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Zebrafish embryos exposed to alcohol undergo abnormal development of motor neurons and muscle fibers

by

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Abstract

Children with Fetal Alcohol Spectrum Disorder have significantly delayed motor skills, and deficiencies in reflex development. The reasons underlying these motor deficits are not fully understood. The purpose of this thesis was to investigate the effect of embryonic exposure to ethanol (EtOH) on motor neuron and muscle fiber morphology and physiology in zebrafish. We observed that EtOH-exposed fish took longer to hatch and exhibited fewer swimming bouts in response to touch. Immunolabelling of motor neurons indicated that EtOH-exposed fish had significantly higher rates of motor neuron axon defects. Examination of muscle fiber morphology revealed that EtOH exposure resulted in significantly smaller muscle fibers. Miniature endplate current (mEPC) recordings from muscle fibers revealed that event amplitudes, rise times, half widths, frequencies and decay times were affected by EtOH exposure. These findings indicate that motor neurons and muscle fibers of zebrafish are affected by embryonic EtOH exposure, which may be related to deficits in locomotion.

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List of Abbreviations

ACh = acetylcholine

AChR = acetylcholine receptor

ANOVA = analysis of variance

AP = action potential

ARBD = alcohol-related birth defects

ARND = alcohol-related neuro-developmental defects

ATP = adenosine triphosphate

CaP = Caudal Primary

CNS = central nervous system

DMSO = dimethyl sulfoxide

dpf = days post fertilization

ECS = extracellular solution

EtOH = ethanol (purified form of alcohol)

FAE = fetal alcohol effects

FAS = fetal alcohol syndrome

FASD = fetal alcohol spectrum disorder

GTP = guanosine triphosphate

HEPES = 4-(2-Hydroxyethyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine

hpf = hours post fertilization

ICS = intracellular solution

iGluR = ionotropic glutamate receptor

IOM = Institute of Medicine

mEPC = miniature endplate current

MiP = middle primary

MS-222 = tricaine methane sulfonate

nAChR = nicotinic acetylcholine receptor

NMJ = neuromuscular junction

PBS = phosphate buffered saline

PBS-T = phosphate buffered saline with tween (0.1%)

pFAS = partial fetal alcohol syndrome

PNS = peripheral nervous system

PTU = phenylthiourea

RA = retinoic acid

RB = Rohon-Beard

ROL = retinol (vitamin A)

RoP = rostral primary

SEM = standard error of the mean

Shh = Sonic hedgehog

TTX = tetrodotoxin

1. Introduction

1.1. Fetal Alcohol Syndrome

Fetal alcohol syndrome (FAS) is currently recognized as the leading cause of mental retardation in the westernized world, and is the most common preventable cause of birth defects (Kumada *et al.*, 2007). FAS in humans is characterized by motor and reflex development delays, pre- and post-natal growth deficiencies, and cranial, facial, joint and cardiac abnormalities (Kalberg *et al*., 2006; Staisey and Fried, 1983; Jones and Smith, 1973; Jones *et al.*, 1973). FAS was first recognized in the United States in 1973 when two physicians, K. Jones and D. Smith, noticed a group of children who were unrelated and from three separate ethnic groups with similar physical and neurobehavioral abnormalities (Jones and Smith, 1973). It was determined that these children had been exposed to excessive amounts of alcohol during pregnancy, which pointed to alcohol as being teratogenic to the developing fetus (Jones *et al*., 1973).

However, it had been long known that alcohol consumption during gestation had adverse effects on the offspring. Ancient Greek and Roman beliefs stipulate that alcohol intoxication at the time of conception would give the couple a ‘damaged’ child (Jones and Smith, 1973; Green, 1974). Similarly, Carthaginian custom forbade couples to drink on their wedding night (Calhoun and Warren, 2007). Even Aristotle and the Old Testament warned of the consequences of alcohol consumption during pregnancy (Krous, 1981; Calhoun and Warren, 2007). Interestingly, although these ancient civilizations were aware of the effects of alcohol on the offspring, they also believed it was consumption during conception that caused the effects, not alcohol consumption during the actual pregnancy. Also, intoxication of the father was thought be as harmful

or more harmful than the mother's intoxication level (Calhoun and Warren, 2007). There was little mention in the literature of the effects of alcohol on children until the 1700s, when English physicians described children born from alcoholic mothers as being weak, feeble and a burden to society (Royal College of Physicians of London, 1726). In 1899, Sullivan concluded that alcohol had a toxic effect on the fetus after reporting the mortality and deformity rate in children born from imprisoned alcoholic women (Sullivan, 1899). FAS was not actually documented in medical journals until 1968 when Lemoine and colleagues described the physical and behavioural effects of pre-natal alcohol on children. However, this publication was not well known and did not present clear diagnostic criteria (Calhoun and Warren, 2007). Therefore, it wasn't until 1973 when Jones and colleagues described several children with similar abnormalities, all with confirmed maternal alcohol consumption, that FAS became recognized (Jones and Smith, 1973; Jones *et al*., 1973; Jones *et al*., 1974).

Although FAS had been characterized, clinicians soon realized that the effects of pre-natal alcohol exposure were variable. The term FAE (Fetal Alcohol Effects) was introduced to denote partial expression of the syndrome (Clarren and Smith, 1978). However, this term was abandoned in 1995 due to its vagueness and misapplication in any situation where behavioural deficits were observed and alcohol abuse was suspected (Calhoun and Warren, 2007; Aase *et al*., 1995). In 1996, the Institute of Medicine (IOM) brought forth a new classification system, which included five categories: FAS with and without confirmed alcohol exposure, partial FAS (pFAS), alcohol-related birth defects (ARBD), and alcohol-related neuro-developmental disorders (ARND). In 2000, Astley and Clarren proposed a separate diagnostic system

that included scales to assess the significance of certain characteristics, which became known as the 4-Digit Diagnostic Code (Astley and Clarren, 2000). The Canadian diagnostic guidelines have since adopted a new system that harmonized the IOM classification system and the 4-Digit Code criteria, which is similar to the Revised IOM Diagnostic Classification System (Calhoun and Warren, 2007; Hoyme *et al*., 2005). The term Fetal Alcohol Spectrum Disorder (FASD) is now more popular in common usage because it generally encompasses the entire range of effects of pre-natal alcohol exposure (Spadoni *et al.*, 2007).

The prevalence of FAS is related to the frequency of alcohol consumption during the pregnancy and varies from population to population. FAS affects 1 to 3 per 1000 newborns while FASD is diagnosed in about 1 in 100 newborns (Barr and Streissguth, 2001). The combined prevalence of genetic disorders, such as Down syndrome, and birth defects, such as spina bifida, is lower than the prevalence of FAS. The incidence for FAS has been reported to be higher in certain regions and ethnic groups (Chudley *et al*., 2007). Reasons for this are thought to be the variable poverty rates (poverty has been shown to increase the odds of alcohol abuse), genetic differences (there are three alleles of the alcohol dehydrogenase (ADH2) gene), a lack of reliable and consistent diagnostic criteria, as well as a lack of training of the primary care givers (CDC, 2005).

The 4-Digit Diagnostic Code identifies these four diagnostic features in FAS patients: growth deficiency, FAS facial phenotype, CNS damage/dysfunction and gestational alcohol exposure (Astley and Clarren, 1996; 2000). The FAS facial phenotype is based on the palpebral fissure size and the shape of the philtrum and the upper lip. The palpabral fissure is the eye opening, where in patients with FAS, it is

shorter from the left corner of the eye to the right corner. The philtrum is the vertical groove above the upper lip, and is smoother in FAS patients. The upper lip also tends to be thinner in FAS patients (Astley and Clarren, 1996; 2000). Microcephaly, a small head circumference, sometimes accompanies these abnormalities (Riley and McGee, 2005). There is controversy whether these facial features remain in adulthood, but the consensus is that the features remain past childhood and can still be used to diagnose FAS, although they are less pronounced after maturation (Chudley *et al*., 2007).

The effects of alcohol exposure on the brain seem to vary based on the amount of alcohol consumed, the peak blood alcohol level attained, the duration of the exposure and at what time during pregnancy the exposure occurred (Chudley *et al*., 2007). Although most FAS patients do not display gross anatomical CNS abnormalities, many have neurological damage which may induce symptoms such as seizures, poor coordination and balance, and visual motor difficulties (CDC, 2005; Chudley *et al*., 2005, 2007). The recommended assessment also includes testing the general intellectual ability (IQ), communication ability, academic achievement, memory, executive functioning and abstract reasoning, and social skills of the patient suspected to have FAS (Chudley et al., 2005). Mental retardation is uncommon in individuals with FASD but is increased in individuals with FAS, approximating 25% (Mattson and Riley,1998; Streissguth *et al*., 1997). However, impaired cognitive functioning has been observed in individuals displaying no facial abnormalities, reinforcing the idea that the effects of pre-natal alcohol exposure are variable (Mattson and Riley, 1998). As a result, many children with FASD do not qualify for special help since traditional cognitive testing does not take into account other neurocognitive deficits but focuses more on

criteria like IQ (Green, 2007). Individuals with FASD who display an average IQ often present deficits in planning, sequencing, reasoning and the ability to inhibit and learn from consequences (Mattson *et al*., 1999; Kodituwakku *et al*., 2001a; Kodituwakku *et al*., 2001b). Difficulties with attention and impulsivity also reflect on the overall academic achievement of individuals with FAS and FASD (Green, 2007). Specific neural areas affected include the ventricles, the corpus callosum, the anterior commissure, the basal ganglia and the cerebellum (Riley and McGee, 2005).

While investigating whether reflex reactions are also affected in FAS children, researchers have found that both pre-motor and motor components were lacking, indicating both central and peripheral mechanisms are affected by pre-natal alcohol exposure (Simmons *et al*., 2002; Riley and McGee, 2005). Delayed motor development and fine-motor defects have been reported in children with FAS, where tremors, a weak grasp, poor eye-hand coordination and slow fine motor speed were observed (Jones and Smith, 1973; Mattson *et al*., 1999; Kalberg *et al*., 2006). Development of fine motor skills were reported to be significantly more delayed compared to gross motor skills although the development of both was delayed in FAS children (Kalberg *et al*., 2006). Balance is particularly affected in individuals with FAS, although this deficit seems to be more central in nature where cerebellar damage is suspected to be the cause (Barr *et al*., 1990; Roebuck *et al*., 1992, 1998; Riley and McGee, 2005). In addition, infants with FAS display poor suckling skills and are easily distracted or fatigued while feeding (Martin *et al*., 1979; Streissguth, 1986). Children who had been exposed to more alcohol pre-natally took longer to initiate sucking and lower the sucking pressure when exposed to a non-nutritive nipple (Martin *et al*., 1979). Though CNS damage is

suspected to play a part in the motor deficits seen, little is known about the effects of alcohol on the peripheral nervous system (PNS).

