferent fibres yet.

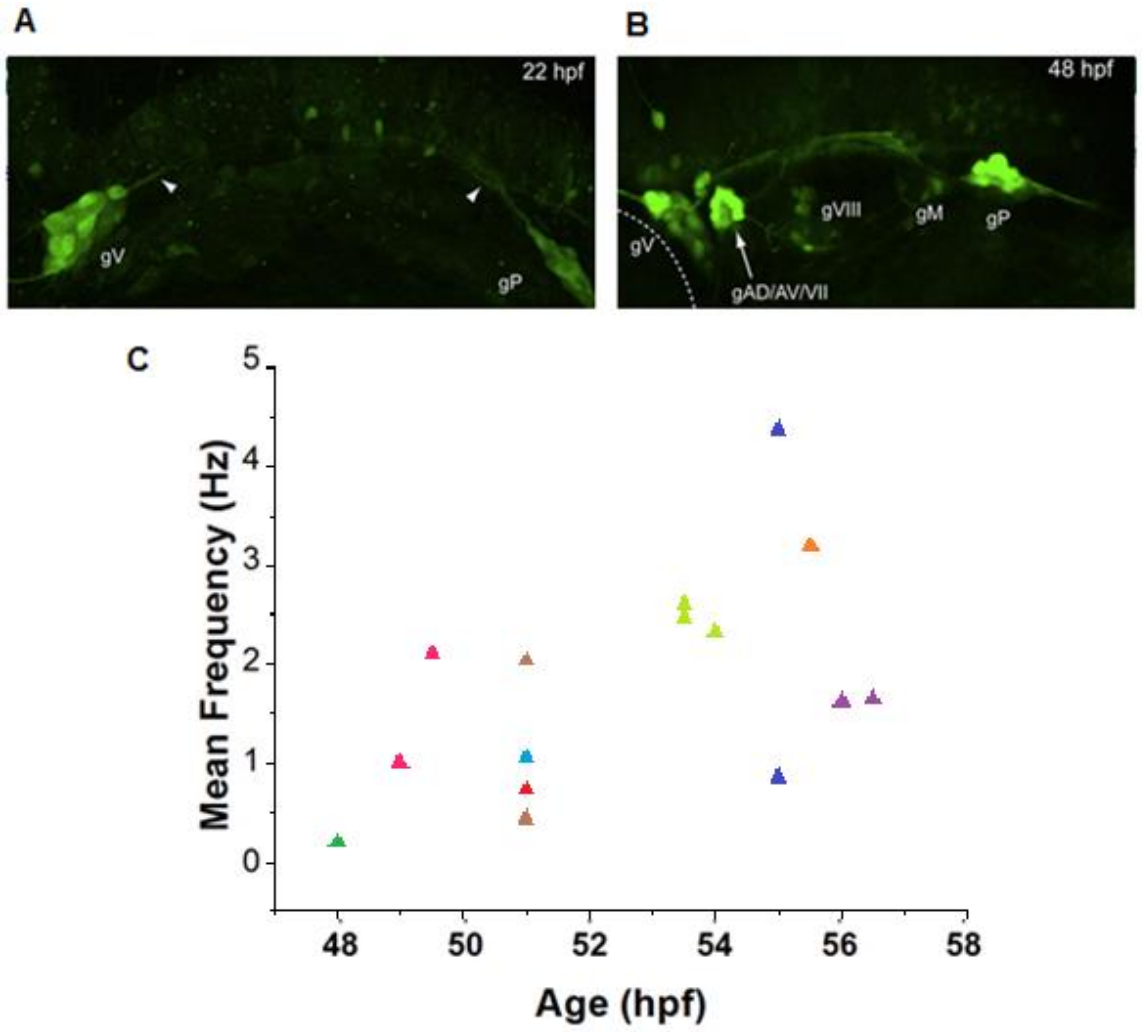

Figure 3.3 Spontaneous activity from 2 dpf

A+B, Comparison of pLLg (gP) at 22 hpt (**A**) and 48 hpt (**B**) labelled with Tg(SKIV2L2:gfp). Also seen is the trigeminal ganglia (gV). Image adapted from Cox *et al.* (2011). **C**, Mean spontaneous firing rates of recordings from the pLLg obtained at 2 dpf. N=9 (Zebrafish), n=15 (cells), Pearson's r=0.532.

3.4.2 Spontaneous activity is not affected by hatching

During the early-larval period, a major developmental feature is the hatching of the zebrafish from the chorion, which occurs at around 2-3 dpf. The time of hatching varies based on individual fish and their stages of development; therefore, hatching can vary between siblings despite being the same age (Kimmel *et al.*, 1995). In order to prepare the zebrafish for electrophysiology, if the fish had not already hatched itself, the fish was manually hatched (pre-hatched) to be restrained with pins (although preference is taken for zebrafish which were naturally hatched). Therefore, it was postulated that spontaneous activity may vary depending on the maturational state of the zebrafish, indicated by if they had developed enough to hatch themselves. Although the functionality of the pLL has not been investigated at this age, or in prematurely hatched zebrafish, the more developed the pLL is may affect the spontaneous firing rate. Previous research identified that rainbow trout (*Oncorhynchus mykiss*) that are prematurely hatched from the chorion are not as well developed as naturally hatched siblings. The premature hatching lead to increased growth and greater activity of the fish compared to trout that were left to hatch naturally themselves. Although rainbow trout spend longer in the chorion than zebrafish (Ninness *et al.*, 2006), it is possible that zebrafish that have hatched independently have more advanced pLL development.

Embryos that were pre-hatched from the chorion did not appear different to embryos that had hatched naturally, and movement seemed minimal from zebrafish in both conditions (not quantified). Extracellular loose-patch recordings were obtained from pre-hatched zebrafish and compared to manually hatched zebrafish at 2 dpf. Although there was variations in spontaneous firing rates, (Figure 3.4A+B) overall no significant difference was found between the mean spontaneous firing rate from either pre-hatched (mean = 1.63 Hz) or naturally hatched zebrafish (mean = 1.88 Hz) (Figure 3.4C). These findings show that the longer exposure to external stimuli to the hair cells of the pLL in the pre-hatched group causes no significant alterations to the firing frequencies of spontaneous activity in the pLLg.

A post-hoc power analysis revealed that on the basis of the mean, between groups comparison effect size observed in the present study, an n of 5 in each group would give a confidence interval of 80%. However, for a confidence interval of 95%, an n of 19 would be needed.

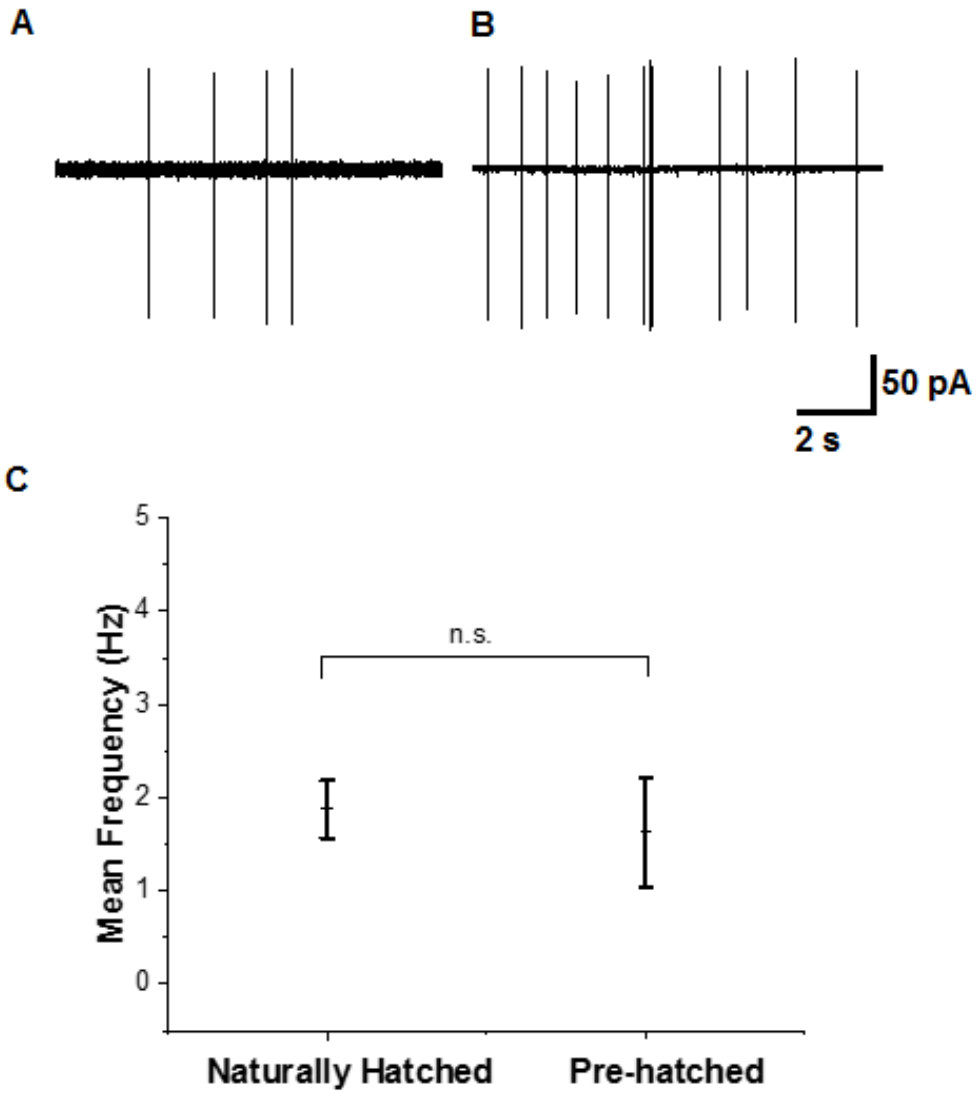

Figure 3.4 Spontaneous activity in naturally *vs* prematurely hatched zebrafish at 2 dpf.

A+B, Two single, 10 second sample traces of extracellular loose-patch recordings obtained from a naturally hatched (**A**, mean ISI = 5192 ms, mean = 0.02 Hz) and pre-hatched (**B**, mean ISI = 606 ms, mean = 1.64 Hz) zebrafish pLLg at 2 dpf. **C,** The mean and SEM of spontaneous activity frequencies of naturally hatched (1.88 ± 0.31 Hz, N = 9) and pre-hatched (1.63 ± 0.59 Hz, N = 6) zebrafish at 2 dpf (two-sample $t(13) = 0.40$, $p = 0.69$) when equal variance is assumed.

A difference in spontaneous firing rates was expected between naturally hatched and pre-hatched zebrafish, as the naturally hatched zebrafish had developed more than the prematurely hatched fish. However, this was not the case and the variation of mean spontaneous firing frequencies was not large at 2 dpf (Figure 3.4C) regardless of hatching. It is possible that any differences between the spontaneous activity of naturally hatched and pre-hatched zebrafish are not large enough to be evident so soon after hatching, unlike the rainbow trout.

3.4.3 Spontaneous activity in early larval stages (<5.2 dpf)

The zebrafish undergoes rapid development from 2-5.2 dpf; therefore, I sought to identify if the spontaneous activity characteristics would alter at an age which the zebrafish is frequently used, to reflect the developing system of the pLL.

Extracellular loose-patch recordings were obtained from the zebrafish pLL until 5.2 dpf. Spontaneous activity is present in the pLLg from 2 to 5.2 dpf (Figure 3.5A). The range of spontaneous firing frequencies increases slightly between 2 to 5.2 dpf. The mean spontaneous firing frequency data had a Spearman correlation coefficient value of $r^s = 0.28$ (Figure 3.5A[$P<0.05$]), which suggests that the mean frequency data follows a slight positive correlation with the age of the zebrafish. A Shapiro Wilk test revealed that the data was not normally distributed; therefore a Kruskall-Wallis test was completed and found that the mean spontaneous firing rates were significantly different at at each age ($\chi^2(14) = 46.23$ $P<0.005$). Therefore, the spontaneous firing rate significantly changes over a 4-day period and the presence of more high-frequency firing increases with age. It hypothesised that the mean spontaneous firing rate increases over time due to increase of hair cells in the pLL.

The spontaneous firing can also be investigated by measuring the inter-spike interval (ISI), which is the time between consecutive spikes. A majority of the mean ISIs occur under 500 ms (43/57) (Figure 3.5B) especially between 100-200 ms, which is in line with recent

findings from other research groups (Song *et al.*, 2018). The highest mean ISI was 3500 ms, which indicates a slow frequency pattern of spontaneous activity. Although the ISIs give a