There have been studies on secondary disabilities, which are disabilities that patients are not born with but develop (Streissguth *et al*., 1996; 1997). Adolescents and adults with FASD were reported to have an increased life-span prevalence of school disruption, getting in trouble with the law, detention/jail/instutional confinement, inappropriate sexual behaviours and alcohol/drug problems (Steissguth *et al*., 2004). Such poor outcomes were increased when the FASD diagnosis was made after 12 years old and when the patient came from an unstable, poor environment (Steissguth *et al*., 2004). Children diagnosed by the age of 6 years old were less likely to develop secondary disabilities (Streissguth *et al*., 1996). Risks of mental health problems are also increased in adults with FASD (Chudley *et al*., 2007). Ideal intervention in adulthood usually consists of a mentor being available on a daily basis to offer structure and order, and function as an 'exterior brain', since most FASD individuals lack judgment and adaptive skills (Chudley *et al*., 2007). During childhood, intervention targets daily routines, the clear presentation of rules, the reduction of distractions in the classroom, and sometimes involves physiotherapy (Green, 2007).

The health and education system have recognized the special needs of children with FASD. However, the cost of these special needs and the toll on human resources, demands more effective identification of the condition and better intervention (Spadoni *et al*., 2007). As such, although efforts are being made to prevent the occurrence of FASD, knowing more about the effects of pre-natal exposure to alcohol will result in improved life quality for the individuals affected by FASD (Green, 2007).

1.2. Animal models of FAS

Shortly after Jones *et al*. (1973) published their findings on FAS in humans, animal models were developed in order to explore the teratogenic effects of EtOH (Riley and McGee, 2005). Although efforts are being made to educate women and deter them from drinking while pregnant, ways to prevent or reduce the impacts of pre-natal alcohol exposure must be explored, as FAS in North America currently results more from compulsive alcohol abuse during pregnancy as opposed to ignorance (Cudd, 2005). Since human studies are limited by ethical constraints, animal models have allowed us further our understanding about FAS. Throughout this text, ethanol (EtOH) will be used to denote the purified form of alcohol, which is typically used in experiments involving animals. Alcohol refers to ethanol mixed with an inconsistent solution.

Experiments with animal models have the advantage of being precisely controlled, while human studies rely on self-reporting and drinking is often associated with tobacco or drug use, which alone have been shown to affect the fetus (Cudd, 2005). In addition, human studies have nutrition and genetics as added variables. However, although animal models are chosen for their similarity to humans, all models present some differences compared to humans and thus the investigator must be aware of these differences in order to reach meaningful conclusions. First of all, brain development occurs at different times during gestation (Dobbing and Sands, 1979). Structures are more vulnerable to damage by ethanol exposure when they are undergoing early and/or rapid development. For example, in rats, the majority of brain development occurs after birth while non-human primates and sheep experience brain development earlier in pregnancy than humans (Cudd, 2005). Therefore, ethanol

exposure must be timed precisely depending on which structure is being studied. Secondly, no animal model has shown all the diagnostic criteria of FAS, resulting in the necessity for multiple models and careful selection of the appropriate model (Cudd, 2005). Also, no animal model displays the same intelligence and behaviours as humans, although behavioural abnormalities such as feeding difficulties and attention deficit has been observed in animals exposed to ethanol pre-natally (Cudd, 2005; Driscoll *et al*., 1990).

Rodents are the most widely used animal models for FAS. Rats are well suited for behavioural studies, although the cerebellum and other neural structures develop after birth (Cudd, 2005). Guinea pigs have a longer gestation period but brain growth is at its maximum pre-natally, allowing researchers to study the effects of ethanol exposure during the third trimester. Rats and mice both have similar advantages: they have a short gestation, gestation is internal, they are relatively inexpensive and they have a strong potential for genetic manipulation (Cudd 2005). Rodent models display very similar neurobehavioural and physiological effects when exposed to ethanol pre-natally compared to humans, including altered brain development and poor motor coordination, while mice exhibit similar facial abnormalities when exposed to ethanol (Driscoll *et al.*, 1990). Rat pups also display feeding difficulties (latency in approaching the dam, reduced suckling pressure, etc.) in early life (Chen et al., 1982). Ethanol pre-natal exposure has been shown to cause altered neuronal migration and excessive cell death, leading to tissue disorganization and microcephaly in mice [and *Xenopus*] and evidence of this is seen in post-mortem human studies of FAS (Miller, 1993, 1996; Yelin *et al.*, 2007a; Niccols, 2007). In addition, when administered during synaptogenesis

(shortly after the birth of rodents, which is equivalent to the third trimester in humans), ethanol had a robust apoptotic neurodegenerative effect in mice and rats (Dobbing and Sands, 1979; Ikonomidou *et al*., 2000; Olney *et al*., 2002), which is thought to explain, in part, the neurobehavioural disturbances observed in children with FAS (Olney, 2004).

Large animals, though more expensive and more difficult to house, have the advantage of being more intelligent, possess a wider range of behaviours and have gestations that are longer (Cudd, 2005). Non-human primates are the most intelligent of animal species, although, like sheep, their maximum brain growth occurs early in gestation compared to humans. The pig, though seldomly used as a model for FAS, is the only species, other than humans, to show a natural preference for alcohol. In other models, ethanol has to be injected or combined with food (Cudd, 2005). The brain growth timing of the pig is the most similar to the human and the animal also displays potential for behavioural studies. Major disadvantages of the pig include the large litters produced and maintenance difficulties (Cudd, 2005).

Non-mammalian models, such as the chick, the zebrafish, the round worm and the fruit fly have been successful in studying FAS (Cudd, 2005). The chick, in particular, has been useful in studying the development of the face and the effects of ethanol on facial features (Cartwright and Smith, 1995). Advantages of non-mammalian models include the low cost of the animals and housing, a short incubation and the lack of a placenta (Cudd, 2005). It has been questioned whether there are interactions between the mother and the placenta, therefore this factor is removed when incubation is external. These models are useful when addressing basic questions involving genetics and development due to the relatively simple nervous systems (Cudd, 2005). However,

concern exists over the fact that higher ethanol concentrations are needed to induce defects similar to the ones seen in humans and mammalian models. Despite this, non-mammalian models, in particular zebrafish, offer advantages not found in other animal models. The remainder of this chapter will focus on the use of zebrafish as a model for FAS.

1.3. Zebrafish ecology and development

Zebrafish (*Danio rerio*) is a well known model for vertebrate development, and is becoming more important as a model for disease and nervous system organization (Lieschke and Currie, 2007; Fetcho 2007). The advantages offered by zebrafish as a model for FAS are significant and allow for a wide range of studies that may be difficult to perform in other preparations. When compared with mammalian models, the main advantages of zebrafish are their quick rate of development, their semi-transparent bodies, and their relatively simple nervous system. In addition, they have a characteristic escape behaviour which can useful for physiological and behavioural assays (Liu and Fetcho, 1999). Moreover, zebrafish embryos develop outside the mother in a chorion or egg casing, thereby allowing one to accurately control the concentration and the time course of ethanol exposure compared with placental organisms. It is important to note that zebrafish embryos exposed to EtOH exhibit similar properties to children with FAS, including defects in eye development, heart rate, apoptosis in the CNS, skeletal morphogenesis and deficits in locomotion (Carvan *et al*., 2004; Bilotta *et al*., 2004).

In nature, zebrafish are primarily found in north-eastern India, Bangladesh and Nepal, around the Ganges and Brahmaputra river basins (Spence *et al*., 2008). Zebrafish typically inhabit slow-moving or still-standing water bodies, as well as the edges of streams and ditches, most often adjacent to rice fields (Jayaram, 1999; Spence *et al*., 2008). These areas are thought to be preferred by the zebrafish because of the abundance of zooplankton, whose growth is stimulated by fertilizers from the rice paddies, and the lack of large predatory fishes in these shallow waters (Spence *et al*., 2007; Spence *et al*., 2006). Zebrafish are characterised by their small size (less than 120 mm in length), the 'danionin notch' in the ventromedial margin of the dentary bone, as well as the coloring of the skin, which alters from light to dark, creating a stripe pattern (Spence *et al*., 2008). Females can lay several hundred eggs every few days and the offspring reach sexual maturity after roughly three months. Eggs are relatively large compared to other fish species (about 0.7 mm in diameter at fertilization) and are also relatively transparent. Development occurs very rapidly: in less than two days after fertilization (dpf), all major organs are developed and shortly after, around 5 dpf, larvae display food seeking and predator avoidance behaviour (Spence *et al*., 2008; Kimmel *et al*., 1995).

Zebrafish developmental stages have been extensively described (Kimmel *et al*., 1995). When incubated at 28.5°C, embryos develop at a predictable pace with only slight variations between embryos. Zebrafish early development can be separated into 8 periods (Kimmel *et al*. 1995). At first, the zygote period represent the stage of the newly fertilized egg, which is followed by the cleavage period when the embryo undergoes 5 rapid cell cycles. The blastula period, from 2.25 hours post fertilization (hpf) to 5.25 hpf,

is when the embryo continues its cell divisions and epiboly begins, which is when the cells spread out over the yolk cell (Kimmel *et al*., 1995). Epiboly continues with the blastoderm spreading over 15% of the yolk cell every hour, which leads into the gastrula period (5.25-10 hpf). At the end of epiboly, muscle and notochord precursors are present and the tail bud forms (Kimmel *et al*., 1995). During the segmentation period (10-24hpf), the muscle somites develop while the embryo elongates and the tail bud becomes more apparent. Somites appear sequentially throughout the segmentation period and one can count them to accurately stage the embryo (Kimmel *et al*., 1995). Most skeletal muscle in the zebrafish embryo arises from the somites (Ingham and Kim, 2005). During this stage (around 16 hpf), neuromeres, which are distinctive CNS swellings that later become brain subdivisions, form and eyes can be seen (Kimmel *et al*., 1995). Around 18 hpf, the Mauthner neuron, an interneuron with a key role in the escape response, receives synaptic input from growing sensory neurons, such as Rohon Beard cells, and it's axon synapses onto spinal primary motor neurons (Kuwada *et al*., 1990; Kimmel *et al*., 1990; Kimmel *et al*., 1995). These primary motor neurons have axons extending into the periphery at 19 hpf and synapse onto muscle fibers, thus completing the reflex arc (Kuwada *et al*., 1990; Kimmel *et al*., 1990). When motor neurons first make contact with muscle fibers, spontaneous contractions are produced (around 17 hpf), which become stronger and more coordinated. By 22 hpf, tail lashes from side to side can be seen (Hanneman and Westerfield, 1989; Kimmel *et al*., 1995). During the pharyngula period (24-48 hpf), the tail continues to lengthen until 31-32 hpf. At this stage, the tail is no longer curved towards the yolk sac and the head straightens out (Kimmel *et al*., 1995). The heart becomes visible and starts beating just before 24

hpf. Pigment formation can be seen around the retinal epithelium and down the length of the embryo (Kimmel *et al*., 1995). Around 26-27 hpf, the Mauthner neuron starts to receive functional inputs from sensory cells (Kuwada *et al*., 1990; Kimmel *et al*., 1990; Ali *et al*., 2000b). The embryos now respond readily to touch and spontaneous body contractions are infrequent. Also, around 27 hpf, the embryos start to respond to touch by swimming, as opposed to just coiling (Saint-Amant and Drapeau, 1998). Around 30 hpf, the heart develops into two distinct chambers although blood circulation will not be seen in the extremities until 54 hpf (Kimmel *et al*., 1995). After this comes the hatching period (48-72 hpf), when branchial arches, which will bear the gills, form and circulation becomes stronger. The embryo becomes more pigmented and the yolk sac appears much smaller since it's nearing depletion (Kimmel *et al*., 1995). Throughout the third day, most embryos hatch out of their chorion and are named larvae by the end of the third day, whether they have hatched or not (Kimmel *et al*., 1995).