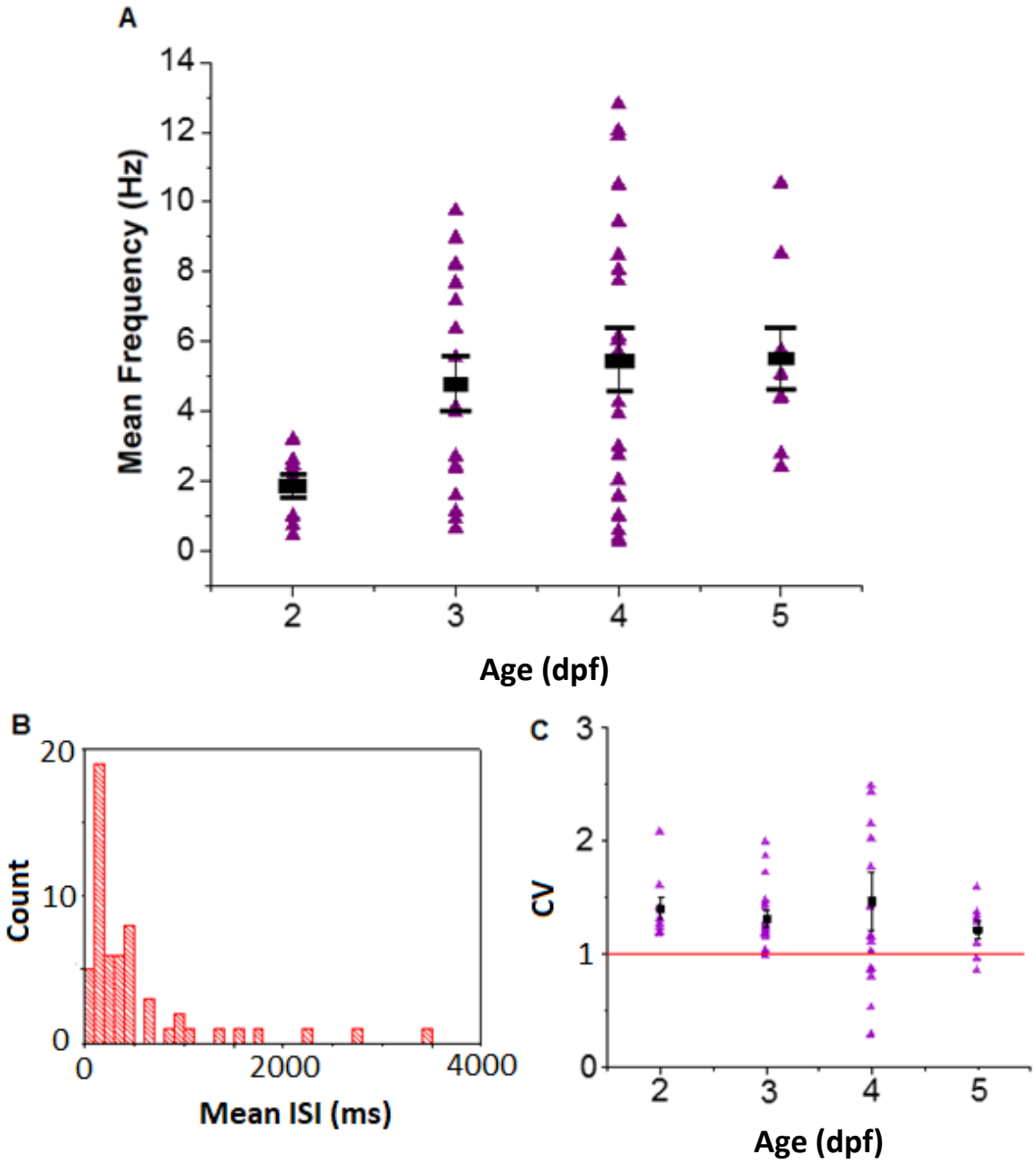

Figure 3.5 Spontaneous activity is present in zebrafish from 2-5.2 dpf

A, The mean frequency of spontaneous activity from each recording (purple) plotted against age with the mean of each age group and standard error (black). 2 dpf (N = 9), 3 dpf (N = 17), 4 dpf (N = 21), 5 dpf (N = 8). The mean duration of all recordings from 2-5.2 dpf was 417 seconds. Spearman correlation coefficient = 0.28, $P<0.05$. Kruskal-Wallis ANOVA $P<0.0005$. **B**, Frequency distribution of frequency of mean inter-spike interval (ms) from all zebrafish aged 2-5.2 dpf with 100 ms bins (mean ISI = 513, N = 57). **C,** Coefficient of variation (CV) plotted against age (dpf). The mean coefficient of variation is not significantly different in any age group. One-way ANOVA, 2 dpf (N = 9), 3 dpf (N = 17), 4 dpf (N = 21), 5 dpf (N = 8).

good indication of how frequent the spikes are, it is important to consider the regularity of the spontaneous spiking as recordings can vary between low and high frequency firing.

The coefficient of variation (CV) is calculated by dividing the standard deviation by the mean inter-spike interval of each recording. The CV is a method used to measure the spread of the ISIs around the mean and therefore the regularity of spontaneous activity firing. The CV of a Poisson distribution is 1; a value <1 is associated with regularity, but > 1 is associated with irregularity and possibly burst-like behaviour (repetitive bouts of high frequency firing followed by intervals of inactivity) (Jones & Jones, 2000; Johnson *et al.*, 2011). All afferent fibre recordings (2-5.2 dpf) have a CV of above 0.28, with the highest recorded value of 2.47 (Figure 3.5C). The CV does not vary significantly across age groups (2-5 dpf, $\chi 2(3) = 0.980, p=0.806$, Kruskal-Wallis ANOVA) and at each age the mean CV values is significantly different to a CV value of 1 (2, 3 and 5 dpf = $P \leq 0.05$; 4 dpf = $P=0.06$ [Wilcoxon signed rank test]). These findings indicate that spontaneous activity obtained from the zebrafish pLL is irregular, and due to the presence of high CV values, may even include burst-like behaviour. Burst-like spontaneous activity has been previously found in other teleost fish afferents such as the midshipman fish (*Porichthys notatus*) (Weeg & Bass, 2002) but has not been reported in the zebrafish larvae before and therefore recordings obtained with burst-like activity were initially disregarded as were thought to be an anomaly. However, after further investigation were seen to be a frequent occurrence and were therefore the recordings with burst-like activity were obtained and included in analysis.

3.4.4 Agarose does not interfere with extracellular recordings from the pLL

To ensure that the use of the new preparation with agarose (for zebrafish >5.2 dpf) was not interfering with the extracellular recordings, the experiments performed in agarose were compared to those in which the zebrafish was pinned down at the age of 4 dpf. It was speculated that the agarose may deflect the cupula, and thus the hair bundles, to cause mechanotransduction depending on the restraint in the agarose.

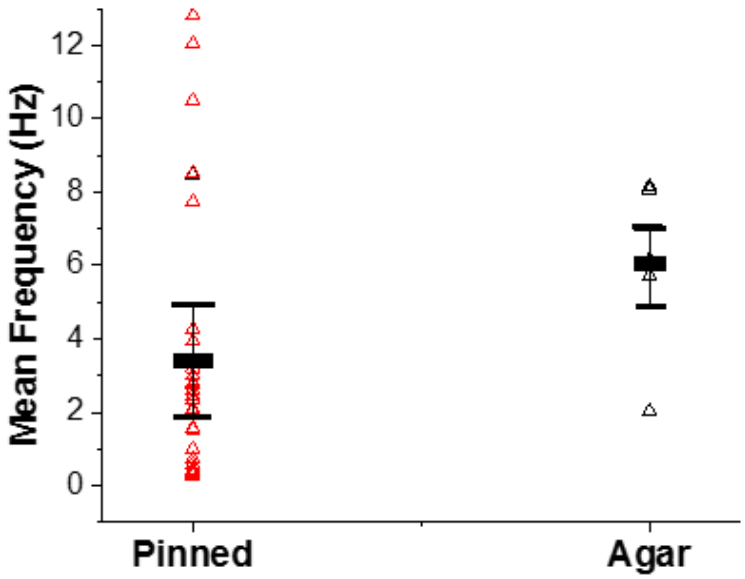

Figure 3.6 Comparison of pinned Vs agarose restraint in 4 dpf zebrafish

The mean spontaneous firing rate of each recording in 4 dpf zebrafish either restrained by pins (N = 26) or in agarose (N = 6). The mean is shown in black with standard error bars. Despite the variation seen in the means between the two methods, a Mann-Whitney test revealed the two groups were not significantly different (U = 119, P≥0.05).

When 4 dpf zebrafish were restrained by pins or agarose, it was found that the mean spontaneous firing rates obtained from recordings in either condition produced no significantly different results (Figure 3.6). The mean spontaneous firing frequency of recordings obtained from pinned zebrafish was 3.5 ± 0.31 Hz, whereas from the agarose restrained zebrafish it was 6.6 ± 0.59 Hz, which although appear dissimilar, the Mann-Whitney test revealed that the two groups were not significantly different. The similarities in spontaneous activity from both preparations suggest that agarose does not interfere with the recordings and mechanotransduction caused by deflections of the kinocilia are not evident. A post-hoc power analysis revealed that on the basis of the mean, the n numbers in each condition were sufficient to give a confidence interval of 80%. However, higher n numbers would be needed to give an ideal confidence interval of 95%.

Although agarose was effective at restraining the 4 dpf zebrafish, it was more suited to older zebrafish (>5.2 dpf) due to their size – removing agarose was technically challenging in younger zebrafish, especially at embryonic stages (from 2-5 dpf zebrafish are 3.1-3.9 mm in length, by 20 dpf zebrafish are 6.2 mm (Kimmel *et al.*, 1995)). Therefore, it was decided that the agarose preparation was not suitable for zebrafish <5.2 dpf.

3.4.5 Spontaneous activity persists into mid-larval stages

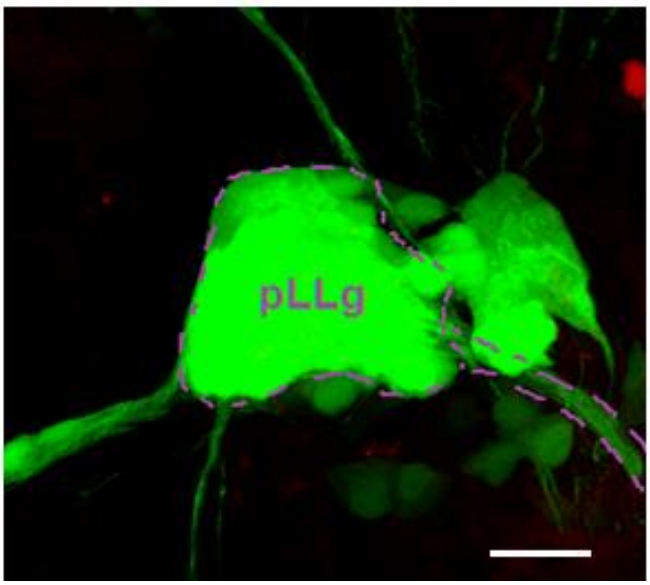

Figure 3.7 GFP in the pLLg

Flattened Z-stack of pLLg from a 12 dpf NeuroDxR-GECO zebrafish, displaying pLLg (GFP, outlined in purple) and various branches. Scale bar = 20 µm.

As the zebrafish grows and develops, the ganglion becomes harder to visualise with a x60 objective as it becomes located more deeply in the fish; and thus, obtaining successful extracellular recordings with the recording electrode was harder over time. Extracellular recordings from the pLLg were obtainable until 18 dpf. The zebrafish uses the gills to breathe after 21 dpf, therefore, intubation would have been required to keep the zebrafish alive if it were necessary to obtain recordings at older ages. To ensure visualisation of the ganglion was optimal under our recording conditions, I used the zebrafish strain TgBAC(*neuroD*:EGFP)

as the enhanced green fluorescent protein (GFP) is expressed in cells with *NeuroD*, a proneural gene (Obholzer *et al.*, 2008; Ochocinska & Hitchcock, 2009) that is prominent in afferent fibres (Figure 3.7). Visualisation of the pLLg with GFP allowed for easier mapping of location of the ganglia over time and is prominent even in older zebrafish with thicker tissue.

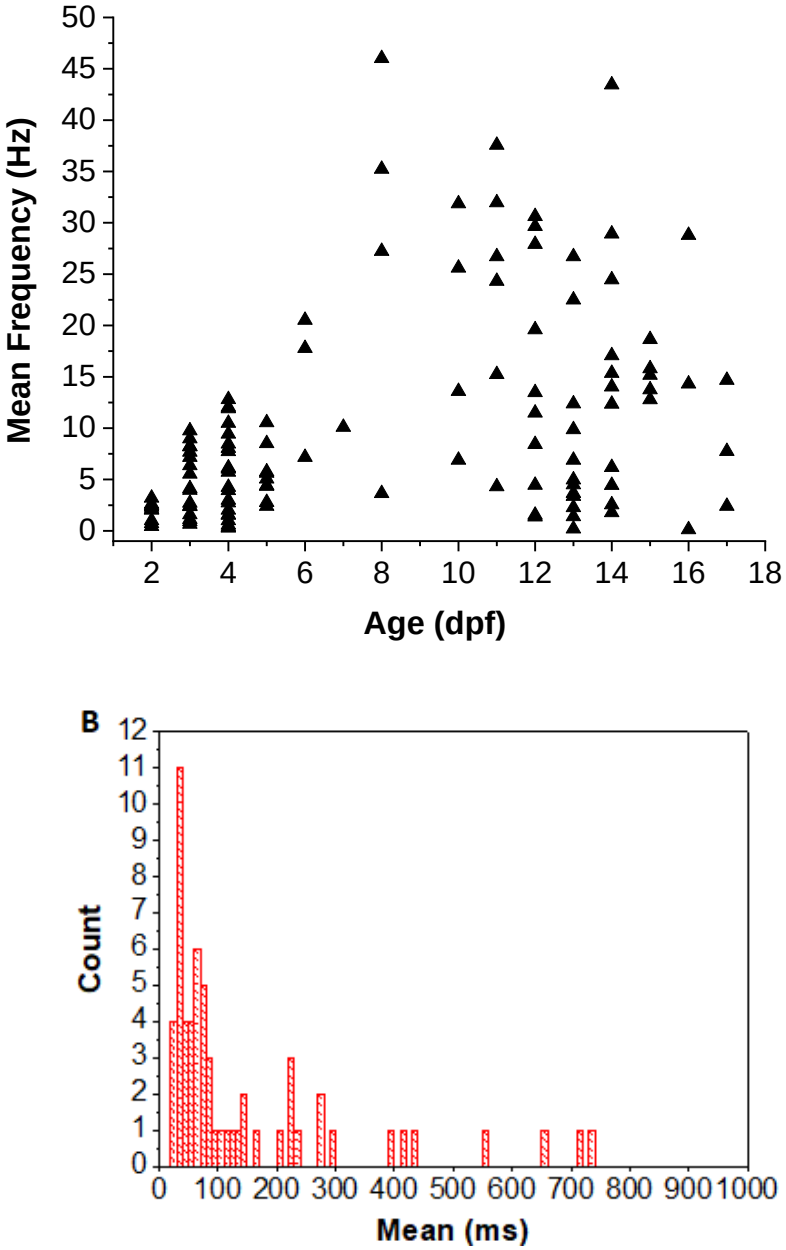

Figure 3.8 Spontaneous activity persists into mid-larval stages

A, The mean spontaneous firing rate of each recording. Spearman correlation coefficient = 0.44 ($P<0.05$).
B, A histogram of the mean inter-spike intervals from zebrafish aged 5.2-18 dpf (ms), N=60.

In the afferent fibres of zebrafish aged > 5.2 dpf, there was a broader range of the mean spontaneous firing frequencies compared to early-larval stages (Figure 3.8A). Although some recordings in > 5.2 dpf afferent fibres display a higher frequency compared to the recordings from < 5.2 dpf zebrafish, lower frequencies are still evident in the older fish. A diversity of mean firing frequencies is evident in the afferent fibres which cannot easily be sorted into high and low firing rates. The mean duration of spontaneous activity in recordings from fish > 5.2 dpf was 516.85 seconds, emphasising that recordings obtained during restraint in agarose are stable. The mean ISIs for zebrafish > 5.2 dpf is similar to < 5.2 dpf, most mean ISIs are under 500 ms (56/60) (Figure 3.8B); however, longer ISIs were more likely to be seen in recordings from <5.2 dpf zebrafish (Figure 3.5B). The lowest mean ISI found in the zebrafish pLL was 21.73 ms, which is similar to that seen in the *Xenopus laevis* lateral line, where no consecutive spikes are seen closer than 20 ms apart (Harris & Milne, 1966). This finding raises similarities between the kinetics of the lateral line of both species and may give an insight into the refractory period of the afferent fibre.

The mean spontaneous firing frequency data was not normally distributed (Shapiro-Wilk $P<0.005$) and had a Spearman correlation coefficient value of $r_s = 0.44$, $P<0.0005$ (Figure 3.8A), which suggests that the mean frequency data follows a slight positive correlation with the age of the zebrafish.

The CV of recordings from 6-17 dpf appear to mostly be irregular as 72% of the values are 1 or above (Figure 3.9A); therefore, the inter-spike interval is equal to a completely randomly distributed process (Softky & Koch, 1993). The mean CV value from all recordings is 1.3. A CV value <1 is thought to represent low firing variability; therefore, CV values >1 are often associated with burst-like behaviour, which has been rarely observed in the zebrafish pLL and not quantified extensively. To investigate this further, I analysed the spontaneous spiking of the 5 recordings with CV values that exceeded 2. Some of these recordings with high CV values appeared to have burst-like activity (Figure 3.9 B+C), which confirmed that burst-like activity is present within the pLL. Although some fibres display a CV higher than 1, and therefore are more likely to have bursting behaviour, bursting behaviour may be present in recordings of spontaneous activity with lower CV values if the bursts are of regular

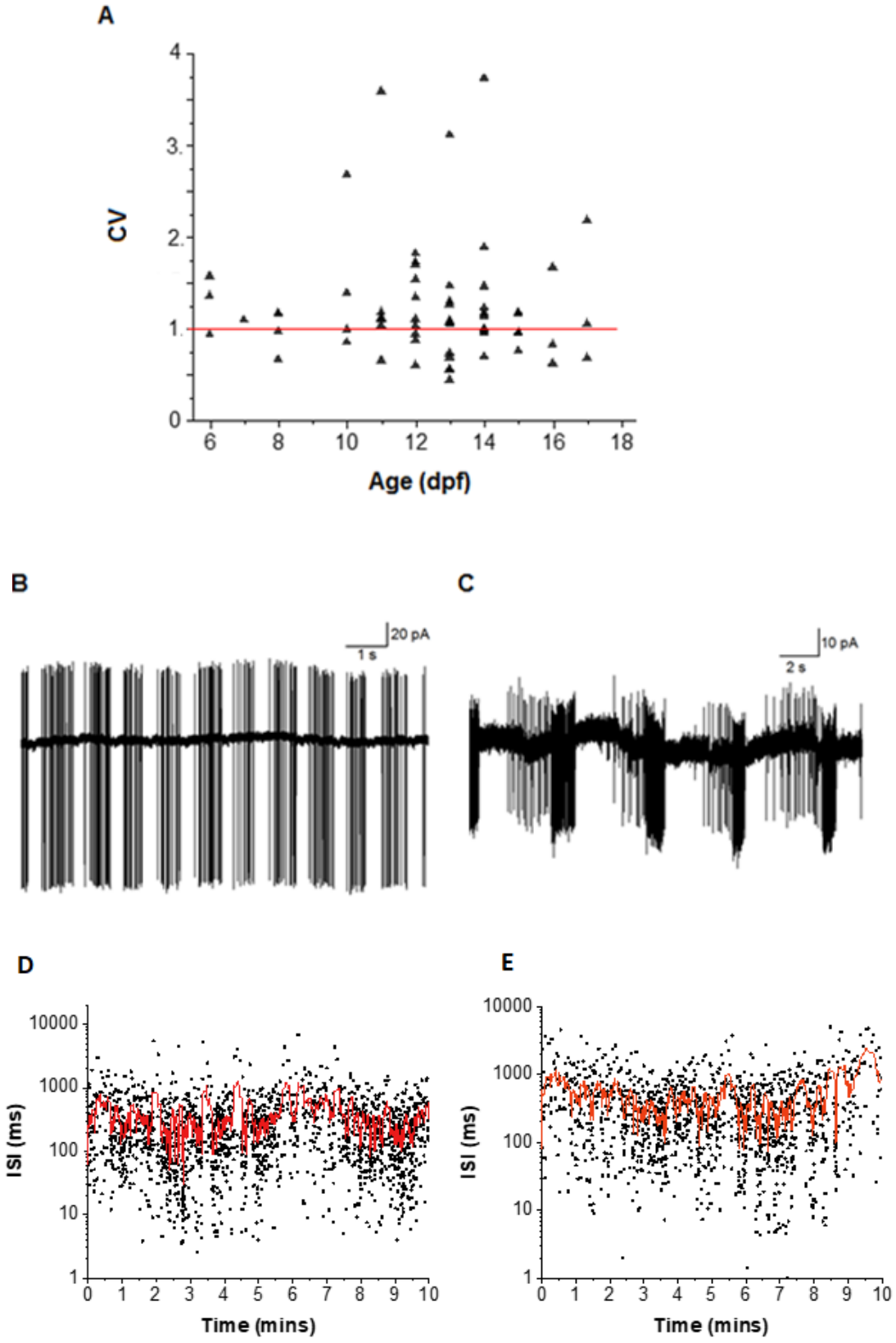

Figure 3.9 Discharge variability

A, The coefficient of variation of each recording of spontaneous activity between 6-17 dpf. The mean CV is 1.23 ± 0.08. Most CV values are above 1 (37/62). **B+C**, Samples of traces with CV values of >2. (B = 2.67, C = 3.59). **D+E**, ISIs plotted against time of two recordings from two 5 dpf zebrafish. Black dots represent an ISI of the recording, the red line represents the moving average.

recordings of spontaneous activity (Figure 3.9 B+C); therefore, a more extensive method of analysis would need to be employed to investigate this occurrence further. The ISIs of two recordings were plotted against time. Areas of high activity can be spotted as the ISIs are clustered together at low ms values. The moving average of these graphs give an insight into burst behaviour as burst-like activity reduces the value of the moving average (for example, at 3 minutes in 3.9D, and at ~9 minutes in 3.9E). given more time, further analysis of the ISIs may allow us to see more patterns in the burst behaviour of spontaneous activity.

All the ISIs of a recording can be plotted against the preceding ISI to show the correlation between successive intervals. As expected from the varying ISI values across different ages, even ISI correlation plots display different relationships. Figure 3.10A-D shows

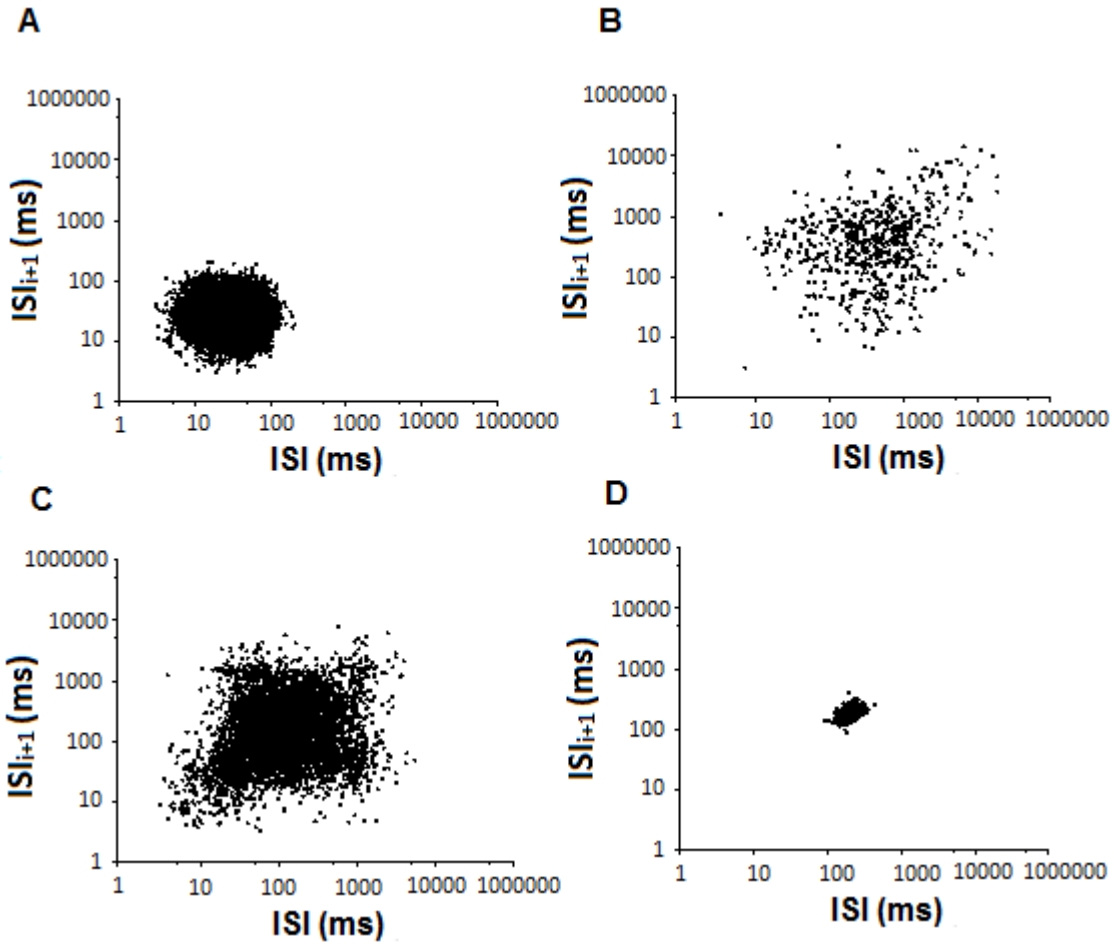

Figure 3.10 Inter-spike interval histograms.

A-D, Four correlational plots of the ISI against the preceding ISI each from one recording from four 12 dpf zebrafish.

four dissimilar ISI correlation plots obtained from different zebrafish at 12 dpf. Figure 3.10A depicts a high frequency cell of high regularity, whereas Figure 3.10B shows a lower frequency cell with irregular activity. Figure 3.10C displays a recording from a cell with a higher frequency but also higher irregularity, and Figure 3.10D has a lower frequency and higher regularity. It is possible that the pLL has not developed spiking patterns that could be used to categorise the afferent fibres into groups, unless there are many groups. These findings bring into question whether afferent fibres can be categorised based on firing patterns, as previously postulated by Liao & Haehnel (2012), or whether individual afferent spontaneous firing frequencies would change over time. More analysis of the ISI plots over time could help to identify if there were many groups of afferent fibres based on spiking patterns of the spontaneous activity.

3.5 Methodological considerations

3.5.1 Location of corresponding neuromast

During development, the quantity of neuromasts in the pLL increases. Using the extracellular loose-patch technique to acquire spontaneous activity data, it is not known what corresponding neuromasts are being innervated from the afferent fibre being recorded from. As a more mature phenotype is seen in the hair cells of the juvenile zebrafish (Olt *et al.*, 2014), it is possible that neuromasts that are born earlier are more mature and therefore have a different spontaneous firing rate as opposed to neuromasts that are born later and have more immature hair cells.

This may explain the larger variation of spontaneous activity frequencies seen in older zebrafish (>5.2 dpf) as it is not possible to discern recordings obtained from afferent fibres that innervate older or younger groups of hair cells in different neuromasts.

3.5.3 Recording acquisition

Although extracellular loose patch recordings measure the cumulative output from hair cells of approximately one or more neuromasts, whole-cell patch clamp recordings of hair cells are more suitable to give a physiological readout of individual hair cell activity. Whole-cell patch clamping would provide more information about input fluctuations and cellular properties that may affect the spontaneous firing rate. However, despite the benefits of whole cell patch clamp, it is a more challenging and time-consuming technique but would investigate other questions about ion channel composition.

Another method which may have been useful to investigate spontaneous activity is calcium imaging. This method is useful for understanding the calcium fluctuations in the cell (to be able to visualise spontaneous activity). Calcium imaging is not as invasive as extracellular loose-patch electrophysiology as no electrodes are used to penetrate the zebrafish for data acquisition and the zebrafish can be restrained in agarose at all ages; therefore, reducing harm from restraining the zebrafish by pins. However, the calcium imaging may not be possible in older zebrafish as the thickness of the tissue may skew the fluorescence observation.

3.6 Summary and Discussion

I have investigated the zebrafish pLL afferent fibre spontaneous activity across embryonic and larval development and found:

- Spontaneous activity in the afferent fibres of the pLL is present from 2 dpf.
- Pre-mature hatching of the zebrafish from the chorion does not affect spontaneous activity in the afferent fibres at 2 dpf.
- From onset of spontaneous activity to the age of protection (5.2 dpf), the variation of spontaneous firing frequencies increases.

- Agarose is a good method to restrain zebrafish >5.2 dpf for extracellular recordings from the pLLg.
- The range of spontaneous firing frequencies >5.2 dpf continues to become more varied with age.

Zebrafish have been proven to be a good animal model in which to indirectly study hair cells in an intact and *in vivo* system. My protocol for restraining >5.2 dpf zebrafish causes minimal damage to the zebrafish and has less interference from anaesthetics and paralytic agents. I have efficiently characterised the broad profile of spontaneous activity in the zebrafish pLL from onset at 2 dpf, to 18 dpf when the zebrafish is approaching juvenile stages and is more mature.

Although in the literature it has been previously suggested that the zebrafish pLL is functional after 48 hpf (Metcalfe *et al.*, 1985; Kimmel *et al.*, 1995; Lopez-Schier *et al.*, 2004), this has not been confirmed through electrophysiology until now. If spontaneous activity is present in the pLLg, this indicates that the hair cells are forming viable connections with the afferent fibres of the pLL, as this activity is not suggested to be intrinsic to the afferent fibre but is due to the release of glutamate from the hair cell in absence of external stimulation (Trapani & Nicolson, 2011). It is important to test the viability of the connections through electrophysiology as it gives more information about cell physiology than other methods used to examine hair cells such as dye labelling.

A variation of mean spontaneous firing frequencies is seen in zebrafish <5.2 dpf over time. This was expected as it is likely to reflect the fast development of the pLL, including increased quantity of hair cells and afferent fibres. The increase in range of frequencies was not correlated with an alteration with the CV with age, suggesting that the spontaneous activity may increase in firing frequency, but remains as regular/irregular as younger zebrafish. I suggest that a larger range of frequencies are seen in the spontaneous activity of zebrafish >5.2 dpf due to the increased number of hair cells and therefore input into the afferent fibres at older ages. Hair cells in neuromasts that originated from the first outgrowth

of cells of the pLL are likely to be older and therefore display a more mature phenotype and also be more numerous (Olt *et al.*, 2014). The maturation of the basolateral membrane potential may also lead to a more mature spontaneous activity profile in older zebrafish which is not seen in early-larval stages and thus a refined pattern of spontaneous activity that has a higher firing frequency. Differences in spontaneous rates between <5.2 dpf and >5.2 dpf zebrafish are therefore correlated with maturational refinements in the pLL, as seen in other species such as the barn owl (Koppl, 1997) and pigeon (Gummer, 1991). Higher spontaneous firing frequencies are associated with more sensitive fibres that need less stimulus to elicit an action potential (Liao & Haehnel, 2012). This indicates that the zebrafish may be able to perceive weaker water stimuli which has behavioural benefits.