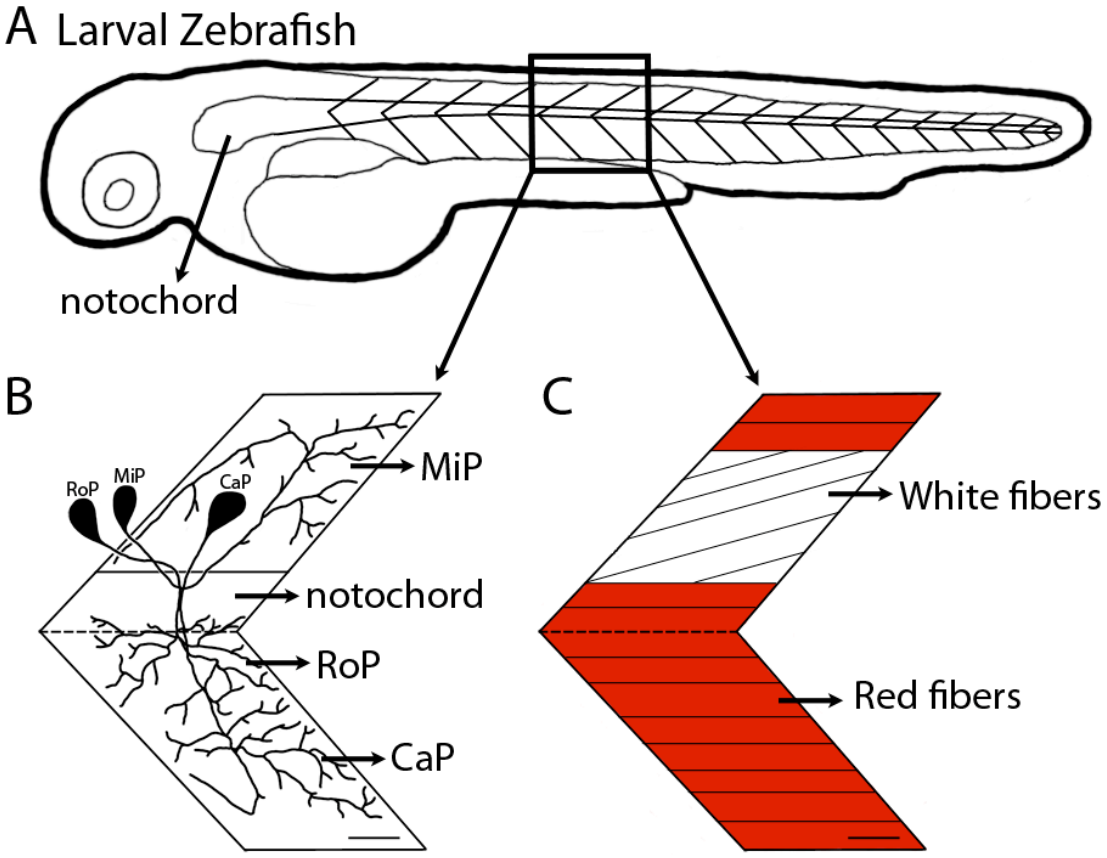
1.4. Zebrafish PNS and neuromuscular junction

Zebrafish mechanosensory perception is first mediated by Rohon-Beard (RB) cells which are primary sensory neurons that form around 18 hpf and reside in the dorsal spinal cord (Kimmel *et al*., 1995; Lewis and Eisen, 2003). Most of these cells undergo apoptosis soon after dorsal root ganglion cell formation (Svoboda *et al*., 2001; Williams *et al*., 2000) while some survive for several more days (Patten *et al*., 2007). The Mauthner cell, a large interneuron in the hindbrain that mediates primarily escape-response behaviours in the zebrafish, receives input from sensory cells such as Rohon-Beard cells and dorsal root ganglion cells (Kimmel *et al.* 1981; Metcalfe *et al.* 1990).

The Mauthner cell then synapses onto interneurons and spinal motor neurons, which innervate skeletal muscle, in order to mediate early embryonic behaviours (Kimmel *et al*., 1995; Kimmel and Westerfield, 1990). Other lower vertebrates, such as *Xenopus* embryos, adult lamprey and adult goldfish, have similar spinal cell types, indicating these animals share common developmental mechanisms (Brustein *et al.*, 2003; Hale *et al.*, 2001; Roberts, 2000).

Zebrafish skeletal muscle is innervated by primary and secondary motor neurons, located in the ventral region of the spinal cord (Kimmel and Westerfield, 1990). There are three primary motor neurons (sometimes 4) per segment which can be identified based on the musculature they innervate and the position of the cell body in the spinal cord (Figure 1). The caudal primary (CaP) motor neuron innervates the lower ventral trunk musculature, while the rostral primary (RoP) motor neuron innervates the upper region of the ventral trunk musculature, and the middle primary (MiP) motor neuron innervates the dorsal trunk musculature (Eisen *et al*., 1986; Eisen, 1991; Figure 1). The first primary motor neurons are born around 9 hpf while the first secondary motor neurons are born around 16 hpf (Myers *et al*., 1986). Secondary motor neurons follow paths similar to the primary motor neurons and are much more numerous compared to primary motor neurons, approximating 24 per segment (Myers *et al*., 1986). Motor neurons start firing action potentials around 24 hpf and display characteristic bursting patterns that change over the course of the first 5 days of development (Saint-Amant and Drapeau, 2000; Buss and Drapeau, 2001). This change in motor neuron firing is likely parallel to the changes seen in swimming behaviour, which is in part mediated by the changing properties of the neuromuscular junction

Figure 1. Diagram depicting a zebrafish larvae (A). (B) The three primary motor neurons present in each segment in zebrafish aged 3 dpf: middle primary (MiP), rostral primary (RoP) and caudal primary (CaP) (adapted from Pike and Eisen, 1990). The dotted line marks the horizontal myoseptum and the solid line marks the ventral aspect of the spinal cord. (C) The two muscle layers found in zebrafish aged 3 dpf, where the surface layer consists of red muscle fibers and the rest of the musculature is composed of white muscle fibers. Scale bar: 20 μm.


A Larval Zebrafish
notochord
B
RoP
MiP
CaP
MiP
notochord
RoP
CaP
C
White fibers
Red fibers

(NMJ; Nguyen *et al*., 1999; Saint-Amant and Drapeau, 1998; Buss and Drapeau, 2001).

Zebrafish larvae possess two types of skeletal muscle fibers: slow tonic (red) fibers and fast twitch (white) fibers (Buckingham and Ali, 2004; Greer-walker and Pull, 1975). Red muscle is only one layer thick and is directly underneath the skin, while white muscle fibers are found in numerous layers underneath the red muscle layer and make up the majority of the musculature (Greer-Walker and Pull, 1975; van Raamsdonk *et al*., 1982; Figure 1C). In adult fish, a third type of muscle fiber can be seen: pink fibers, which display intermediate properties (van Raamsdonk *et al*., 1982). Contrary to other vertebrates, zebrafish muscle is polyinnervated, where white fibers are innervated by one primary motor neuron and 2-3 secondary motor neurons while red fibers are innervated by a few secondary motor neurons only (Eisen, 1991; Westerfield *et al*., 1986; de Graaf *et al*.,1990; Liu and Westerfield, 1988). The muscle fibers perform different functions, where white fibers are inactive during slow swimming episodes and red fibers are de-recruited during fast swimming (Buss and Drapeau, 2002).

White fibers express Na^+ currents and high amplitude K^+ currents while red fibers lack Na^+ currents and express low amplitude K^+ currents (Buckingham and Ali, 2004; Coutts *et al*., 2006). As a result, red fibers are incapable of producing an action potential and are therefore probably tonic, non-twitch fibers (Morgan and Proske, 1984). In embryos, red and white fibers are extensively electrically coupled, although coupling decreases after hatching (Buss and Drapeau, 2000; Waterman, 1969; Buss and Drapeau, 2002; Luna and Brehm, 2006). Coupling in *Xenopus* and mammalian skeletal muscle fibers disappears in early life, although, in some species, such as the lamprey, skeletal muscle remains coupled even into adulthood. In zebrafish red muscle fibers,

coupling is maintained for a longer time period compared to white fibers (Buss and Drapeau, 2002; Luna and Brehm, 2006). This is thought to promote muscle depolarization, because current would be able to reach more distal electrical junctions by the transfer of current between tail segments, thus synchronizing the muscle contractions for undulatory swimming (Jaramillo *et al*., 1988; Bennett, 1966; Luna and Brehm, 2006).

During neuromuscular transmission, the neurotransmitter acetylcholine (ACh) is released by spinal motor neurons at the NMJ, where it binds to post-synaptic nicotinic ACh receptors (nAChRs), which are clustered at the NMJ (Figure 2; Dale *et al*., 1936; Katz, 1966). Miniature endplate currents (mEPCs) result from the spontaneous release (i.e. in the absence of pre-synaptic action potentials) of the contents of one pre-synaptic vesicle into the synaptic cleft, which activates nAChRs, allowing sodium, potassium and calcium to flow across the membrane (Figure 2; Sanes and Lichtman, 1999). The mEPCs produced by this flow of ions across the membrane can be recorded electrophysiologically by patching onto the muscle fibers with a glass pipette (Figure 3A). Tetrodotoxin (TTX) is added to the bathing solution during recordings to prevent action potentials (AP) in order to record only the spontaneous release of ACh (Figure 2). By analyzing the recorded events, one can examine the activation and de-activation kinetics of receptors and the release frequency of the pre-synaptic vesicles.

When contact is first made between the motor neurons and the muscle fibers, synaptic activity commences almost immediately (Kimmel *et al*., 1995; Sanes and Lichtman, 1999). However, the synapse is far from being mature and transmission is relatively inefficient (Sanes and Lichtman, 1999). When comparing mEPC events

Figure 2. Diagram of a vertebrate neuromuscular junction. Acetylcholine (ACh) contained in vesicles is released pre-synaptically from motor neurons spontaneously or as a result of an action potential (AP), although tetrodotoxin (TTX) can be used to block APs. Once released, ACh molecules make their way across the synaptic cleft and bind to nicotinic ACh receptors (nAChRs), which then activate to allow ions to flow across the muscle fiber endplate membrane.