The Pearson correlation coefficient confirmed a low positive association between age of the zebrafish and mean spontaneous firing rate, suggesting that as the zebrafish ages, the pLL afferent fibres are more likely to have a higher mean spontaneous firing rate. The k-means analysis sorted the mean frequencies of the spontaneous activity recordings into two groups. Two groups of afferent fibres were also suggested by previous literature as birth order influences the thresholds and spontaneous firing rates of the afferent fibres (Liao & Haehnel, 2012; Pujol-Marti *et al.*, 2012). Although the k-means was successful into sorting the mean firing rates into two groups, the developmental reason for this clustering is not clear. However, the mean spontaneous firing rates from zebrafish <5.2 dpf reside in the same group. Thus, it may be that the mean values <5.2 dpf that have also been sorted into this group represent recordings from afferent fibres that are younger or are contacting less hair cells as the frequency of spontaneous activity is lower.

It is not known if the variation of mean spontaneous firing rates would continue to become more diverse with further aging of the zebrafish. I would assume that there is a limit at which spontaneous activity does not increase in frequency any further, as increases in hair cell quantity slows down after the formation of stitches and scales (Williams & Holder, 2000; Gompel *et al.*, 2001). Zebrafish must be able to distinguish the difference between evoked and spontaneous activity in order to perform the correct tasks in response to an evoked stimulus and so spontaneous activity cannot exceed a certain frequency. Another

spontaneous activity property that I have identified is the burst-like activity present in some recordings. Spontaneous activity firing in bursts has not been reported in the zebrafish pLL in the literature, and therefore the function is not known. I did not investigate the function of the burst-like behaviour; however, in the rat visual system bursting is evident in the ganglion neurons before the onset of vision for refinement of synaptic connections to higher order projections (Galli & Maffei, 1988). Thus, it is hypothesised that the bursting may be present at the onset of the afferent fibre forming a contact with the hair cells in order to refine higher order contacts and improve somatotopy. It would be interesting to identify if there was a pattern in bursting behaviour in the pLL to further understand the function of the behaviour.

3.7 Future work

To understand the ranges of spontaneous firing frequencies seen in older zebrafish (>5.2 dpf) further, labelling the afferent fibre would allow us to quantify the amount of hair cells needed to elicit the frequency of spontaneous activity as the number of hair cells an afferent fibre innervates could be correlated to the spontaneous firing rate. As spontaneous activity in the pLL is thought to be a cumulative signal from many hair cells, how much effect can one hair cell's spontaneous release of neurotransmitter elicit spontaneous activity in the corresponding afferent fibre? Previous work has stimulated the hair cells of a neuromast whilst consequently recording from an afferent fibre with extracellular electrophysiology in larval fish to identify which neuromast the afferent fibre innervates (Trapani & Nicolson, 2011). However, as the >5.2 dpf zebrafish were embedded in agarose in my preparation and the number of neuromasts are plentiful at older ages, this could be very challenging and would involve a change in the method.

It would be interesting to also investigate if the properties of the hair cell influence the spontaneous rate in the afferent fibre. This could be conducted by whole cell patch clamp of a hair cell and simultaneous extracellular loose-patch recording of the afferent fibre in the ganglion or in the pLL nerve itself. From this we could conclude if the immature hair cells are

innervated by the afferent fibre, and whether the different levels of hair cell maturity cause a change in the spontaneous activity.

3.8 References

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Chapter 4 The effects of damage to the posterior lateral line afferent fibres by copper treatment

4.1 Introduction – Regeneration of hair cells in the zebrafish pLL

Zebrafish, like amphibians and birds, have an extraordinary capacity to regenerate hair cells after a multitude of insults (Williams & Holder, 2000). Despite the morphological and functional similarities of hair cells of the zebrafish posterior lateral line (pLL) and hair cells of the inner ear of mammals (Pichon & Ghysen, 2004; Nicolson, 2017), the human inner ear is not capable of hair cell regeneration. Therefore, after insult to the ear - by noise trauma, chemotherapy agents or aging - hearing loss is usually permanent.

Regeneration of hair cells in the zebrafish pLL has been previously researched mainly to understand if it is possible to prevent toxicity of compounds that are toxic to human hair cells. However, there is still a lot we don't know about the damage and regeneration process, such as how certain ototoxic compounds enter hair cells and cause hair cell death (Coffin *et al.*, 2013). Previous work investigating hair cell regeneration in the zebrafish pLL has mainly focussed on transgenic fish with optical reporters in the hair cells or dyes to observe the process, such as FM1-43 or YO-PRO1, that label hair cells (Hernandez *et al.*, 2006; Ma *et al.*, 2008; Owens *et al.*, 2008). There are limitations to imaging studies using dyes and transgenic fish as it is only possible, for example, to visualise morphology and basic mechanotransduction (MET) channel viability. Therefore, it is not clear what the biophysical properties of the regenerating hair cells are and the impact of regeneration on the afferent fibre spontaneous activity. Few other methods are available in zebrafish that allow us to functionally assess the hair cells and afferent fibres during regeneration. In mammals we are able to perform Auditory Brainstem Responses (ABRs) and Distortion Product Otoacoustic Emissions (DPOAEs) to examine hair cell functionality and auditory neuronal connectivity in a non-invasive manner, which is not possible in the zebrafish. However, the zebrafish pLL is an excellent system in which to research regeneration of hair cells *in vivo*.

The hair cells of the zebrafish have kinocilia that are ~14 µm in length when mature (Kindt *et al.*, 2012), which are possible to be visualised with a x60 objective, whereas the stereocilia are much shorter (around 1.5 µm in length (Kindt *et al.*, 2012)). Therefore, the

presence of stereocilia isn't usually easily visualised with DIC imaging even under high magnifications. As the kinocilia can take days to reach normal lengths during regeneration (Linbo *et al.*, 2006), it is hard to visually differentiate hair cells from supporting cells at early stages until the kinocilia are longer; therefore, it is hard to determine hair cells at early stages of regeneration or when they become functional (Wang *et al.*, 2017). There are strains of zebrafish which have genetically inserted hair cell-specific markers that allow for visualisation of hair cells prior early on in development. The use of the Tg(*myo6b*:R-GECO) zebrafish strain is used primarily for the genetically encoded calcium ion indicator (GECI) function; however, it is also useful due to the red fluorescent protein tag found specifically in cells expressing *myosin6b*. *Myosin6b* is one of two isoforms of the unconventional *myosin6* gene that is present in the hair cells (Avraham *et al.*, 1995). Expression of *myosin6b* in the zebrafish pLL is seen as early as 24 hours post fertilisation (hpf) in the precursors to the hair cells in the otic placode by in situ hybridisation; therefore, hair cells are visible (Seiler *et al.*, 2004) before they are functional (see Chapter 3 for initiation of function of the lateral line) which avoids the need of a functional MET channel to label the hair cells which is required for most hair cell dyes.

Although focus has mainly been directed towards the regenerating hair cells, reforming connections with the afferent fibres is also an important factor to consider in regeneration. Afferent fibres reliably form connections with hair cells of the same polarity (Obholzer *et al.*, 2008), even when the hair cells are regenerating (Nagiel *et al.*, 2008) allowing the zebrafish pLL to continue to function as it was before hair cell damage. Previous research has found that when larval zebrafish (3 dpf) are treated with 10 µM copper sulphate ($CuSO_4$), progenitors start to appear within 6 hours hpt (Nagiel *et al.*, 2008), which is similar to regeneration of hair cells after neomycin treatment (an ototoxic compound frequently used for zebrafish regeneration studies) (McHenry *et al.*, 2009). Hair cells become polarised within 12 hours after copper treatment and by 48 hpt it is suggested that all hair cells of one polarity are contacted by the afferent fibre (Nagiel *et al.*, 2008). This research suggests that the regeneration process is rapid. However, other groups speculate that it may take up to 72 hours for hair cells of a neuromast to completely regenerate (Williams & Holder, 2000; Harris *et al.*, 2003; Lopez-Schier & Hudspeth, 2006). Speculation about hair cell regeneration is

usually based on morphological appearances of hair cells; therefore, we do not know exactly when the hair cells and afferent fibres form functional synapses.

Behavioural studies have been conducted to correlate hair cell presence with functional output (Wang *et al.*, 2017). During regeneration after neomycin treatment it is seen that the more hair cells that are present, the larger the strength of the startle response (McHenry *et al.*, 2009), suggesting that the increase in hair cells causes an increase in sensitivity or efficiency of the pLL-related behavioural responses. Rheotaxis, another pLL-related behaviour, is reported to return to normal in larval zebrafish that have been exposed to neomycin by 48-52 hpt even though hair cell regeneration is not seen to be complete until 72 hpt (Ma *et al.*, 2008). However, sensitivity to perform an escape response to stimuli is not increased by increasing hair cell number in a neuromast after 4 hair cells are present (McHenry *et al.*, 2009). This finding is surprising and questions why the zebrafish have +20 hair cells per neuromast at older ages (Olt *et al.*, 2014) if it doesn't functionally help to complete behaviours more efficiently. As these findings were found in 5 dpf zebrafish, it is possible that as the number of hair cells increases, the proportion of cells needed to elicit an appropriate startle response also increases; therefore, the hair cells quantity in older fish is not unnecessary for behaviour. The larger quantity of hair cells present in neuromasts at older ages may also increase sensitivity, therefore it is important to compare the regeneration differences between developmental stages to further answer these questions.

Other sensory inputs, such as the visual system, contribute to the behaviour seen in rheotaxis studies during regeneration. Therefore, it is thought that electrophysiology could help in investigating the functionality during regeneration more thoroughly and without disturbance from other sensory modalities. It is known that destroying the hair cells of the auditory or vestibular system eradicates spontaneous activity in birds and mammals (Kiang *et al.*, 1976; Salvi *et al.*, 1994; Muller *et al.*, 1997), which confirms that the hair cell is responsible for the spontaneous activity seen in the afferent fibre. As previously discussed in chapter 3, spontaneous activity is present in the afferent fibres of the pLL of 2-18 dpf zebrafish. The frequency of spontaneous activity in afferent fibres varies more greatly over time which is suggested to be due to the increased number of hair cells which some afferent

fibres receive input from. Hair cells have a constant turnover throughout the life of the zebrafish (Williams & Holder, 2000), but as neuromasts and afferent fibre connections are established, it is thought that spontaneous activity would remain stable as hair cell turnover is a constant and slow process (Cruz *et al.*, 2015). However, at present no work has been conducted to investigate if spontaneous activity alters in response to renewal or regeneration of hair cells. Therefore, we do not know at what time point the hair cell is re-innervated by the afferent fibre and spontaneous activity returns, or at what frequencies spontaneous firing occurs during regeneration.

As previously mentioned, one compound that has been used in regeneration studies in zebrafish is copper. Copper is an important element for life; however, it is also a known toxic compound in many different forms. Research in many aquatic organisms including the zebrafish has found copper to be toxic to the gills, liver (Craig *et al.*, 2007) and lateral lines (Hernandez *et al.*, 2006; Linbo *et al.*, 2006; Mackenzie & Raible, 2012). Despite the toxicity, copper also has beneficial properties, such as it is used widely for its antimicrobial properties and in many aspects of shipping and aquatic mining. Antifouling paints which contain copper in the shipping industry are used to prevent accumulation of sea organisms on the hull of ships; however, the copper coating eventually wears off the ships and into the water which is becoming an increasing risk to aquatic life (Tornero & Hanke, 2016). Previous research has found that after copper oxide nanoparticle exposure in sea urchins, spermatozoa see a decrease in the mitochondrial membrane potentials, increased fragmentation and elevated reactive oxygen species quantities (Gallo *et al.*, 2018). When copper oxide nanoparticles were compared to $CuSO_4$ in the common carp, *Cyprinus carpio*, both forms were toxic to the carp especially in the liver and kidneys. However, $CuSO_4$ is dissolved and therefore can cause more severe damages than copper oxide nanoparticles (Hoseini *et al.*, 2016). The developmental effects of copper oxide nanoparticles have been investigated in zebrafish and have also found that there is an increase in oxidative stress, like in the sea urchin spermatozoa (Ganesan *et al.*, 2016) which could be a possible mechanism for hair cell damage.

It is evident that $CuSO_4$ is toxic to zebrafish; however, how $CuSO_4$ enters and accumulates in hair cells is not clear. The hair cells of the zebrafish pLL are exposed to

external fluids, allowing for easy entry of compounds into the hair cell through bath application (Hernandez *et al.*, 2006). Many ototoxic agents that are studied in zebrafish are investigated to find out how to block the entry into the hair cells by application of a competitive non-ototoxic drug. Many compounds enter and accumulate through the mechanotransduction channel (MET) (Gale *et al.*, 2001), as it is a large pored non-selective ion channel (Corey & Hudspeth, 1979; Farris *et al.*, 2004), or through apical endocytosis (Richardson *et al.*, 1997). It has been speculated that $CuSO_4$ enters cells through copper transporters (CTR) (Kaplan & Maryon, 2016), which are expressed in zebrafish from embryogenesis (Mackenzie *et al.*, 2004). Interestingly, the platinum-based chemotherapy agent cisplatin also is thought to enter hair cells through CTRs (Ishida *et al.*, 2002). However, other mechanisms of uptake are likely to exist for $CuSO_4$ and cisplatin. From concentrations as low as 10 µM $CuSO_4$, hair cells die through a necrosis pathway and when the mechanotransduction channel is blocked by amiloride, hair cell death still occurs, suggesting that the mechanotransduction channel either isn't a mechanism of entry or is not the predominant route for $CuSO_4$ (Olivari *et al.*, 2008).

As copper can be toxic at high concentrations it is important for cells to be able to regulate copper levels; therefore, mechanisms of efflux are also important. Copper efflux has been researched in lower organisms such as bacteria. Intracellular concentrations of copper in *Escherichia coli* are thought to be regulated by an ATP-dependent proteolysis mechanism (Bittner et al., 2017). Copper efflux has also been investigated in human cells, as copper transporters have been implicated in certain cancers (Howell et al., 2010). The ATPases ATP7A and ATP7B sequesters and causes efflux of copper from mammalian cells (Lutsenko & Petris, 2003) which has been consequently identified in rat cochlear cultures (Ding et al., 2011) but not investigated in the zebrafish lateral line.

4.2 Hypotheses and aims

The first aim of this chapter was to establish a protocol in which hair cells could be ablated and regeneration could be mapped both visually and in combination with electrophysiology in early-larval (3-5.2 dpf) and mid-larval (12-15 dpf) zebrafish.

The second aim was to identify when the hair cells start to regenerate and compare this to the spontaneous activity found in the afferent fibre.

The third aim was to investigate whether development of the pLL had any effect on regeneration of hair cells and spontaneous activity properties by comparing early-larval and mid-larval stages.

It is expected that hair cells of the pLL will regenerate within 48 hours as shown previously (Hernandez *et al.*, 2006) and will hence be functional at this stage through presence of spontaneous activity in the pLLg, like is seen in embryonic development (chapter 3). It is not known when the hair cells become functional during regeneration and whether they do so prior to forming a synaptic connection with the afferent fibre.