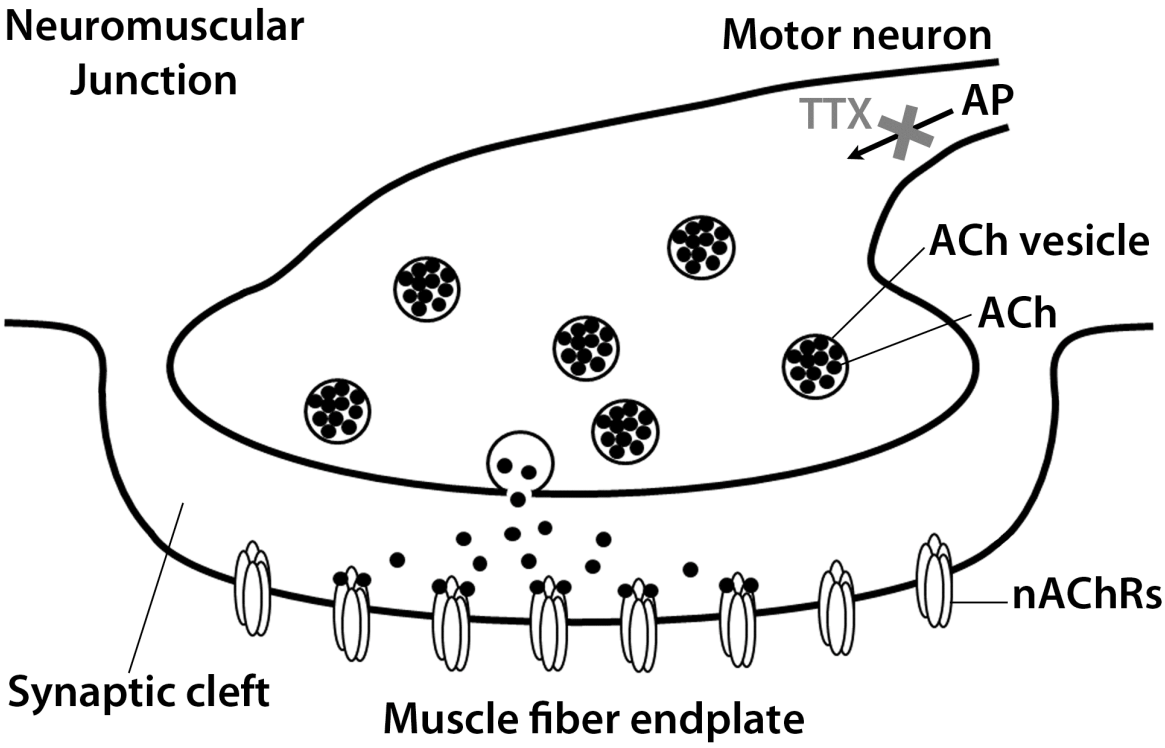
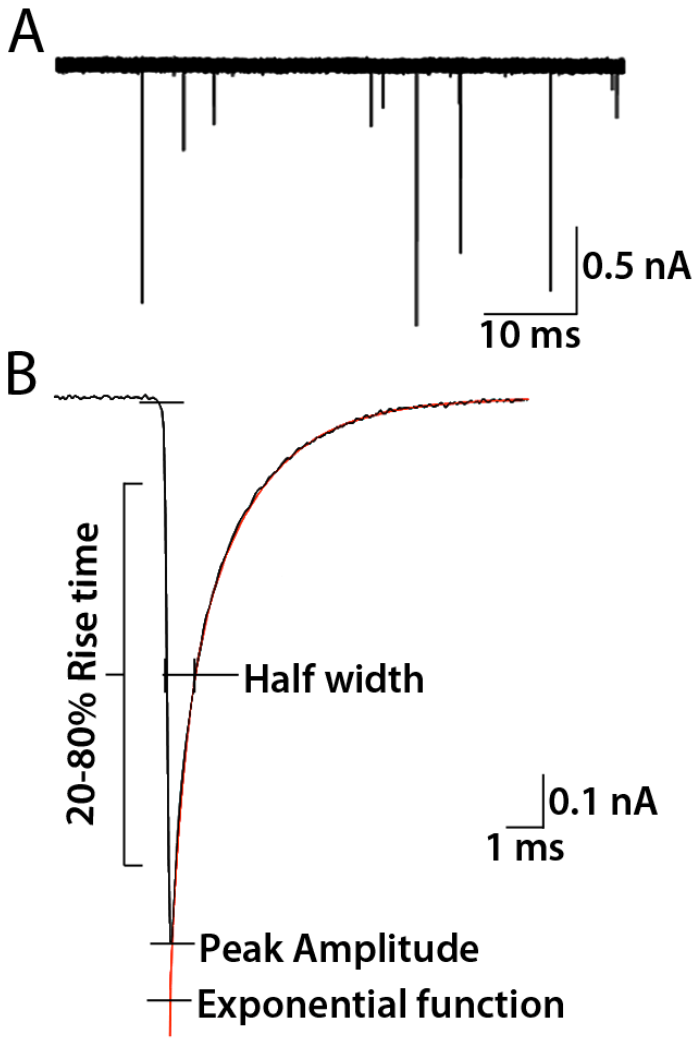

Neuromuscular
Junction
Motor neuron
TTX
AP
ACh vesicle
ACh
nAChRs
Synaptic cleft
Muscle fiber endplate

Figure 3. Representative trace of a mEPC recording from 3 dpf control larvae (A). (B) Average of the events detected in (A) showing the properties that are measured using averaged events during mEPC analysis: amplitude, rise time, half width and decay time constants, which are obtained with the exponential function. The recording was captured in the presence of TTX and carbonoxolone.


A
0.5 nA
10 ms
B
20-80% Rise time
Half width
0.1 nA
1 ms
Peak Amplitude
Exponential function

captured at 24 hpf, 3 dpf and 6 dpf, Nguyen *et al.* (1999) reported an increase in frequency and amplitude, and faster event kinetics. The faster, larger synaptic currents are thought to reflect the larvae's new found ability to swim in fast bursts after hatching (Nguyen *et al*., 1999; Saint-Amant and Drapeau, 1998; Buss and Drapeau, 2001). This increase in synaptic transmission efficiency is thought to be the result of several developmental changes, such the decrease in electrical coupling between fibers, a wider synaptic cleft, faster clearing of ACh in the synapse (by acetylcholinesterase (AChE)) and a faster rate of closing of nAChRs (Drapeau *et al*., 2001; Nguyen *et al*., 1999). Furthermore, as the synapse matures, ACh is released in lower concentrations and is cleared faster while nAChR become more densely packed at the endplate (Drapeau *et al*., 2001).

1.5. Zebrafish as a model for FAS

Recent studies have explored the possibility of using zebrafish embryos as an animal model for pre-natal alcohol exposure (Carvan *et al*., 2004; Bilotta *et al*., 2002, 2004; Matsui *et al*., 2006; Bradfield *et al*., 2006; Arenzana *et al*., 2006; Dlugos and Rabin, 2007; Li *et al*., 2007). Gross morphological effects of EtOH exposure on zebrafish embryos include pericardial edema (accumulation of fluid in the pericardial sac of the heart), axial malformations (clubbed/misshapen extremities), otolith defects, axial blistering and delayed development. Other effects seen include a reduced eye diameter, craniofacial and skeletal deformities, cardiac malformations, altered heart rate, hyperactivity, microcephaly and reduced length of the fish (Carvan *et al*., 2004;

Matsui *et al.*, 2006; Arenzana *et al.*, 2006; Dlugos and Rabin, 2007; Li *et al.*, 2007; Lockwood *et al*., 2004; Bilotta *et al*., 2002, 2004; Tanguay and Reimers, 2008).

Several studies have explored the effects of early EtOH exposure on the visual system of the zebrafish specifically, since 90% of children diagnosed with FAS display some kind of ocular problem (Arenzana *et al*., 2006; Matsui *et al*., 2006; Dlugos and Rabin, 2007; Bilotta *et al*., 2002; Stromland 1985, 1987). EtOH exposure from 2 dpf to 5 dpf at low concentrations (1%) caused altered photoreceptor function, even though the fish displayed no physical or retinal deformities, and a higher concentration (2%) disrupted photoreceptor and optic nerve development (Matsui *et al*., 2006). When exposed to 2.4% EtOH from 5 hpf to 24 hpf, zebrafish aged 2 dpf displayed an incidence of cyclopia of 50%, where the fish displaying cyclopia had delayed retinal development and an increased mortality rate (Aranzana *et al*., 2006). When exposed to 0.1%-1% EtOH from 0 dpf to 3 dpf, zebrafish aged 6 dpf displayed a significant decrease in the volumes of the photoreceptor, inner nuclear and ganglionic layers, which increased in severity in a dose-dependent manner (Dlugos and Rabin, 2007).

Ethanol exposure has also been shown to cause behavioural and neuronal changes in zebrafish embryos. Carvan and collegues (2004) reported an altered startle response and learning and memory deficits in zebrafish as a result of early exposure to low concentrations of EtOH. They also noticed increased cell death in the CNS. When exposed to 1% EtOH from 22 to 24 hpf, 6 month old zebrafish exhibited an altered behaviour in response to images of conspecifics and predators, where the exposed fish did not make an attempt to join conspecifics and did not swim away from an image of a predator (Fernandes and Gerlai, 2009), demonstrating that exposure to low EtOH

concentrations during a short period can have lasting behavioural effects. Early EtOH exposure has also been shown to induce defects in optic nerves and motor neurons (Parng *et al*., 2007). EtOH exposure may also be related to the loss of Sonic hedgehog (Shh) signal transduction (Li *et al.*, 2007), which is important for the normal development of motor neuron progenitors and somites.

1.6. Research objectives and hypothesis

The purpose of this thesis was to examine the effects of early ethanol exposure on cells involved in locomotion. Kalberg *et al.* (2006) hypothesized that delays in motor development seen in children with FAS may be related to specific neurobehavioral deficits. Changes in motor coordination have been observed in humans and other animal models with FAS (Kalberg *et al.*, 2006; Driscoll *et al.*, 1990), and an altered startle reflex response has been observed in zebrafish as a result of early EtOH exposure (Carvan *et al*., 2004). Therefore, I wanted to determine if cells involved in locomotion are affected by early EtOH exposure, and I hypothesized that embryos exposed to ethanol would have deficits in locomotion and abnormalities in motor neuron and possibly muscle fiber morphology. I also hypothesized that exposure to EtOH would alter the NMJ location and mEPC kinetics at the NMJ. In order to examine this, I first examined the swimming behaviour of control and EtOH-exposed fish. I then examined motor neuron and muscle fiber morphology using immunohistochemistry. I also looked at synaptic location and physiology using immunohistochemistry and electrophysiology, respectively.

2. Material and Methods

2.1. Animals and ethanol exposure

Wild type Zebrafish embryos were raised at 28.5°C and staged as previously described (Kimmel *et al.*, 1995; Westerfield, 2000). Embryos were exposed to various ethanol concentrations (1%, 1.5%, 2%, 2.5% and 3% v/v or approximately 171, 257, 342, 428 and 514 mM) at 8 hours post fertilization (hpf), by adding EtOH to the egg water (0.1mg/ml of Instant Ocean in distilled water). The exposure medium was then replaced at 1 dpf with fresh egg water. For immunohistochemistry studies, pigment formation was blocked by adding 0.003% phenylthiourea (PTU) to the egg water at 24 hpf. All protocols were carried out in compliance with guidelines described by the Canadian Council for Animal Care (CCAC) and the University of Alberta.

Gross deformities were observed at 3 dpf where the number of larvae exhibiting pericardial edema, axial abnormalities and/or cyclopia was counted for each treatment. Also, the number of fish that had hatched out of the chorion was recorded on each day until 7 dpf, as well as the number of fish still alive.

2.2. Swimming behaviour

Larvae (3 dpf) were anaesthetized in 0.02% tricaine (MS-222; Sigma Chemical, St. Louis, MO) and immobilized in low melting point agarose (1%; Sigma Aldritch, St Louis, MO) (Saint-Amant and Drapeau, 1998). The agarose was cut away at the level of the yolk sac to allow the tail to move freely while keeping the head immobilized. Preparations were washed with anaesthetic-free egg water and allowed to recover for 10 minutes. Swimming was evoked by touching the mid section of the embryo with thin

fishing line. I then counted the number of larvae that exhibited swimming behaviour after the initial touch response. Swimming was easily distinguishable from the touch response based on the angle of the tail undulations, where the tail movements during the touch response were very large and lasted only 1-2 cycles (Liao and Fetcho, 2008). Tail movements were captured with an Olympus DP72 camera at 42 ms intervals and were subsequently analyzed by visually distinguishing the large angle tail flicks during the escape response, from the small angle sinusoidal tail movements that occur during swimming.

2.3. Immunohistochemistry

For labelling with the anti-acetylated tubulin antibody, larvae at 3 dpf were fixed overnight at 4°C in Dents fixative (80% methanol, 20% DMSO) and then rehydrated in 75%, 50% and 25% methanol the next day, followed by 3 h of washing in PBS-T (phosphate buffered saline (PBS) and 0.5% Tween-20; Hanington *et al.*, 2008). PBS consisted of (in mM): 150 NaCl, 8 NaH_2HPO_4, 2 $NaH_2PO_4 \cdot 2H_2O$, and had a pH of 7.2. Larvae were then incubated in a blocking buffer for one hour, which contained 20% bovine serum albumin (BSA) and 10% goat serum in PBS-T. They were incubated overnight at 4°C in anti-acetylated tubulin monoclonal antibody (1:500; Sigma-Aldrich; Hanington *et al.*, 2008; Table 1). The next day, larvae were washed with PBS-T and then incubated blocking solution for an hour, followed by overnight incubation at 4°C in a goat anti-mouse IgG coupled to Alexa Fluor 488 (1:1000; Molecular Probes). Preparations were then washed for 7 h in PBS-T, followed by mounting in glycerol for viewing.

Table 1. Antibodies

Antibody	Source	Primary antibody concentration	Secondary antibody concentration***	Epitope	References
Anti-acetylated tubulin	Sigma-Aldrich*	1:500	1:1000	Acetylated tubulin	Hanington *et al*. (2008)
znp-1	DSHB**	1:250	1:1000	Primary motor neuron axons	Trevarrow *et al*. (1990)
zn-8	DSHB**	1:250	1:1000	DM-GRASP, secondary motor neuron axons	Fashena and Westerfield (1999)
F59	DSHB**	1:50	1:1000	Myosin heavy chain, red muscle cells	Crow and Stockdale (1986)
F310	DSHB**	1:250	1:1000	Myosin light chain, white muscle cells	Crow and Stockdale (1986)
3A10	DSHB**	1:250	1:1000	Neurofilament-M; primary motor axons	Serafini *et al*. (1996)
SV2	DSHB**	1:100	1:2000	Endocrine synaptic vesicle transmembrane glycoprotein	Buckley and Kelly (1985)

* Sigma-Aldrich, St. Louis, MO

** Developmental Studies Hybridoma Bank, University of Iowa, Iowa city, Iowa

*** The secondary antibody goat anti-mouse IgG coupled to Alexa Fluor 488 (Molecular Probes) was used when labelling with the anti-acetylated tubulin, znp-1, zn8, F59 and F310 primary antibodies, while goat anti-mouse IgG coupled to Cy3 (Jackson ImmunoResearch) was used when labelling with the 3A10 and SV2 primary antibodies.

For labelling with the znp-1, zn-8, F59 and F310 antibodies, larvae at 3 dpf were fixed in 2% paraformaldehyde for 1-2 hours (h) and washed with PBS for 2-3 h. The preparations were then permeabilized for 30 mins in 4% Triton-X 100 containing 2% BSA and 10% goat serum. Larvae were incubated for 48 h at 4°C in either mouse monoclonal antibody znp-1 at 1:250, mouse monoclonal antibody zn-8 at 1:250, mouse monoclonal F59 at 1:50 or mouse monoclonal F310 at 1:250 (Table 1). Larvae were then washed for 2-3 h in PBS and incubated in the secondary antibody for 4 h at room temperature. The secondary antiserum was goat anti-mouse IgG coupled to Alexa Fluor 488 (1:1000; Molecular Probes). Larvae were then washed with PBS for 7 h before being cleared in 70% glycerol.

For double labelling studies, procedures followed the same protocol as above although the primary antibodies used were either mouse monoclonal 3A10 (Serafini *et al*., 1996; DSHB, U of Iowa) at 1:250 or mouse monoclonal SV2 (Buckley and Kelly, 1985; DSHB, U of Iowa) at 1:100 and the secondary antibody used was goat anti-mouse IgG coupled to Cy3 (Jackson ImmunoResearch) at 1:1000 for 3A10 and at 1:2000 for SV2. Once the secondary antibody had been removed, tissues were incubated in Alexa Fluor 488-conjugated α-bungarotoxin (1 μM; Molecular Probes) for 30 mins. The larvae were then washed for 7 hours with PBS, followed by mounting in glycerol for viewing.

2.4. Electrophysiology

Prior to dissections, 3 dpf fish were anaesthetized in 0.02% tricaine (MS-222; Sigma Chemical, St. Louis, MO) in normal extracellular solution (ECS). The ECS consisted of (in mM): 134 NaCl, 2.9 KCl, 2.1 $CaCl_2 \cdot 2H_2O$, 1.2 $MgCl_2 \cdot 6H_2O$, 10 HEPES, 10 D-glucose. The pH was adjusted to 7.8 using 1N NaOH and the final osmolarity was adjusted to 290±2 mOsm.

Dissections consisted of pinning the larvae through the notochord onto a sylgard-lined glass coverslip in a recording chamber and removing the skin covering the tail muscle with fine forceps. Red and white muscle fibers were distinguishable under Nomarski Differential Interference Contrast (DIC) based on orientation (Buckingham and Ali, 2004). The first layer of muscle consists of red fibers, which run parallel to the notochord, while white fibers can be found immediately under red fibers and run at a slight angle to the notochord. Lucifer yellow (0.1%) was added to the pipette-filling solution to verify fiber identity after individual recordings and to ensure that the fiber was not electrically coupled.

Patch-clamp electrodes were pulled from borosilicate glass and had resistances between 0.95 and 1.6 MΩ after being filled with an intracellular solution. The intracellular solution consisted of (in mM): 130 CsCl, 8 NaCl, 10 HEPES, 10 EGTA, 2 $CaCl_2 \cdot 2H_2O$, to which Mg-ATP and Li-GTP was added fresh daily. The pH of the intracellular solution was adjusted to 7.4 using CsOH and the final osmolarity was adjusted to 290±2 mOsm. During all whole cell recordings, the larvae were continuously perfused (1-2 ml/min) with a tricaine-free extracellular recording solution containing tetrodotoxin (TTX; 1 μM), to block action potentials, and carbonoxolone (100 μM), to

block gap junctions in order to reduce electrical coupling (Personius and Balice-Gordon, 2001; Luna *et al*., 2004).

Whole-cell currents were recorded at a holding potential of -60mV using an Axopatch 200B amplifier (Axon Instruments) and were low-pass filtered at 10 kHz and digitized at 50 kHz. Immediately after establishment of the whole-cell recording mode, series resistance was compensated by 80-90%, using the amplifier's compensation circuitry. Experiments were aborted if the series resistance changed by more than 20%.

2.5. Analysis and statistics

All larvae were imaged on a Zeiss LSM confocal microscope and photographed under a 20x objective. Images were compiled using Zeiss LSM Image Browser software and shown as z-stacks of the trunk unless specified. Because I was unable to differentiate between CaP and RoP motor neurons, due the close proximity of their innervations fields and their axons, I referred to them as the ventral motor neurons, and, for simplicity, I referred to the MiP motor neuron as the dorsal motor neuron. To determine the effects of EtOH exposure on motor neurons, each axon was given an overall score of 1 to 4 (Table 2), where 1 denotes severe defects and 4 denotes no defects (Carrel *et al*., 2006; McWhorter *et al*., 2008). For each larva, 6-8 ventral and dorsal axons were examined and the proportion of observed motor neuron axons with each score (1, 2, 3 or 4) was calculated for each larvae. A total of 246 fish were examined for immunohistochemistry (86 control larvae, 49 1.5% EtOH-exposed larvae, 61 2% EtOH-exposed larvae and 61 2.5% EtOH-exposed larvae). The average proportion of axons with each score was calculated for control and exposed larvae.

Table 2: Classification of ventral motor neuron axon defects

Score	Axon morphology
1	Severe defects such as complete absence, early truncation of the axon, innervation of the neighbouring muscle segment or presence of an extra axon between segments.
2	Moderate defects such as an abnormal axon morphology
3	Mild defects such as mildly misshapen axons
4	No defects

Listed are motor neuron axon defects observed in zebrafish larvae aged 3dpf, labelled with the anti-acetylated tubulin, znp-1 or zn-8 antibody, with the corresponding score. This classification table is based on previous classification schemes (Carrel *et al*., 2006; McWhorter *et al*., 2008), with modifications.

Acquired images of tissues labelled with F59 and F310 were analysed using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA, USA). For each fish examined, the angle was determined after tracing the outline of the segments, using Adobe Photoshop CS4, and three hemi-segments were randomly chosen for measurements. For each hemi-segment examined, the number of fibers was counted, and the length and width of three fibers were measured, where the width of the fibers was measured at three locations along their length and averaged. Measurements were not averaged per fish or per hemi-segment. The fibers measured were chosen based on their position within the hemi-segment, where a fiber from near the centre line, the middle and the outer edge of the hemi-segment were analyzed. For analysis using F310, measurements was performed on one image slice taken midway through the white muscle, due to the thickness of the white muscle layer. For all immunohistochemistry experiments, at least three fish were examined per trial and at least three trials were conducted for each antibody used. Negative controls were also conducted and no unusual background was detected (data not shown).

Synaptic events were detected and analysed with Axograph 4.6 (Axon Instruments). All events were visually inspected and separated into two types of events (slow and fast events, which will be discussed in the results section 3.5) based on amplitude and rise time. Events with uneven baselines or overlaying events were discarded. mEPC properties were measured from the averaged events obtained fror each individual recording. The peak amplitude was measured from the base of the averaged mEPCs to their peak while the rise time was measured from 20% to 80% of the peak amplitude (Figure 3B). The half width was measured at 50% of the peak

amplitude (Figure 3B). The decay time course was analysed by fitting the averaged events with a sum of exponential curves (Figure 3B), from which the decay time constant(s) (τ) was obtained.

All values are reported as means ± SEM (standard error of the mean). Significance was determined using a one way ANOVA ($p<0.05$). Statistical analysis was done using SigmaPlot 11.0 (SPSS) and Microsoft Office Excel 2007.

3. Results

3.1. Gross morphology, mortality and locomotion

Zebrafish embryos were exposed to 1%, 1.5%, 2%, 2.5% and 3% (v/v) (or approximately 171, 257, 342, 428 and 514 mM) EtOH from 8 hpf to 24 hpf, and general morphological changes were observed at 3 dpf (Figure 4,5). I chose this exposure time course (8-24 hpf) because I wanted to examine the effects of EtOH exposure on cells involved in locomotion, such as motor neurons and muscle fibers (Figure 4). Genesis of the primary neurons occurs from 10 to 12 hpf in the zebrafish embryo, followed by the elongation of the primary axons a few hours later, at 20 hpf (Kimmel *et al*., 1991; Kimmel and Westerfield, 1990). Furthermore, somite formation begins at 10 hpf and ends at 24 hpf (Hanneman and Westerfield, 1989), and muscle fibers begin to form shortly after (1-2 h) somite formation (Devoto and Melançon, 1996). EtOH concentrations were chosen based on previous studies that reported morphological and behavioural effects as a result of zebrafish exposure to concentrations between 1.5% and 2.9% (Arenzana *et al*., 2006; Bilotta *et al*., 2004; Figure 4).