I hypothesised that the spontaneous activity will return as the hair cells regenerate and will not reach similar frequencies to control animals until hair cells of treated fish have reached the same numbers as control neuromasts. As the hair cells become more mature and numerous, the spontaneous activity properties will alter, as is seen during early stages of the pLL development (detailed in chapter 3). Therefore, zebrafish treated at older ages may take longer for spontaneous activity to reach similar frequencies as age-matched controls.

4.3 Methodology

Full details of the methodology used for this chapter is detailed in chapter 2. Briefly I will explain the rationale for the process.

In order to identify hair cells during regeneration, a zebrafish strain (Tg(Myo6b:R-GECO) was used with a genetically encoded hair cell marker. Kinocilia and stereocilia are absent in the neuromasts of copper treated neuromasts (Linbo *et al.*, 2006) and therefore, it is important to be able to see the hair cells prior to kinocilia development to be able to differentiate them from supporting cells. The Tg(Myo6b:R-GECO) zebrafish were outcrossed with TgBAC(NeuroD:EGFP) through pair mate for visualisation of the regenerating hair cells (red), afferent fibres and pLLg (green). These fish produced viable young and displayed no phenotype. The regeneration of hair cells in the Tg(Myo6b:R-GECOxNeuroD:EGFP) strain is confirmed after copper treatment at 3 dpf through the presence of red hair cells due to the expression of R-GECO. The hair cell specific promoter *myosin6b* ensures that the R-GECO is expressed early on in the hair cell development (even before rearrangements of the cells after mitosis)(Mirkovic *et al.*, 2012), which allows for easier hair cell counting if the hair cells are visible but do not yet obtain the kinocilia.

$CuSO_4$ was used as the ototoxic agent to ablate hair cells as no systemic effects are seen in larvae exposed to $CuSO_4$ until a higher dose of 50 µM is used (Hernandez *et al.*, 2006). The protocol had also been previously tested (Dr Katie Kindt, Bethesda, USA). Previous research also suggested that neurites of the afferent fibre - that are located below hair cells - were unaffected by $CuSO_4$ treatment (Hernandez *et al.*, 2006).

I compared the spontaneous activity at early- and mid- larval stages of development to identify if the number of hair cells present in a neuromast would influence the spontaneous activity. Early-larval zebrafish were treated with copper at 3 dpf as recovery was expected within the age of protection (48 hpt) and spontaneous activity could be obtained using existing protocols. Mid-larval zebrafish (treated at 12 dpf) were chosen as a good comparison

to the early-larval zebrafish for $CuSO_4$ treatment, as this is at an age where the zebrafish are more mature and have a higher quantity of hair cells per neuromast, but extracellular loose-patch recordings are not difficult to obtain whilst in the agarose due to tissue density.

Zebrafish of either age group were exposed to 10 µM $CuSO_4$ for two hours in an incubator, washed and returned to fresh E3/aquarium tank. At 0 hpt, a few zebrafish from each group were culled and fixed in 4% paraformaldehyde (PFA) for confocal microscopy to confirm absence of hair cells. Zebrafish were prepared for electrophysiology as detailed in previous chapters (2+3) at various time points during regeneration. After extracellular loose-patch recording, the zebrafish were culled and fixed for confocal microscopy. Z-stacks and images were obtained using confocal microscopy for hair cell quantification from neuromasts L1-5. Three neuromasts were imaged from each fish and a preference was made for neuromasts more anteriorly located.

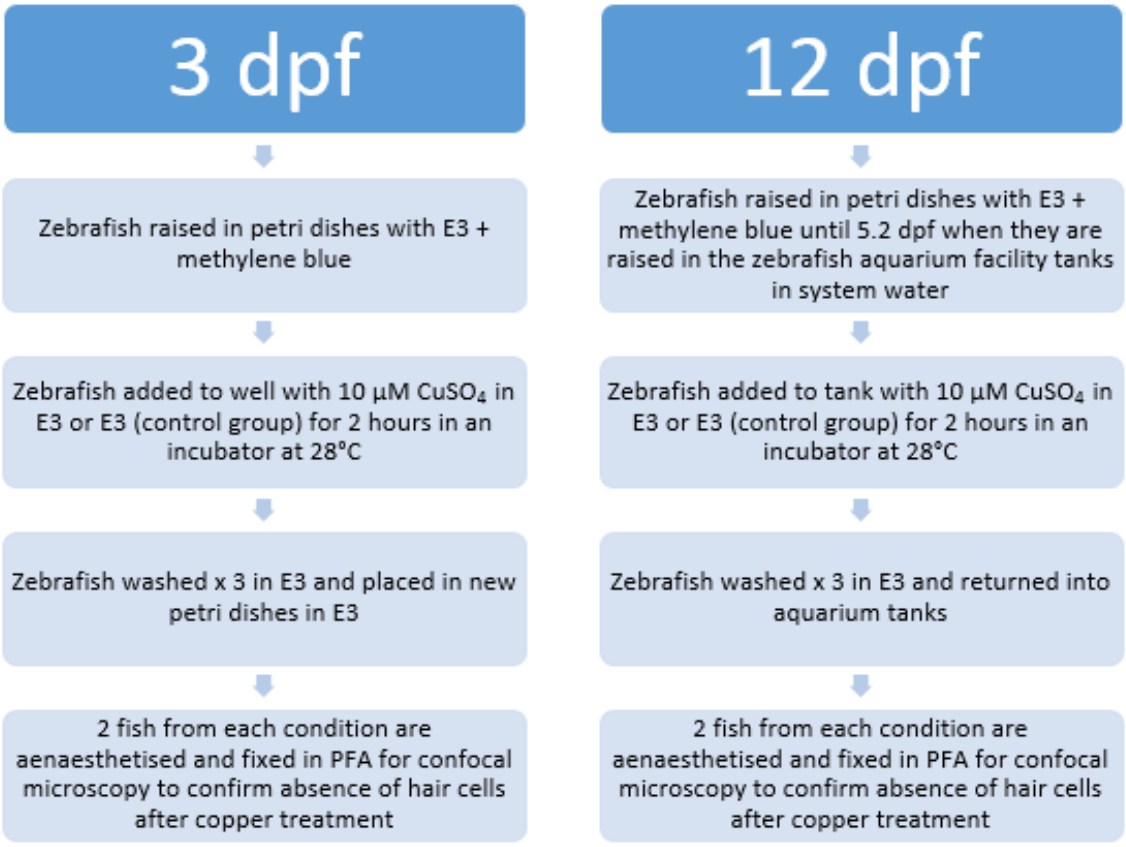

Figure 4.1 Protocol workflow

4.4 Results

4.4.1 Early larval stages (<5.2 dpf)

4.4.1.1 Hair cell count

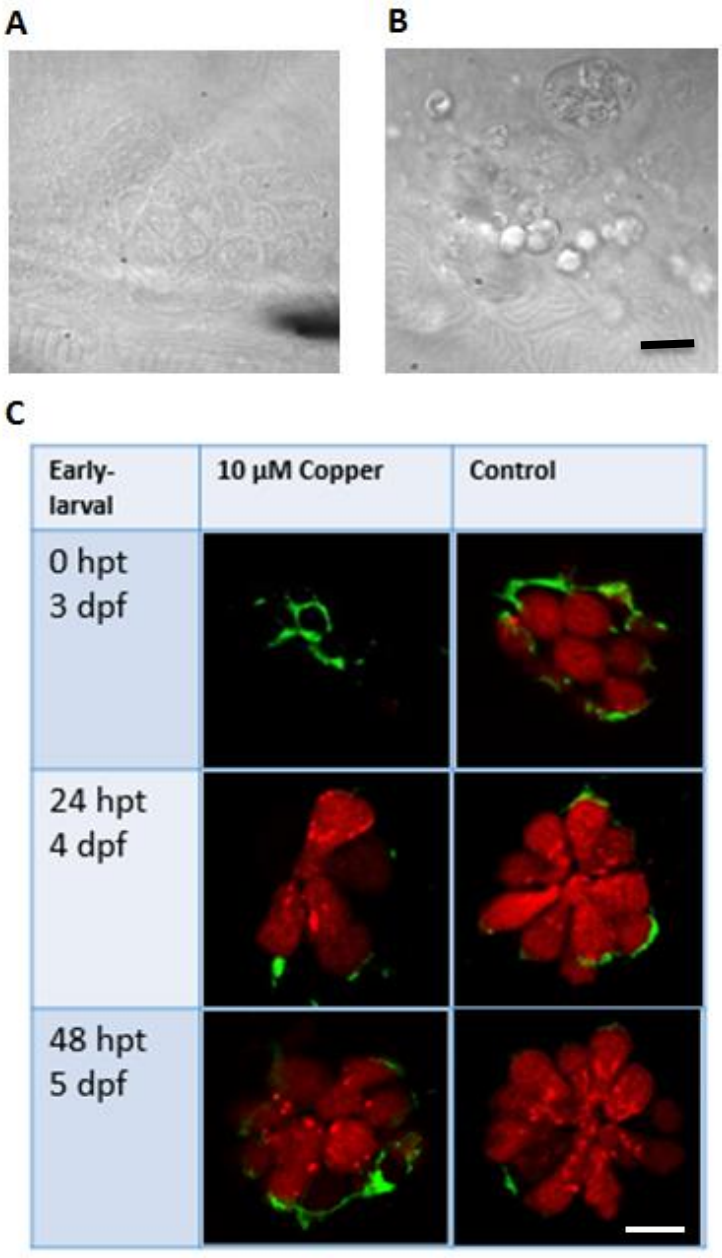

Figure 4.2 Hair cell regeneration after copper exposure.

DIC images of control neuromasts (**A**) and 10 µM $CuSO_4$ treated zebrafish neuromasts (**B**) at 0 hpt. **C**, Images of neuromasts and afferent fibres (green) from 10 µM $CuSO_4$ treated zebrafish (left panels) and control zebrafish (right panels) aged 3 dpf. Within 48 hpt, hair cells (red) have regenerated to a similar quantity as age-matched controls. Scale bar = 10 µm.

After exposure to 10 µM $CuSO_4$, two zebrafish from the control and copper group were culled and fixed in 4% PFA. Once mounted on a slide, three neuromasts of the pLL from each zebrafish were imaged on a confocal microscope. At 0 hpt most hair cells of the pLL are abolished in 3 dpf zebrafish (Figure 4.2). Zebrafish were less likely to initiate a startle response to vibrations in the petri dish compared to controls (not quantified). Usually fish of this age are capable of an escape response with a distance of at least one body length (Kimmel *et al.*, 1974). This finding confirmed the protocol was efficient at ablating hair cells in a quick and efficient manner.

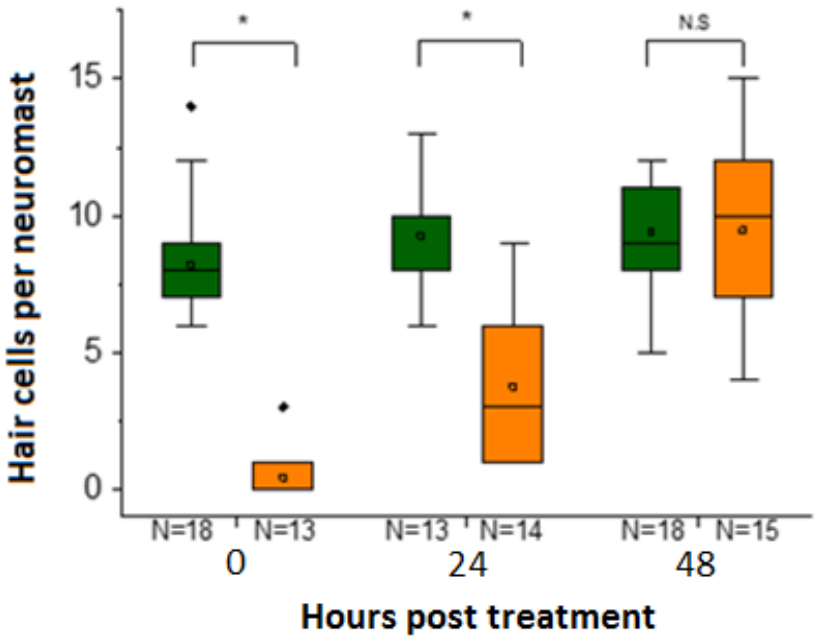

Figure 4.3 Hair cells per neuromast after copper treatment

Hair cells per neuromast calculated at 0, 24 and 48 hpt in copper treated (orange) and control (green). Zebrafish were treated with copper at 3 dpf. Boxes = upper and lower quartiles and median, whiskers = minimum and maximum values, open squares = mean, black diamonds = outliers. Statistics calculated by two-way ANOVA.

In contrast to previous findings by Hernandez *et al.* (2006), hair cells were present due to regeneration within 24 hours after 10 µM $CuSO_4$ (Figure 4.2C), whereas Hernandez *et al.* (2006) did not observe regeneration in the pLL until 48 hpt and hair cells did not reach the same levels as age-matched controls until 72 hpt. Hernandez *et al.* (2006) assessed viability by uptake of DiAsp dye which confirms an open MET channel (Hernandez *et al.*, 2006), which contrasts to my protocol that examined R-GECO expression under a *myosin6b* promoter, which may arise earlier than DiAsp hair cell entry via the MET channel. After manual

quantification of hair cells in treated and control groups (Figure 4.3), a two-way ANOVA determined that there was a significant interaction between the effects of $CuSO_4$ on the hair cell quantity of the pLL and the length of time after exposure to copper ($F(2, 106) = 8.361$, $P < 0.001$). In the post-hoc analysis using Šidàk's multiple comparisons test, it was confirmed that the quantity of hair cells in the control and copper treated zebrafish were significantly different at 0 and 24 hpt, but not at 48 hpt. These results suggest that the regenerated hair cells in the copper treated zebrafish reach similar numbers to age-matched controls within 48 hpt. However, it is not known through confocal imaging if the afferent fibres have formed appropriate connections with the newly regenerated hair cells to initiate spontaneous activity. It was also confirmed using a one-way ANOVA between hair cell quantities per neuromast at 0, 24 and 48 hpt that the means of the three groups were significantly different ($P<0.005$) and a Levene's test suggested that the population variances were not significantly different ($P=0.086$).