When zebrafish embryos were exposed to EtOH increasing concentrations of EtOH, 3-dpf fish displayed a reduced body length, smaller eye diameter and smaller head size (Figure 5). Fish exposed to concentrations above 1.5% EtOH displayed an increased incidence of pericardial edema (Figure 6A; $p<0.05$). In larvae exposed to 2.5% and 3%, fish displayed significantly more axial malformations and instances of cyclopia (Figure 6A; $p<0.05$). These effects increased in severity and frequency in a dose dependent manner. Larvae exposed to 1% EtOH exhibited no significant morphological defects. Larvae exposed to 2.5% and 3% EtOH exhibited a significantly

Figure 4. Diagram comparing the exposure windows and concentrations of EtOH used in various studies, when determining the effects of EtOH exposure on zebrafish embryos and larvae.

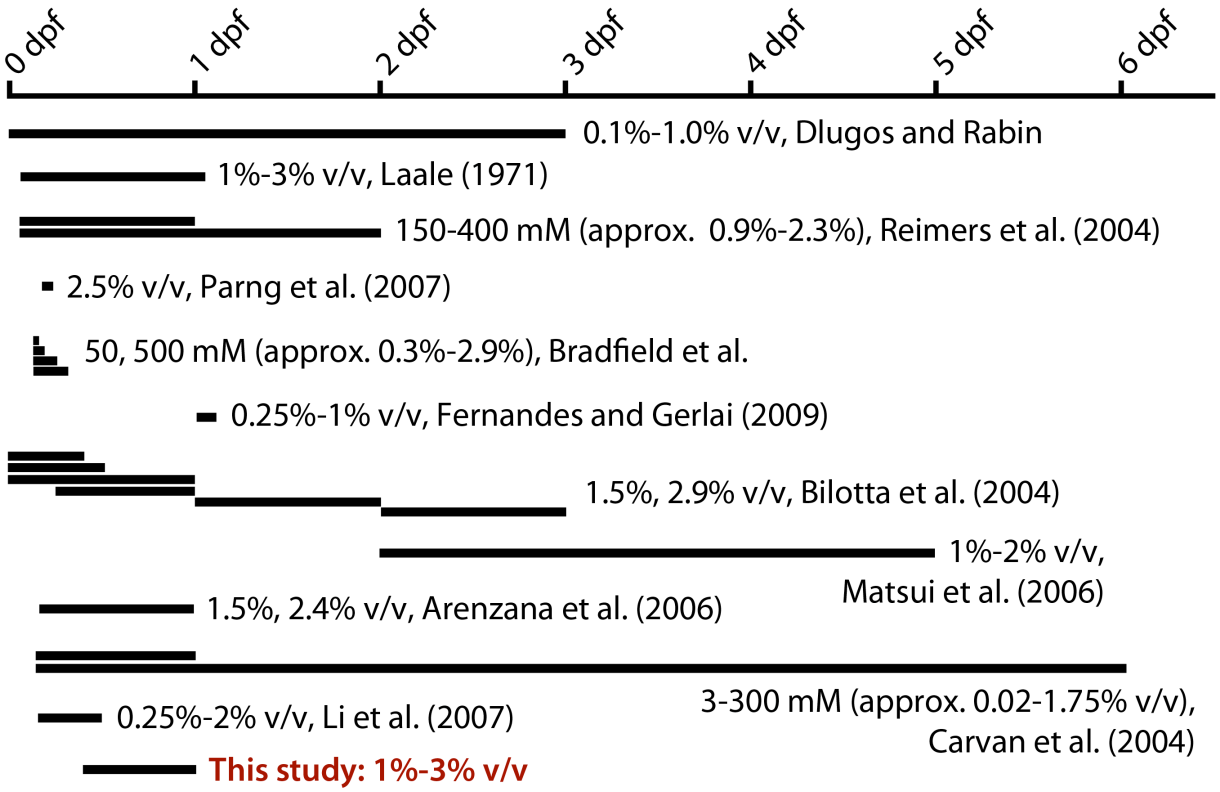

0 dpf
1 dpf
2 dpf
3 dpf
4 dpf
5 dpf
6 dpf
0.1%-1.0% v/v, Dlugos and Rabin
1%-3% v/v, Laale (1971)
150-400 mM (approx. 0.9%-2.3%), Reimers et al. (2004)
2.5% v/v, Parng et al. (2007)
50, 500 mM (approx. 0.3%-2.9%), Bradfield et al.
0.25%-1% v/v, Fernandes and Gerlai (2009)
1.5%, 2.9% v/v, Bilotta et al. (2004)
1%-2% v/v,
Matsui et al. (2006)
1.5%, 2.4% v/v, Arenzana et al. (2006)
3-300 mM (approx. 0.02-1.75% v/v),
Carvan et al. (2004)
0.25%-2% v/v, Li et al. (2007)
This study: 1%-3% v/v

Figure 5. Effect of EtOH exposure on zebrafish morphology. Embryos were exposed to 0% (A), 1% (B), 1.5% (C), 2% (D), 2.5% (E) or 3% (F) EtOH from 8 hpf to 24 hpf and then allowed to develop in normal EtOH-free egg water. All images were captured at 3 dpf. Embryos exposed to higher EtOH concentrations exhibit smaller eyes, an altered tail length, curved spines and pericardial edema. Scale bar: 20 μm.

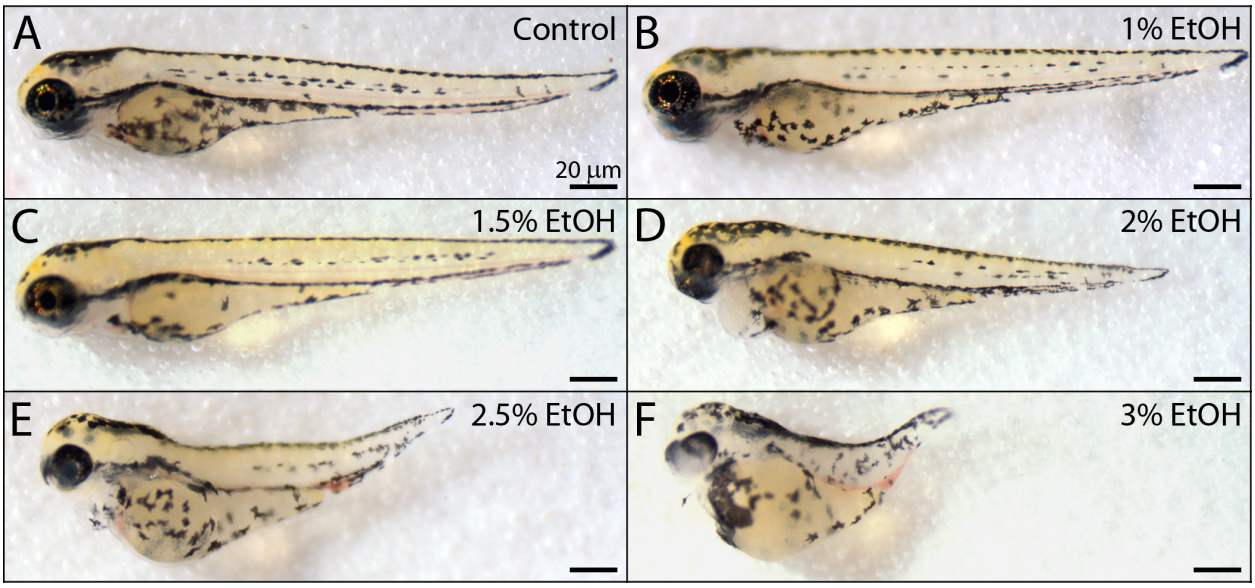
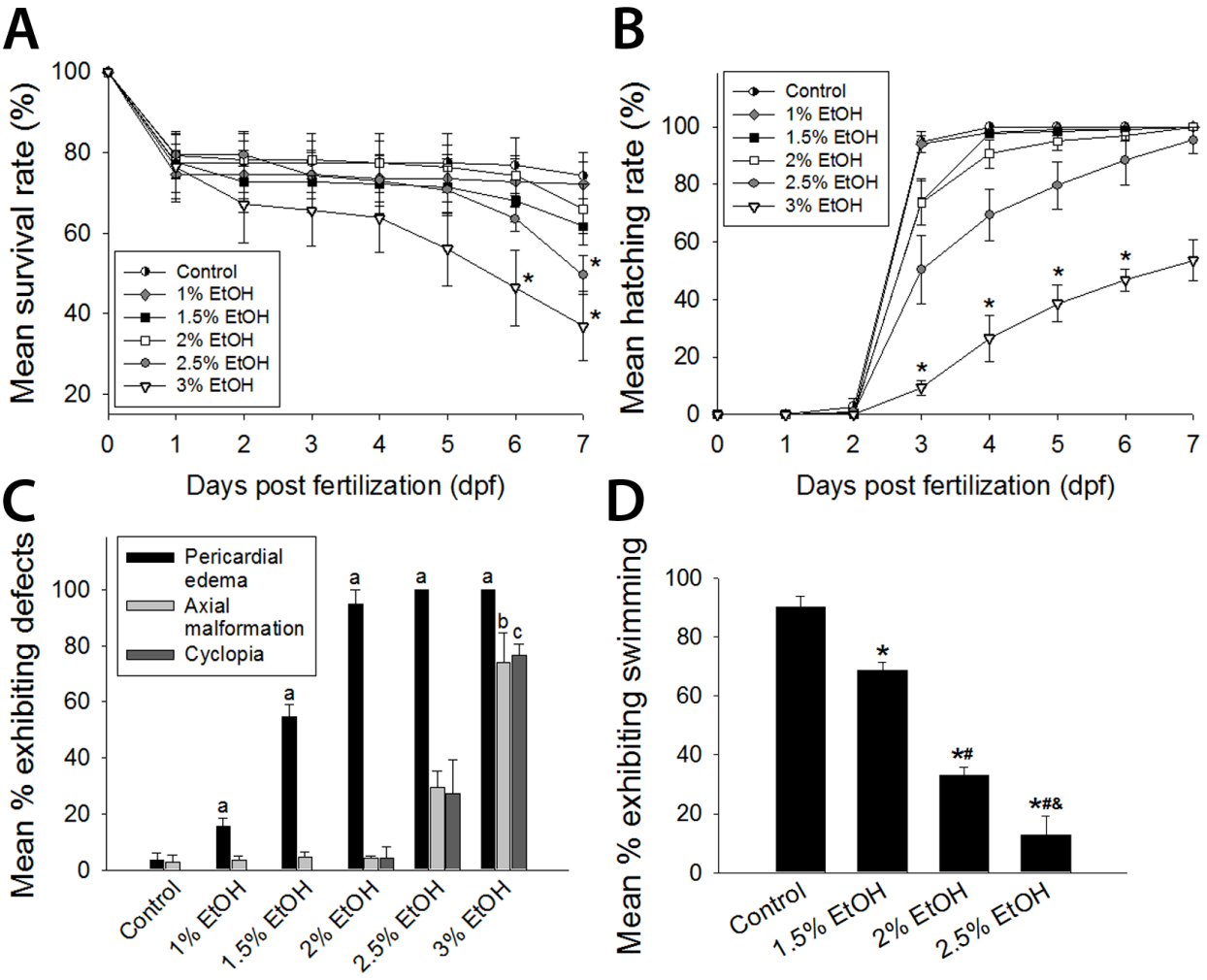

A
Control
20 µm
B
1% EtOH
C
1.5% EtOH
D
2% EtOH
E
2.5% EtOH
F
3% EtOH

Figure 6. Effect of EtOH exposure on zebrafish survival rate, hatch rate, morphology and swimming behavior. (A-B) Line graph showing the rate of survival (A) and the rate of hatching (B) during the first week of development (n=140, 144, 140, 163, 157 and 155 fish examined for 0%, 1%, 1.5%, 2%, 2.5% and 3% EtOH-treated fish respectively). (C) Bar graph showing the rate of deformities in EtOH-treated and untreated fish at 3 dpf (n=147, 155, 146, 165, 156 and 142 fish examined for 0%, 1%, 1.5%, 2%, 2.5% and 3% EtOH treated fish respectively). (D) Bar graph showing effect of EtOH treatment on zebrafish swimming response following touch to the trunk at the level of the yolk sac. Embryos were exposed to normal egg water (n=108 fish), 1.5% (n=76 fish), 2% (n=66 fish) or 2.5% EtOH (n=68 fish) from 8 hpf to 24 hpf and swimming was captured at 3 dpf and subsequently analyzed. [a] Significantly different from % controls regarding pericardial edema, $p<0.05$; [b] significantly different from % controls regarding axial malformations, $p<0.05$; [c] significantly different from % controls regarding cyclopia, $p<0.05$. * Significantly different from control, [#] significantly different from 1.5% EtOH exposed larvae, [&] significantly different from 2% EtOH exposed fish, $p<0.05$. Scale bar: 20 μm.


A
Mean survival rate (%)
Days post fertilization (dpf)
Control
1% EtOH
1.5% EtOH
2% EtOH
2.5% EtOH
3% EtOH
B
Mean hatching rate (%)
Days post fertilization (dpf)
C
Mean % exhibiting defects
Pericardial edema
Axial malformation
Cyclopia
Control
1% EtOH
1.5% EtOH
2% EtOH
2.5% EtOH
3% EtOH
D
Mean % exhibiting swimming
Control
1.5% EtOH
2% EtOH
2.5% EtOH

decreased survival rate, where more than half of the fish exposed to 3% EtOH had died by 7 dpf (Figure 6B; $p<0.05$). To determine if locomotion was affected by EtOH exposure, I first determined the number of fish that hatched on each day of development (Figure 6C). All surviving untreated embryos hatched by 4 dpf, and at least half of 1%, 1.5% and 2% EtOH-treated fish hatched by 4 dpf (Figure 6C). Meanwhile, larvae exposed to 2.5% EtOH took longer to hatch and over half of larvae exposed to 3% EtOH did not hatch by 7 dpf (Figure 6C). Based on these observations, I conducted the rest of the study on larvae exposed to 1.5%, 2% and 2.5% EtOH only.

I recorded swimming episodes that occurred immediately after the escape response, following touch of the trunk of the fish (Saint-Amant and Drapeau, 1998) to determine if EtOH exposure affected swimming behaviour (Figure 6D). When compared to controls, exposed larvae exhibited significantly fewer bouts of swimming. Approximately 68±3% of fish treated with 1.5% EtOH exhibited swimming after responding to the touch stimulus (Figure 6D). This fell to only 33±3% and 13±6% for fish exposed to 2% and 2.5% EtOH, in comparison with 90±4% for the untreated fish (Figure 6D; $p<0.05$ between all treatments). Furthermore, we noticed that unrestrained, EtOH-treated fish generally exhibited fewer swimming episodes compared with controls. These results were consistent with our hypothesis and prompted us to perform an examination of the morphology of the motor neurons and muscle fibers.

3.2. Motor neuron morphology

Motor neuron axon morphology was scored from 1 to 4 as described in Table 2, where a score of 4 was attributed to axons with no defects and a score of 1 was

attributed to axons with severe defects. To determine if axons throughout the CNS were affected by exposure to ethanol, cells with the anti-acetylated tubulin antibody (Wilson *et al*., 1990; Ton *et al*., 2006). Ethanol exposure induced a number of morphological defects throughout the CNS, but this study focused on motor neuron and muscle fiber morphology. When compared with controls, larvae exposed to EtOH exhibited altered ventral and dorsal motor neuron axon morphology. For example, exposed larvae exhibited ventral projections that curved abnormally within the ventral musculature and in some cases, terminated prematurely. Dorsal axons projected at aberrant angles and curved rostrally across the spinal cord (Figure 7A-D). Quantification of this data is given in Figure 7E-F, which indicates the relative percentages of axons with normal trajectories and innervation patterns in controls and EtOH-exposed fish. A score of 4 denoted no defects while a score of 3 denoted mild defects, a score of 2 denoted moderate defects and a score of 1 denoted severe defects. Exposure to higher levels of EtOH (2% and 2.5%) resulted in a greater proportion of fish exhibiting moderate defects (Figure 7; $p<0.05$). For instance, fish exposed to 2.5% EtOH (n=117 axons) exhibited 25±4% moderate defects in ventral axons compared with control values (n=133 axons) of 2±1% for moderate. 1.5% EtOH-treated fish only exhibited a significant increase in the number of mild defects of ventral axons. Furthermore, fish exposed to 2.5% EtOH had significantly fewer branches projecting from the main ventral axon (6.0±0.2 branches; n=117 axons) compared with control larvae (6.8±0.1 branches; n=133 axons; Figure 7A-D,G; $p<0.05$).

In order to differentiate between primary and secondary motor neurons, zebrafish larvae were immunolabelled with the znp-1 antibody, a specific marker for primary

Figure 7. Anti-acetylated tubulin labelling in untreated controls and EtOH-treated fish at 3 dpf. Fish were exposed to 0% (A, n=133 axons in 21 fish), 1.5% (B, n=67 axons in 11 fish), 2% (C, n=88 axons in 13 fish) and 2.5% (D, n=117 axons in 16 fish) EtOH. Typical abnormalities such as curved axons and aberrant branching patterns, seen in EtOH exposed larvae are shown by arrows for ventral motor neurons and by arrowheads for dorsal motor neurons. (E-F) Bar graphs showing the percentage of ventral (E) and dorsal (F) motor neurons scored with a value of 1, 2, 3 or 4 based on Table 2, where '1' represents severe defects and '4' represents no defects. (G) Bar graph quantifying the mean number of branches projecting from the ventral motor neuron axon (n=88-117 axons). [a] Significantly different from control score 4, [b] Significantly different from control score 3. [c] significantly different from control score 2, $p<0.05$. * Significantly different from control, $p<0.05$. Scale bar: 20 μm.

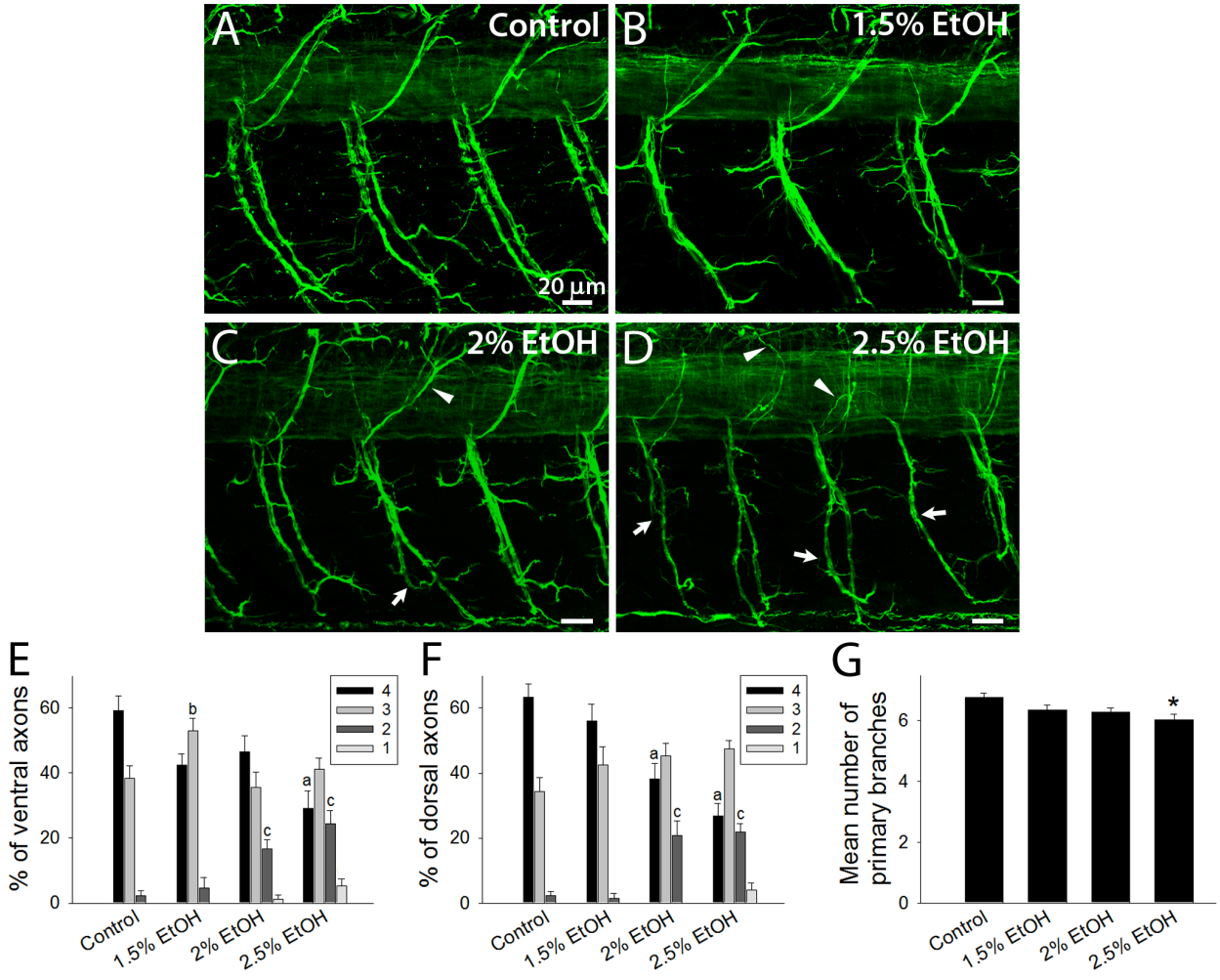
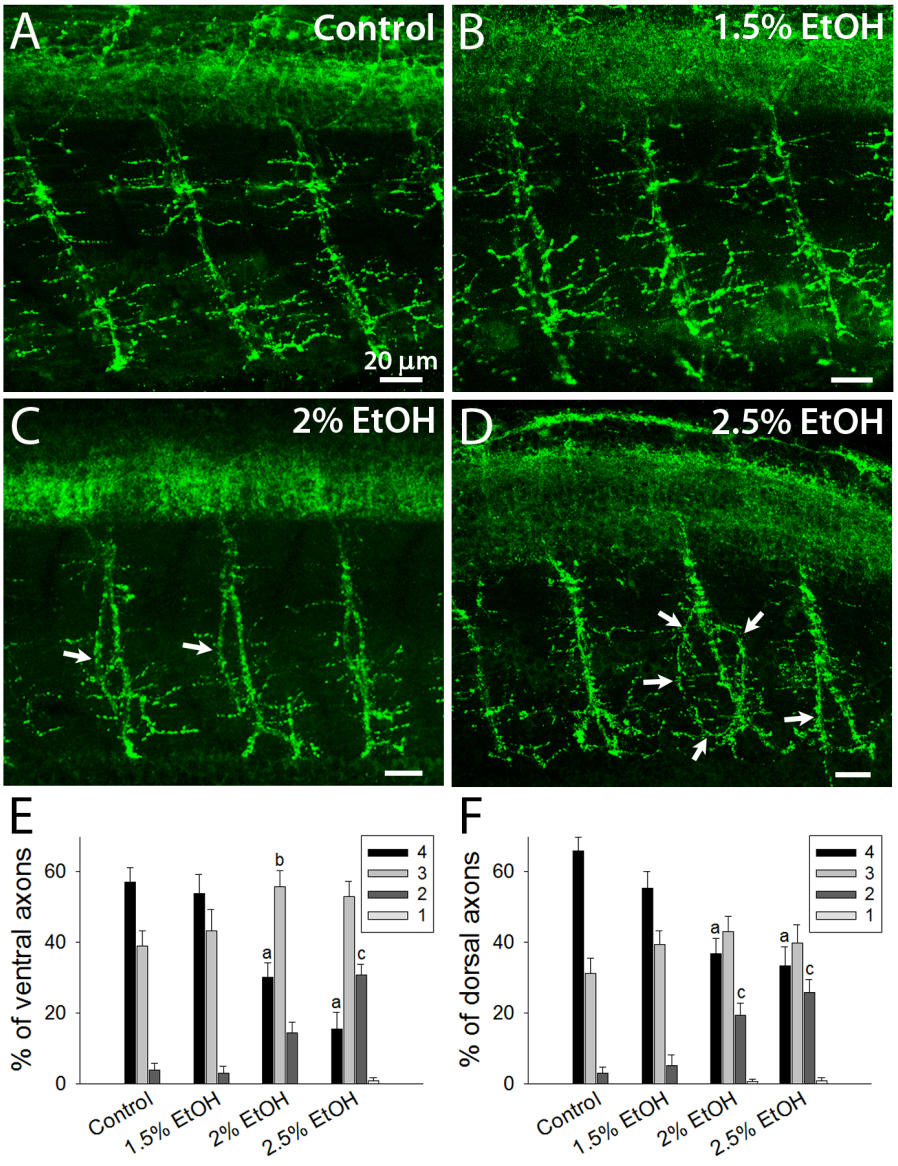