4.4.1.2 Spontaneous activity

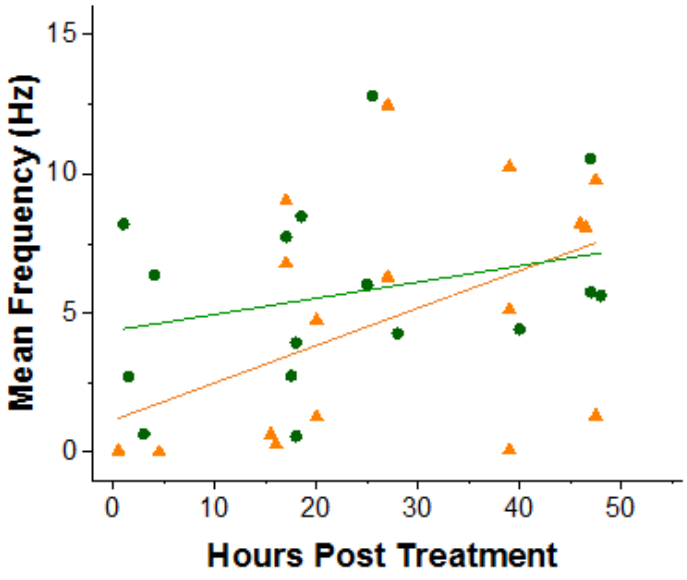

Figure 4.4 Spontaneous activity after copper treatment.

Mean frequency of spontaneous firing from recordings from $CuSO_4$ treated (orange) and control zebrafish (green) during regeneration of hair cells. Linear fit of each condition shown as a line of the corresponding colour. N = 64, Pearson's r = 0.50 ($CuSO_4$), 0.28 (control). According to the F-test the slopes are not statistically different (P=0.35).

The presence of hair cells with appropriate morphology during regeneration does not indicate whether the cells are yet functional. Extracellular loose-patch recordings revealed that at 0 hpt, the absence of hair cells in the 3 dpf $CuSO_4$ treated zebrafish leads to a complete disappearance in spontaneous activity (Figure 4.4). If low frequency spontaneous activity is present ~0 hpt, it is assumed that this is due to a few resilient hair cells that have survived the copper treatment which are not assumed to have a healthy physiology. Hair cells begin to arise within 24 hpt, which correlates with the emergence of spontaneous activity (Figure 4.4). By 1 dpt spontaneous activity is present and it displays similar firing frequencies to age-matched controls and persists at similar frequencies. The Pearson's correlation coefficient shows that the spontaneous firing frequency in the control animals has a slight positive correlation over time (r = 0.28) (as seen previously in chapter 3), whereas in the $CuSO_4$ treated group, the correlation between hpt and frequency of spontaneous activity is more positively correlated (r = 0.50) as the increase in hair cells between 0-50 hpt is far greater in the $CuSO_4$ treated group than the increase in normal development during this period.

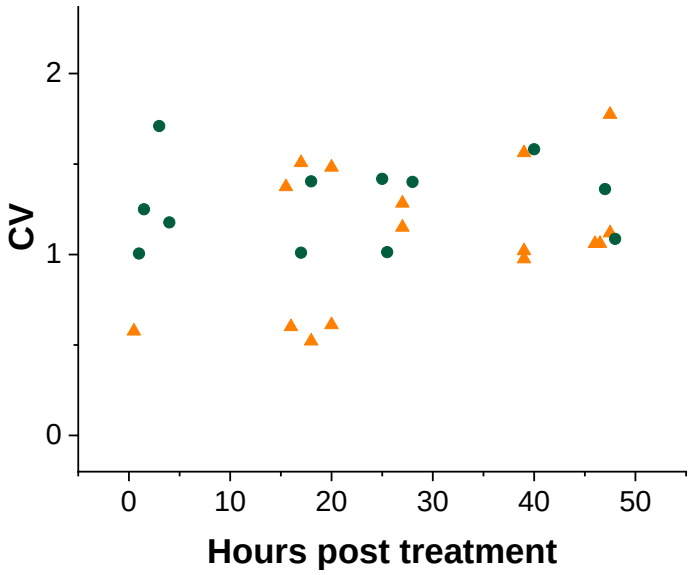

Figure 4.5 Coefficient of variation during regeneration.

Coefficient of variation (CV) of spontaneous activity plotted against time (hpt) in $CuSO_4$ treated (orange) and control (green) zebrafish pLLg.

The CV was calculated to identify if the regularity of the spontaneous activity would vary during regeneration (Figure 4.5). The CV does not vary significantly across treatment

groups (copper treated: $\chi2(2) = 0.538$, $p=0.768$; control: $\chi2(2) = 1.03$, $p=0.598$; Kruskal-Wallis ANOVA). The mean CV values in both the copper-treated and control groups is significantly different to a CV value of 1 (both groups $P\leq0.05$ [Wilcoxon signed rank test]). The results in Figure 4.5 show that there is a low dispersal of inter-spike intervals (ISIs) around the mean. The CV values do vary but this is not correlated with treatment condition. As the CV values seen during regeneration are similar to those seen during normal development in chapter 3, it is thought that with further regeneration and development of the pLL that the CV values would not change.

4.4.2 Mid-larval stages (>12 dpf)

4.4.2.1 Hair cell count

The same protocol was used to treat 12 dpf zebrafish with 10 µM $CuSO_4$ as in the 3 dpf treatment. However, at this age the zebrafish are past the age of protection; therefore, the zebrafish must be raised in the aquarium facility tanks from 5.2 dpf (as described in chapter 3). Amendments were made to the 3 dpf $CuSO_4$ treatment protocol to adhere to Home Office regulations. For $CuSO_4$ treatment, zebrafish were removed from the tank with a tea strainer and placed into a smaller tank covered in tin foil with 300 mL of E3 + $CuSO_4$. Control zebrafish were siblings of the $CuSO_4$ treated zebrafish but were raised in a separate tank in the aquarium facility. Control zebrafish were also placed into a smaller tank with E3. These tanks were then placed in an incubator (~28°C) for two hours before zebrafish were scooped into new tanks containing E3 three times as a washing step before returning to the aquarium facility flow-out tanks until needed for electrophysiology.

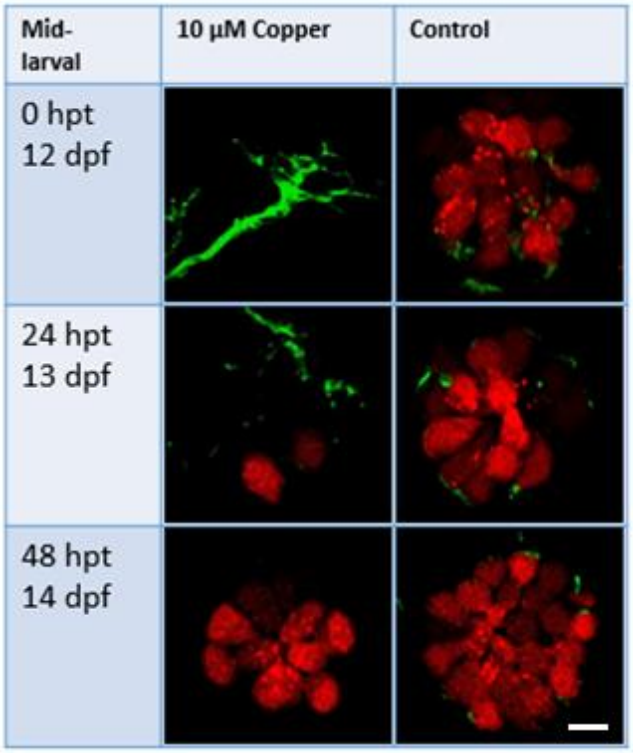

Figure 4.6 Hair cell regeneration after copper exposure at 12 dpf

Images of neuromasts and innervating afferent fibres (green) from 10 µM $CuSO_4$ treated zebrafish (left panels) and control zebrafish (right panels) aged 12 dpf. Within 48 hpt, hair cells (red) have begun to regenerate. However, at 48 hpt hair cells have not reached the same levels as age-matched controls. Scale bar = 10 µm.

Zebrafish treated at 12 dpf with 10 µM $CuSO_4$ also had an absence of hair cells at 0 hpt (Figure 4.6) which was significantly different to hair cell quantities seen in control zebrafish (two-way ANOVA post-hoc analysis, P = <0.0001) (Figure 4.7). By 24 hpt, hair cell regeneration is evident (Figure 4.6), however at this time point the number of hair cells has not reached the same values as age-matched controls and therefore are still significantly different (two-way ANOVA post-hoc analysis, P = <0.01). At 48-52 hpt, number of hair cells had increased but did still not reach similar levels to age-matched controls (two-way ANOVA post-hoc analysis, P = <0.05), but a one-way ANOVA between hair cell quantities per neuromast from copper treated fish only at the three time points revealed that the means at each time point were significantly different (P<0.002). However, a Bonferroni post-hoc comparison revealed that the hair cell quantities per neuromast between zebrafish at 24 hpt and 48 hpt were not significantly different (P>0.05), whereas between 0 hpt and 24 hpt were

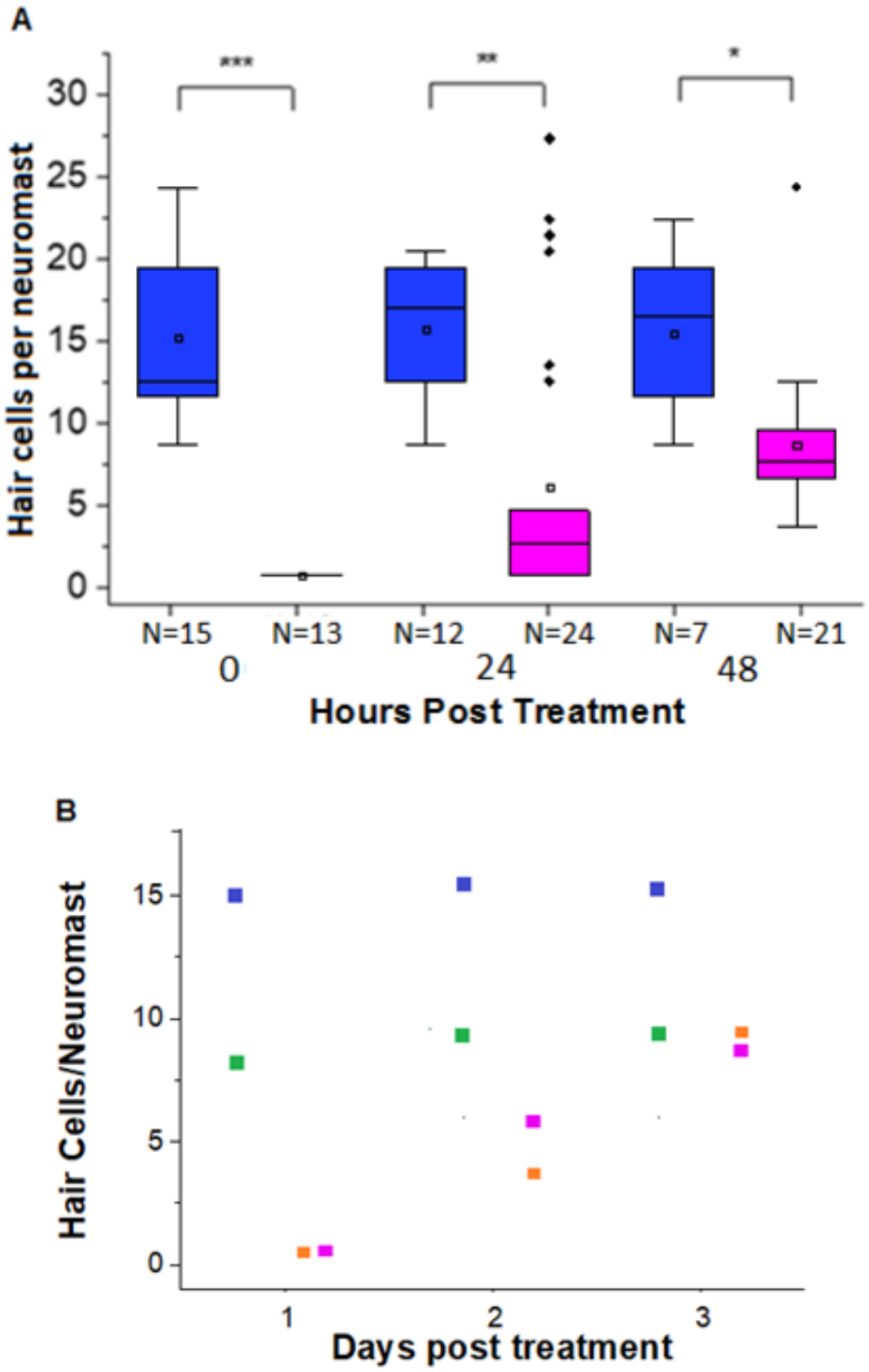

Figure 4.7 Hair cells per neuromast after copper treatment at 12 dpf

A, Hair cells per neuromast calculated at 0, 1 and 2 dpt in copper treated (pink) and control (blue). Zebrafish were treated with copper at 12 dpf. Boxes = upper and lower quartiles and median, whiskers = minimum and maximum values, open squares = mean, black diamonds = outliers. Statistics calculated by two-way ANOVA. **B**, The mean hair cells per neuromast of 3 dpf treated zebrafish (green=control, orange=copper) and at 12 dpf (blue=control, pink=copper)

significantly different ($P<0.05$). Although the post-hoc comparison suggested that there were

no significant differences between hair cell quantity at 24 and 48 hpt, it is thought that if another time point was chosen and measured, there would be more hair cells that have regenerated and thus a significant difference in hair cell quantity. These findings could suggest that although zebrafish treated at 3 dpf recover hair cell number within 2 dpt, 12 dpf treated zebrafish take longer to recover from copper treatment as they have more hair cells to regenerate per neuromast.

4.4.2.2 Spontaneous Activity

No spontaneous activity is present in 12 dpf $CuSO_4$ treated zebrafish in the first few hours after treatment (Figure 4.8), similar to what is found at 3 dpf as there are no remaining hair cells. Spontaneous activity is present within 24 hpt, correlating with the beginning of hair cell regeneration. However, at 48 hpt spontaneous activity does not reach similar frequency ranges to age-matched controls that is seen in 3 dpf treated zebrafish. This is likely to be due to the difference in the number of hair cells in 3 and 12 dpf zebrafish. Hair cell counts in

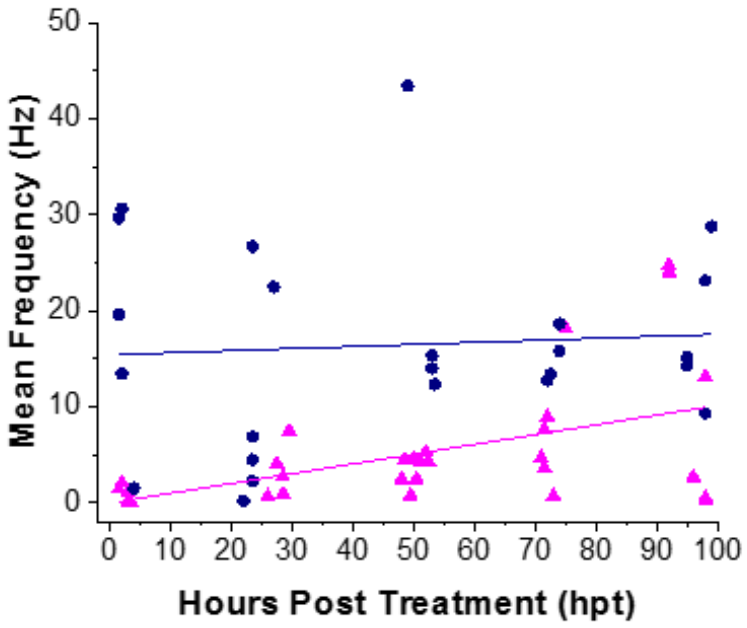

Figure 4.8 Spontaneous activity after copper treatment at 12 dpf

Mean frequency of spontaneous firing from recordings from copper treated (magenta) and control zebrafish (blue) during regeneration of hair cells. Linear fit of each condition shown as a line of the corresponding colour. N = 57, Pearson's r = 0.49 (copper), 0.07 (control). According to F-test results, the slopes are not statistically different (P=0.24).

zebrafish treated at 3 dpf are similar at 2 dpt between the $CuSO_4$ and the control group; therefore, the mean frequency of spontaneous activity is also similar. As a result of this finding, I decided to investigate the spontaneous activity at later time points after $CuSO_4$ treatment to identify if the frequency would increase to similar levels as controls as the number of hair cells increases, or if it was halted in an immature state.

By 4 dpt spontaneous firing frequencies in copper treated fish have reached similar frequencies to age-matched controls (Figure 4.8). The number of hair cells that need to regenerate to return to the same levels as control fish is greater than in the early-larval fish and therefore the time course is longer. The Pearson's correlation coefficient shows that there is almost no positive correlation between the frequency of spontaneous activity and the age of the fish in the control condition, suggesting that hair cell numbers do not vary much over these four days. Interestingly, the correlation between spontaneous frequency and hpt is more strongly correlated over the 4 dpt in treated zebrafish. These findings are similar to that found in 3 dpf treated zebrafish, as hair cells seem to regenerate over a stable time course. However, at 3 dpf control hair cells are being produced at a faster rate than at 12 dpf.

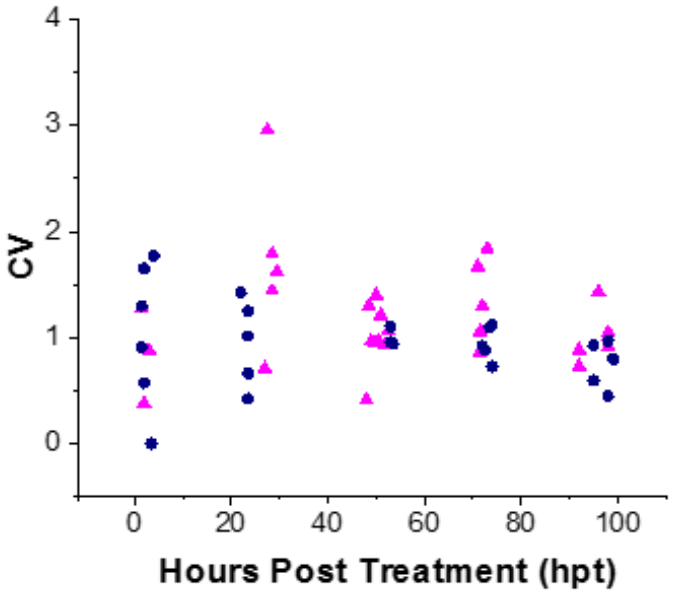

Figure 4.9 Coefficient of variation after copper treatment at 12 dpf

Coefficient of variation of all spontaneous activity recordings obtained from zebrafish treated at 12 dpf (pink) and controls (blue).

The CV displays similar results to zebrafish treated at 3 dpf in both copper treated and control groups. Although a slight decline in CV values is seen in the control zebrafish values over time, this was not significantly different, as CV values in chapter 3 of control fish of this age range does not decline. Therefore, the regularity appears to stay stable during regeneration as previously found in other conditions. A one-way ANOVA revealed that the CV vales of control and copper treated fish were significantly different from 1 ($P<0.05$).

4.5 Methodological considerations

Although zebrafish appeared to regain normal behaviours quite rapidly after copper treatment, it has been suggested that other fish have other sensory effects to copper exposure, such as impaired olfactory responses (Tilton *et al.*, 2008; Tierney *et al.*, 2010). However, as we are only observing the pLL, then our results only show impact from the copper to this system. The concentration of copper used was as low as possible to avoid zebrafish harm, but at a concentration that is high enough to ablate hair cells at both early- and mid- larval zebrafish. Copper is among the highest researched toxic metals in fish; however, the mechanisms of toxicity are still relatively unknown (Tierney *et al.*, 2010). In order to cause less harm to the zebrafish, another ototoxic agent could be used with less system-wide impact.

I observed that there is slight neurite retraction under the neuromasts following copper exposure. It is not known how $CuSO_4$ enters and accumulates into hair cells; therefore, how the dying hair hell causes afferent fibre retraction is also unknown. During regeneration the neurites re-grow their usual basket-like structures underneath the hair cells and are able to reform appropriate connections with them as spontaneous activity is present. It is clear that the pLL is highly adaptable to damage and regeneration is not hindered by the neurite retraction. It would be interesting to explore the impact on neurite retraction on the regeneration of the hair cells, especially in regard to active zone formation at the synapse. It has been previously shown that an excess activation of glutamate receptors can lead to hair cell death in zebrafish (Sheets, 2017) and afferent fibre damage in the cochlea (Puel *et al.*,

1994; Chen *et al.*, 2009). Therefore, it is possible that there is an excess release of glutamate released during hair cell death causes afferent fibre excitotoxicity and thus neurite retraction.

4.6 Summary

Copper sulphate appropriately ablates most hair cells of the pLL at 10 µM after 2 hours of exposure in 3 and 12 dpf zebrafish. Hair cell regeneration becomes visible by 24 hpt. However, it takes longer for 12 dpf $CuSO_4$ treated zebrafish to reach similar hair cell numbers to age-matched controls after treatment.

Spontaneous activity is present 1 dpt after exposure to $CuSO_4$ in both early-larval and mid-larval zebrafish. Early-larval treated zebrafish had similar spontaneous firing rates to age-matched controls during regeneration, whereas it took the 12 dpf treated zebrafish longer to regenerate hair cells to the same quantity as age-matched controls and therefore spontaneous firing frequencies were not similar until 4 dpt.

4.7 Discussion

Zebrafish are a good animal model to investigate hair cell regeneration *in vivo*. My data shows that $CuSO_4$ is capable of ablating hair cells of the pLL, as demonstrated by lack of behavioural escape responses and confocal microscopy of neuromasts with absent hair cells. After $CuSO_4$ treatment at 3 dpf and 12 dpf, hair cells have started to regenerate and spontaneous activity is present in the afferent fibres within 24 hpt. This demonstrates that at 1 dpt some hair cells have regenerated rapidly and have synaptic machinery that is efficient enough to elicit spontaneous activity. This is a fast process which helps the zebrafish to regain pLL associated behaviours quickly after hair cell damage.

As 12 dpf $CuSO_4$ treated zebrafish took longer for spontaneous activity in the afferent fibres of the pLL to reach similar frequencies as age-matched controls; I assume that this is due to 12 dpf zebrafish having a larger number of hair cells per neuromast compared to early-larval zebrafish, thus taking longer to regenerate the same amount of hair cells as age-matched controls. As spontaneous activity is present in the afferent fibres when few hair cells have regenerated, it is likely that the zebrafish can use the pLL for necessary behaviours prior to all hair cells regenerating, but when the hair cell numbers have recovered, the behaviour may become more refined.

Not all ototoxic compounds affect the hair cells in the same manner. For example, it is known that some ototoxic compounds, such as aminoglycosides, selectively enter and accumulate into the hair cell via the MET channel (Marcotti *et al.*, 2005). Therefore, it would be interesting in screening studies that use known compounds to block ototoxic compound entry through the MET channel to see if the hair cells are affected by using extracellular loose-patch electrophysiology in the pLLg. Extracellular recordings allow for more detailed information about the hair cell-afferent fibre synapse and function than imaging studies using dyes such as FM1-43; therefore, it is a good tool to use in combination with imaging to further understand ototoxicity research to identify if the hair cells are functioning physiologically, rather than just observing if hair cells are present and the MET channels are open like in previous imaging studies.

For a more detailed examination of the hair cell regeneration, whole-cell patch clamp of the regenerating hair cells would give an insight into the ion channel composition and exocytosis capabilities. For example, is the hair cell capable of exocytosis prior to the formation of kinocilia and MET channels? This could also be addressed using calcium indicators and stimulation devices such as a fluid jet (discussed further in chapter 5).

Whereas I have focused on the pLL, it is known that the aLL is more resilient to ototoxic agents (Hernandez *et al.*, 2006). A similar experiment could be employed to investigate this phenomenon further. In combination with whole-cell patch clamping of hair cells of the aLL,

it could be identified why the hair cells of the aLL are less susceptible to ototoxic damage due to factors such as the open probability of the MET channel or a different expression of ion channels. Extracellular loose-patch recordings from the aLL ganglion have not yet been attempted.

4.9 References

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Chapter 5 General Discussion

This study has used extracellular loose-patch electrophysiology in the zebrafish posterior lateral line (pLL) *in vivo*, complemented by confocal microscopy, to characterise spontaneous action potential activity in the developing zebrafish, and during hair cell regeneration. These are areas of research that have not yet been thoroughly investigated using electrophysiology techniques; therefore, my work gives an insight into the developmental features of the pLL and how it adapts to hair cell loss. I have detailed the investigation of the onset of spontaneous activity in the zebrafish pLL afferent fibres and recorded spontaneous activity past previously reported ages using the extracellular loose-patch technique. The spontaneous activity displays a wider variation of frequencies in older zebrafish (>5.2 dpf) and has more high-frequency spontaneous firing than early-larval zebrafish (<5.2 dpf). This is likely to reflect the development that the pLL undergoes during this time period through the increase of hair cells and arborisation of afferent fibres. Variations in the spontaneous firing rate are also seen during regeneration of hair cells in zebrafish. Early-larval zebrafish regain similar spontaneous firing frequencies to age-matched controls within 48 hours after $CuSO_4$ treatment, whereas the neuromasts of mid-larval zebrafish have more hair cells to regenerate and hence take longer to regain frequencies similar to age-matched controls. Spontaneous activity in the afferent fibres of the pLL has not been investigated at this age range before and nor has hair cell regeneration in zebrafish been investigated using electrophysiology. Here I will discuss implications, limitations and future work that could be undertaken to further understand my findings.

5.1 Development of spontaneous activity in the posterior lateral line of zebrafish

5.1.1 Spontaneous activity findings and speculations

Previously, it was speculated that hair cells of the pLL were not able to transduce any sensory input until 3-4 dpf, as this was when the hair cells were thought to become innervated by the afferent fibres (Raible & Kruse, 2000). This is also shown by behavioural observations as zebrafish with mutations in hair cell-specific proteins do not usually display

disrupted phenotypes until 3-4 dpf (Nicolson *et al.*, 1998). However, it is clear from my findings that as spontaneous activity is present from 2 dpf, the afferent fibres must be innervating the hair cells as the spontaneous activity is not intrinsic to the fibre (Trapani & Nicolson, 2011). Spontaneous activity may be present before the hair cell can perform mechanotransduction, which was not confirmed in the present study. However, what is certain is that at 2 dpf hair cells have formed a synapse with the afferent fibre and are therefore ready to elicit action potentials.

As more action potential recordings were obtained from the pLL afferent, I postulated that the higher frequency of spontaneous activity in older fish was due to some afferent fibres receiving more input as they are innervating a growing amount of neuromasts. Although I related spontaneous activity mostly to the increase in hair cell number during development, it is important to remember that the afferent fibres also develop and refine contacts to hair cells. The afferent nerve follows the primordium during migration down the flank of the fish to deposit cells that will become the neuromasts of the pLL (Metcalfe *et al.*, 1985; Kimmel *et al.*, 1995). However, further afferent fibres differentiate after this initial outgrowth (Fame *et al.*, 2006) and innervate new and previously existing neuromasts. We know that the afferent fibres only form contacts of hair cells of the same polarity (Faucherre *et al.*, 2009), but the arborisation and branching of afferent fibres is extensive and sometimes not predictable (Dow *et al.*, 2018). I have observed that afferent fibres will branch from innervating one neuromast to contact another neuromast that is already innervated by another branch of the afferent nerve. Therefore, we cannot easily conclude from this thesis of work how specific afferent innervation patterns of the pLL is impacting the spontaneous activity.

Not only is it not entirely clear how the afferent fibre innervation pattern influences spontaneous activity during development, but we also do not know how the different hair cell biophysical properties are likely to influence the spontaneous activity. During development of the hair cell, the basolateral membrane properties become more mature, which is related to maturation of the cell and efficiency of neurotransmitter release. Hair cells with a more immature phenotype are found in the periphery of the neuromast (Olt *et al.*,

2014), which is related to the area which new hair cells appear (Lopez-Schier & Hudspeth, 2006; Wibowo *et al.*, 2011). I have postulated that the quantity of hair cells will influence the frequency of spontaneous firing, but it is also possible that a change in basolateral membrane properties of hair cells could also cause changes to the spontaneous firing rate especially at more mature ages when the proportion of mature hair cells is higher.

5.1.2 Are there two types of afferent fibres and is this evident throughout development?

One theory in the field of zebrafish pLL research is that there may be two types of afferent fibres. This theory is based on soma size due to birth order, as earlier born afferent fibres will have larger soma diameter, a lower input resistance and lower spontaneous firing rates compared to later born afferent fibres (Liao & Haehnel, 2012; Pujol-Marti *et al.*, 2012). Although some evidence shows that this may seem to hold true in larval zebrafish, it is not known if there is a functional significance of this and if it translates into older ages. The only evidence from my data to support the theory that there may be two types of afferent fibres based on spontaneous activity properties is from the K-means analysis (chapter 3). The K-means analysis split the mean spontaneous firing frequencies of each recording from 2-18 dpf into two groups based on their relationship to each other. The second, higher frequency group, did not emerge until after the age of protection (>5.2 dpf); therefore, does not correlate with previous research from other groups that suggests two types of afferent fibres are evident in larval stages (Liao & Haehnel, 2012; Pujol-Marti *et al.*, 2012). The lower frequency spontaneous activity group is present at all ages investigated. However, it may be that the variation of spontaneous rates is not clearly segregated and is correlated to the range of stimuli the afferent fibres can detect, which increases with time. Each afferent fibre contacts a growing number of hair cells during development (Olt *et al.*, 2014), especially after the onset of stitch formation (Ledent, 2002). To further investigate afferent fibre properties, soma size of individual afferent fibres could be plotted over time to identify if smaller diameter somas eventually become larger and if this correlates with spontaneous firing frequencies.

Afferent fibres can also be categorised based on the location of neuromast that they innervate (Gompel *et al.*, 2001b), which was not conducted during this study. Neuromasts that are deposited by the primordium first are more likely to be innervated by afferent fibres that contact the Mauthner cell, whereas later born (secondary) neuromasts are innervated by afferent fibres that do not contact the Mauthner cell (Pujol-Marti *et al.*, 2012). Afferent fibres that innervate hair cells in neuromasts near the tail are associated with being able to respond to stronger stimuli than those of the pLL located more anteriorly (Liao & Haehnel, 2012). Therefore, the spontaneous activity in the afferent fibres may be different depending on what their innervation pattern and function is. The variations in spontaneous firing based on the location of the cell the afferent fibre innervates has also been found in the cochlea. The inner hair cells have different spontaneous firing patterns based on their location along the cochlea which is thought to drive tonotopic patterning in the corresponding neurons of the auditory system (Johnson *et al.*, 2011); therefore, this may be the case with the afferent fibres of the pLL. This could explain functionally if afferent fibres that contact the tail neuromasts are more heavily involved in the escape response (Olszewski *et al.*, 2012); therefore, these afferent fibres detect stronger stimuli than those near the head. As it was not known in this present study which afferent fibre innervated which neuromast during spontaneous activity recordings, I cannot confirm whether different afferent fibres that innervate neuromasts located in different locations have altered spontaneous firing rates. As somatotopic organisation is seen in the pLLg (Sato *et al.*, 2010), recordings were obtained from different locations in the pLLg. This ensures afferents that innervate neuromasts located at different positions along the fish are recorded from to obtain a good representation of spontaneous activity from the pLLg.

It is not possible to conclude whether there are different types of afferent fibres with varying firing properties to be able to respond to different strengths of stimuli or if the variation in mean spontaneous firing rates is due to different developmental stages. The afferent fibres, and thus spontaneous activity, may constantly be undergoing changes due to new production of hair cells, neuromasts and also hair cell turnover. From previous literature and my findings, the afferent fibres are clearly plastic to changes and are likely to remain so during maturation as well as development (Nagiel *et al.*, 2008; Olt *et al.*, 2014).

5.1.3 Spontaneous burst-like activity

One aspect of my research which was intriguing was spontaneous firing with burst-like behaviour as it has not previously been reported in the literature in the afferent fibres of the zebrafish pLL. Burst-like activity is present in spontaneous activity of other systems to convey information about sensory stimuli (Krahe & Gabbiani, 2004; Jay *et al.*, 2015) and facilitate development (O'Donovan, 1999). It was evident from the raw data that burst-like activity was present in some recordings. It is not known if the pLL afferent fibres display a pattern of burst-like spontaneous activity and what may determine this feature. I speculate that as the hair cells have different properties based on their location in the neuromast (Olt *et al.*, 2014) their varying outputs may lead to different patterns of spontaneous activity, some of which may display burst-like activity as seen in the cochlea (Johnson *et al.*, 2011). However, more work is needed to quantify the burst-like behaviour and identify the functional importance of this activity. In other neuronal systems that display burst-like spontaneous activity have been found to share common features such as duration of burst spikes and presence of certain receptors (Mazzoni *et al.*, 2007). In order to be able to compare the activity I have found in the zebrafish pLL to other bursting systems, intra- and inter- spike intervals of the bursting spikes would be calculated and then identification of modulatory mechanisms, such as receptors, would help elucidate the functional significance of this unusual activity.

5.2 The regeneration of hair cells and consequences to spontaneous activity

The accessibility of the hair cells of the pLL allows for rapid destruction and regeneration of hair cells using ototoxic compounds *in vivo*. Avian species are also capable of hair cell regeneration in various tissues; however, damage and repair to hair cells is slower than in zebrafish and cannot be investigated *in vivo* (Monroe et al., 2015). Imaging studies have proven to help advance regeneration research greatly, but is only possible in the zebrafish pLL *in vivo*. I have used electrophysiology to investigate hair cell regeneration and have found it to be insightful in ways that imaging alone could not provide.

Previous research has speculated that after ototoxic exposure hair cell regeneration is complete between 48-72 hpt (Hernandez *et al.*, 2006; Mackenzie & Raible, 2012). My study has shown, using extracellular loose-patch electrophysiology, that the afferent fibres form synapses with hair cells within 24 hpt as spontaneous activity is present. I speculate that even though hair cell numbers have not reached a similar quantity to age-matched controls at 24 hpt, the hair cells that are present are functional due to the presence of spontaneous activity. There is also a difference in spontaneous activity frequencies zebrafish treated with $CuSO_4$ at different ages. Early-larval zebrafish are capable of regenerating hair cells to the same levels as age-matched controls quicker than mid-larval zebrafish which I believe to be due to the former having a smaller quantity of hair cells per neuromast to regenerate. Consequently, the recovery of the spontaneous activity to similar frequencies as age-matched controls is delayed in mid-larval zebrafish, as more hair cells are required to elicit similar frequencies of spontaneous activity. However, hair cell quantity may not be the only factor influencing the differences in spontaneous firing rates during regeneration. It would be beneficial to understand the progression of the biophysical properties of hair cells during regeneration as this may elucidate factors that may alter spontaneous firing rate within the cell during regeneration. Whole cell patch-clamp electrophysiology has previously been conducted in developing zebrafish past the age of protection and has elucidated the different properties that hair cells develop as they mature (Olt *et al.*, 2014). Therefore, this technique could be employed to further understand the regeneration process of hair cells by patching hair cells at various points during regeneration. This could also discover how regeneration of hair cells differs from development in terms of ion channel expression and exocytosis efficiency.

My study focuses on the pLL due to the accessibility of the pLLg for electrophysiology and the greater amount of previous research compared to the anterior lateral line (aLL) (Hernandez *et al.*, 2006; Mackenzie & Raible, 2012). However, it would be interesting to identify whether the aLL displays a similar pattern of spontaneous activity to the pLL. It is known that the aLL is more resistant to ototoxicity; therefore, higher concentrations of ototoxic compounds are needed to cause hair cell damage compared to in the pLL. As many ototoxic agents enter hair cells of the pLL through the mechanotransduction (MET) channels of the hair cells (Alharazneh *et al.*, 2011), then the hair cells of the aLL may have a lower open

probability at rest, allowing less ototoxic compounds to enter the cell and a possible reduction in spontaneous activity. The aLL may also have a different spontaneous firing frequency as the aLL is faster to respond to stimuli that produces an escape response than the pLL in goldfish (Mirjany & Faber, 2011), which suggests a difference in hair cell or afferent fibre physiology. The aLL ganglia (aLLg) are located anterior to the pLLg, on the head of the fish; therefore, access with an electrode to the aLLg may be problematic and may be best investigated with genetically encoded calcium indicators (GECIs) rather than electrophysiology.

Further investigation into the origins of spontaneous activity in the zebrafish pLL may provide us with insights into how spontaneous activity can help to restore functionality of hair cells after regeneration by strengthening contacts with higher order projections in human ears to ensure the recovery of hearing efficiently.

5.3 Implications to zebrafish research

Zebrafish allow us to study the hair cells and afferent fibres *in vivo*, which is unlike any other animal model. Work conducted in older zebrafish is usually done in vitro and little *in vivo* work is conducted apart from behavioural studies due to the licencing and difficult practicalities of the experimental set up. For example, zebrafish >21 dpf use their gills and therefore the zebrafish must be intubated so they can breathe during *in vivo* studies. It is important to correlate behaviour to cellular and systematic developments at suitable ages to investigate functional consequences. Research in the zebrafish is mostly conducted under 5.2 dpf due to Home Office (UK) licencing laws. However, the use of zebrafish at older ages has wider implications. If zebrafish are to be used as an animal for model for human hearing loss and deafness research, the age which the zebrafish pLL is investigated is important as the hair cells larval pLL will not be as mature and therefore not as transferrable to other sensory systems with hair cells. The use of the embryonic and larval zebrafish pLL will still be useful in answering certain questions of research that are not impacted by further development of the system.

Extracellular loose-patch electrophysiology allows us to measure the summed output of hair cells contacting one afferent fibre. I have only used this method to record spontaneous activity at rest from the pLLg, but it is possible to use this technique to record evoked activity (Trapani & Nicolson, 2011). Spontaneous firing rates have been used to identify synaptic release properties in mammals (Merchan-Perez & Liberman, 1996; Furman *et al.*, 2013) as well as zebrafish (Sheets *et al.*, 2017), which allows us to understand more about the hair cells of these systems which can later lead to advancements in hair cell loss and disorder in humans. However, there is still a lot we do not know about the properties of stimulated activity in the zebrafish pLL.

As previously discussed, larval zebrafish are beneficial for high-throughput screening due to their transparency, they are easy to breed and are small. However, work from this current study and Olt *et al*. (2014) highlight that the use of <5.2 dpf zebrafish for high-throughput screening is only representative of a small and immature window of hair cell and pLL development. Many high-throughput screens with zebrafish for hearing and deafness research involve screening libraries of compounds for otoprotective agents that could then be confirmed in mice for its protective properties before carrying forward to human trials (Ou *et al.*, 2010). Use of zebrafish at a more physiologically relevant age may provide a higher quality of results that have greater implications, despite the longer time course of the experiment.

5.4 Limitations

Although the upmost care was taken to ensure that the techniques used were optimal for answering the aims of this study, all experimental procedures have certain benefits and pitfalls. Firstly, although extracellular loose-patch technique was efficient for recording spontaneous activity in the afferent fibres in real-time, the recordings were not correlated with amount of hair cells innervated, and from what location along the fish. This would have helped us to identify more of the functional consequences to changes in spontaneous activity over time. Nevertheless, using extracellular loose-patch electrophysiology for examining

regeneration had not previously been considered; therefore, this technique was able to tell us more about the regeneration of hair cells and the contacts with the afferent fibres than imaging alone. Secondly, the use of tricane methnsulfonate (MS-222) could have been avoided in place for a less toxic anaesthetic such as benzocaine (Olt *et al.*, 2016a). However, MS-222 was used as it is the more popular anaesthetic in zebrafish research; therefore, it would indicate that my research had more comparability to existing and future spontaneous activity recordings with zebrafish. Thirdly, there are of course other factors that may interfere with spontaneous activity in the pLL. This present study does not take into account the efferent fibres (Haehnel-Taguchi *et al.*, 2018) and the effect that they may have on the hair cells and therefore spontaneous activity. Efferent fibres inhibit hair cells when necessary (Brown & Nuttall, 1984); therefore, during strong stimulation of the canal neuromasts if the burbot fish (*Lota lota*), the efferents have a greater inhibitory effect on the hair cells (Flock & Russell, 1973). At rest, when efferent fibres are inhibited, the spontaneous firing frequency increases (Flock & Russell, 1973). Therefore, the efferents may also modulate the pLL at rest. Nevertheless, it is unknown under what circumstances the efferents would be triggered to inhibit hair cell activity in the zebrafish pLL at rest.

The ages of zebrafish used in this study was limited by Home Office Animals (Scientific Procedures) Act 1986. Although it is possible to record from the hair cells of that pLL in zebrafish >21 dpf *in vivo* (Olt *et al.*, 2016a), it would be extremely technically challenging to record from the pLLg above this age using the extracellular loose-patch technique. The hair cells of the pLL neuromasts remain superficial into adulthood, whereas I observed that the pLLg appears to become more deeply located in the fish over time. Therefore, to record spontaneous activity *in vivo* from the pLLg in adult zebrafish surgery would be required, or a better optical reporter to visualise the pLLg more efficiently in thick tissue. This would allow investigation of spontaneous activity into older ages to identify whether it continues to refine with age or if it is limited to certain firing frequencies.

5.5 Future work

It is still unclear what the purpose of spontaneous activity in the zebrafish pLL is beyond establishing somatotopy in the pLLg and the hindbrain. If its only purpose is to ensure the afferent fibre establishes precise contacts with higher order projections, why does it persist into later developmental stages and why does it display such a range of frequencies? Spontaneous activity has been shown in the olfactory system of mice to help establish and maintain sensory neural maps (Yu *et al.*, 2004). However, it is more likely that factors such as the *$Ca_V1.3$* calcium channels are essential for synapse maintenance. Zebrafish with *$Ca_V1.3$* mutations do not survive into adulthood (Nicolson *et al.*, 1998); however, in mice it has been shown that this channel is required for maintaining the hair cell-afferent fibre synapse (Nemzou *et al.*, 2006). To examine the purpose of spontaneous activity further, the best method would be to investigate mutations in zebrafish or to block spontaneous activity. However, so far it has not been proven possible to abolish spontaneous activity in the zebrafish pLL for long without harming the zebrafish or rendering the hair cells of the pLL unable to respond to stimuli. For example, cobalt chloride blocks spontaneous activity but becomes toxic at high concentrations and long-term exposure (2-3 days) (Stewart *et al.*, 2017).

The present study has provided us with an insight into the development of spontaneous activity in the pLL and how spontaneous activity recovers during regeneration of hair cells. Although we now know that spontaneous activity is present when hair cells have regenerated before 24 hpt, we do not know exactly what point the hair cells become functional during regeneration, nor if they do so prior to forming contacts with the afferent fibre. This study of work has led to a wide range of potential future experiments which could be undertaken, some of which have previously been discussed. The main experiments that are most pertinent to furthering this research are as follows: firstly, using GECIs to identify hair cell and afferent fibre responses during regeneration in specific hair cells/neuromasts. Secondly, to use whole-cell patch-clamp electrophysiology to identify the phenotypes of regenerating hair cells, as described above.

Although using extracellular loose-patch electrophysiology I was able to identify when the sum of the regenerated hair cells was great enough to elicit spontaneous activity, it is not known when individual hair cells become functional during regeneration. Some groups suggest hair cell regeneration is present from 6 hpt (Nagiel *et al.*, 2008), but this is based on morphological features alone. The use of zebrafish strains with genetically encoded calcium indicators (GECIs) in the hair cells and afferent fibres makes it possible to identify whether the hair cells are functional during regeneration, and if they have formed suitable connections with the afferent fibres to elicit an action potential. Hair cells in a neuromast of the pLL can be stimulated by a fluid jet and the calcium fluctuations - due to the depolarisation of hair cells - can be measured (Sheets *et al.*, 2012; Zhang *et al.*, 2018). Therefore, if a zebrafish strain was used to examine regeneration with GECIs in the hair cells and afferent fibres, it would be possible to determine if the hair cell is functional at what time point and if the timing correlates with afferent fibre responses. It is clear that the use of electrophysiology in combination of functional imaging using GECIs would detail more details about the pLL afferent fibre and hair cell relationship.

5.6 Conclusion

In this study, I have established a method for recording extracellular activity from the afferent fibre somas of the posterior lateral line ganglion in zebrafish from 2-18 dpf. I have shown that the frequency of spontaneous activity, but not regularity (shown by CV), becomes more varied with age of the zebrafish. In combination with previously established protocols, it is evident that this technique has allowed me to gain insight into the development of the pLL spontaneous activity. My findings will cause thought into the functioning of the hair cells and afferent fibres during development and how this implicates other investigative work.

5.7 References

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