A
Control
20 µm
B
1.5% EtOH
C
2% EtOH
D
2.5% EtOH
E
% of ventral axons
60
40
20
0
4
3
2
1
a
b
c
c
a
c
Control
1.5% EtOH
2% EtOH
2.5% EtOH
F
% of dorsal axons
60
40
20
0
4
3
2
1
a
c
a
c
Control
1.5% EtOH
2% EtOH
2.5% EtOH
G
Mean number of primary branches
6
4
2
0
*
Control
1.5% EtOH
2% EtOH
2.5% EtOH

Figure 8. Primary motor neuron morphology in larvae at 3 dpf following exposure to 0% (A, n=105 axons in 19 fish), 1.5% (B, n=64 axons in 11 fish), 2% (C, n=107 axons in 16 fish), 2.5% (D, n=89 axons in 11 fish) EtOH and labelled with znp-1. Photos shown were optimized to demonstrate ventral motor neuron abnormalities seen in EtOH exposed fish (shown by arrows). (E-F) Bar graphs showing the number of ventral (E) and dorsal (F) motor neurons scored a 1, 2, 3 or 4 based on Table 2, where 1 represents severe defects. [a]Significantly different from control score 4, [c] significantly different from control score 2, $p<0.05$. Scale bar: 20 μm.


A
Control
20 µm
B
1.5% EtOH
C
2% EtOH
D
2.5% EtOH
E
% of ventral axons
0
20
40
60
4
3
2
1
a
b
a
c
Control
1.5% EtOH
2% EtOH
2.5% EtOH
F
% of dorsal axons
0
20
40
60
4
3
2
1
a
c
a
c
Control
1.5% EtOH
2% EtOH
2.5% EtOH

motor neuron axons (Figure 8; Trevarrow *et al.*, 1990). Larvae exposed to EtOH exhibited primary motor neuron axons that were curved, projected at aberrant angles, or branched abnormally, or any combination of these (Figure 8A-D). Larvae exposed to 2% and 2.5% EtOH displayed a significantly greater number of primary motor neuron axons with defects in both the dorsal and ventral regions of the trunk (Figure 8). For instance, fish exposed to 2.5% EtOH exhibited 31±3% moderate defects in their ventral axons (n=89 axons) compared with controls (4±2% moderate defects, n=105 axons). Fish treated with 1.5% EtOH did not exhibit any motor neuron defects (Figure 8; n=64 axons; $p>0.05$). Consistent and reliable counting of the number of primary branches projecting from the primary motor neuron axons was not feasible.

In order to assess the effects of EtOH exposure on secondary motor neurons, larvae were immunolabelled with the zn-8 antibody, which recognizes DM-GRASP on the surface of secondary motor neurons (Figure 9; Fashena and Westerfield, 1999). Secondary motor neurons follow a path similar to the one taken by primary motor neurons (Figure 1B), although they do not extend as far and possess fewer peripheral branches (Myers *et al*., 1986). An exposure concentration of 1.5% EtOH did not have a significant effect on the axons of secondary motor neurons (Figure 9E, F). When compared to controls, 2% and 2.5% EtOH-exposed fish displayed ventral motor neuron abnormalities such as altered axon branching and excess curvature, while dorsal motor neurons displayed abnormalities such as aberrant projection angles (Figure 9A-D). For instance, quantification of these results indicate that 2.5% EtOH-exposed fish had significantly more ventral secondary motor neuron axons with moderate defects (18±4%; n=82 axons) compared to controls (3±1% with moderate defects; $p<0.05$;

Figure 9. Secondary motor neuron morphology in larvae at 3dpf following exposure to 0% (A, n=122 axons in 20 fish), 1.5% (B, n=54 axons in 9 fish), 2% (C, n=82 axons in 12 fish), 2.5% (D, n=75 axons in 10 fish) EtOH and labelled with zn-8. Typical abnormalities such as curved axons and aberrant branching patterns, seen in EtOH exposed larvae are shown by arrows for ventral motor neurons and by arrowheads for dorsal motor neurons. (E-F) Bar graphs showing the number of ventral (E) and dorsal (F) motor neurons scored a 1, 2, 3 or 4 based on tables 2, where 1 represents severe defects. [a] Significantly different from control score 4, [c] significantly different from control score 2, $p<0.05$. Scale bar: 20 μm.

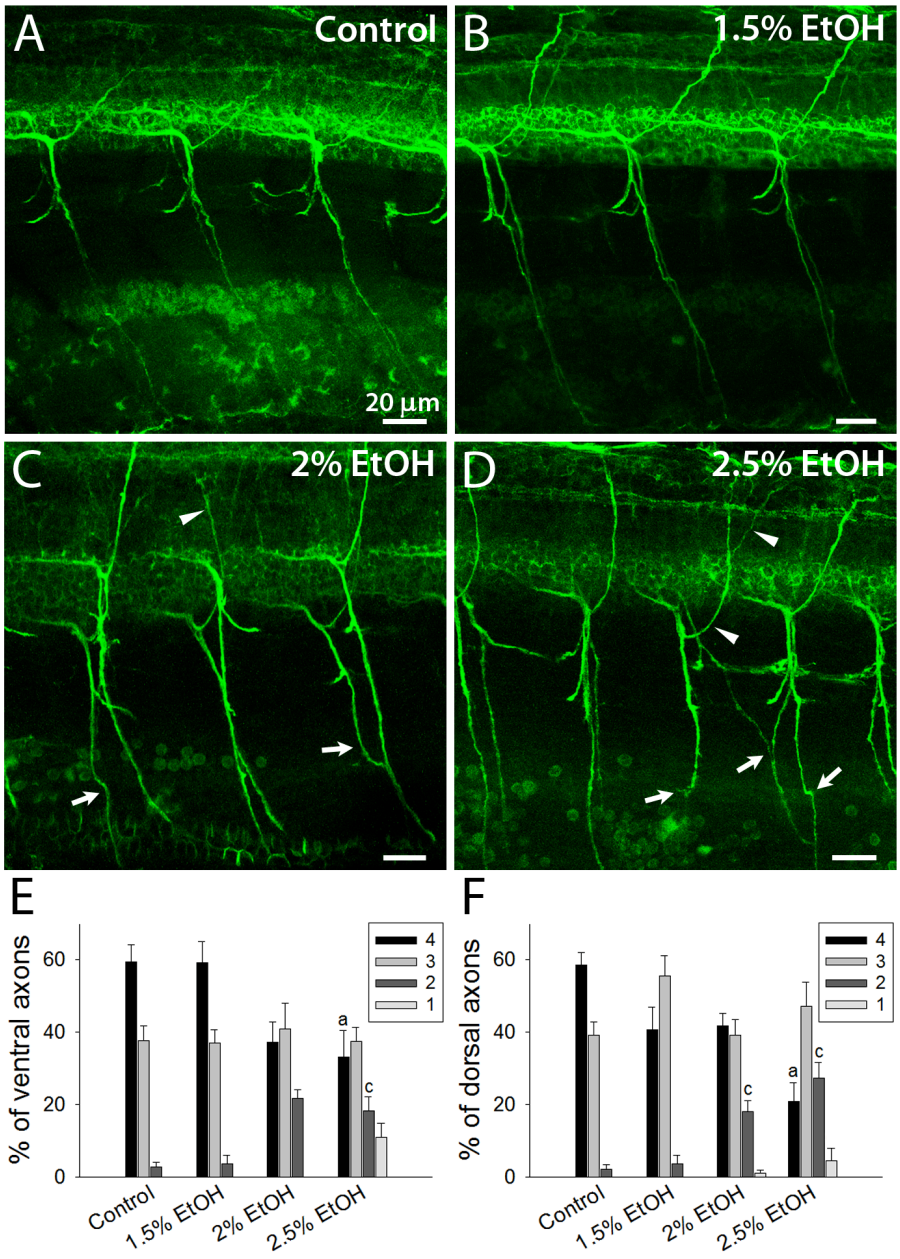

A
Control
20 µm
B
1.5% EtOH
C
2% EtOH
D
2.5% EtOH
E
% of ventral axons
60
40
20
0
4
3
2
1
a
c
Control
1.5% EtOH
2% EtOH
2.5% EtOH
F
% of dorsal axons
60
40
20
0
4
3
2
1
a
c
Control
1.5% EtOH
2% EtOH
2.5% EtOH

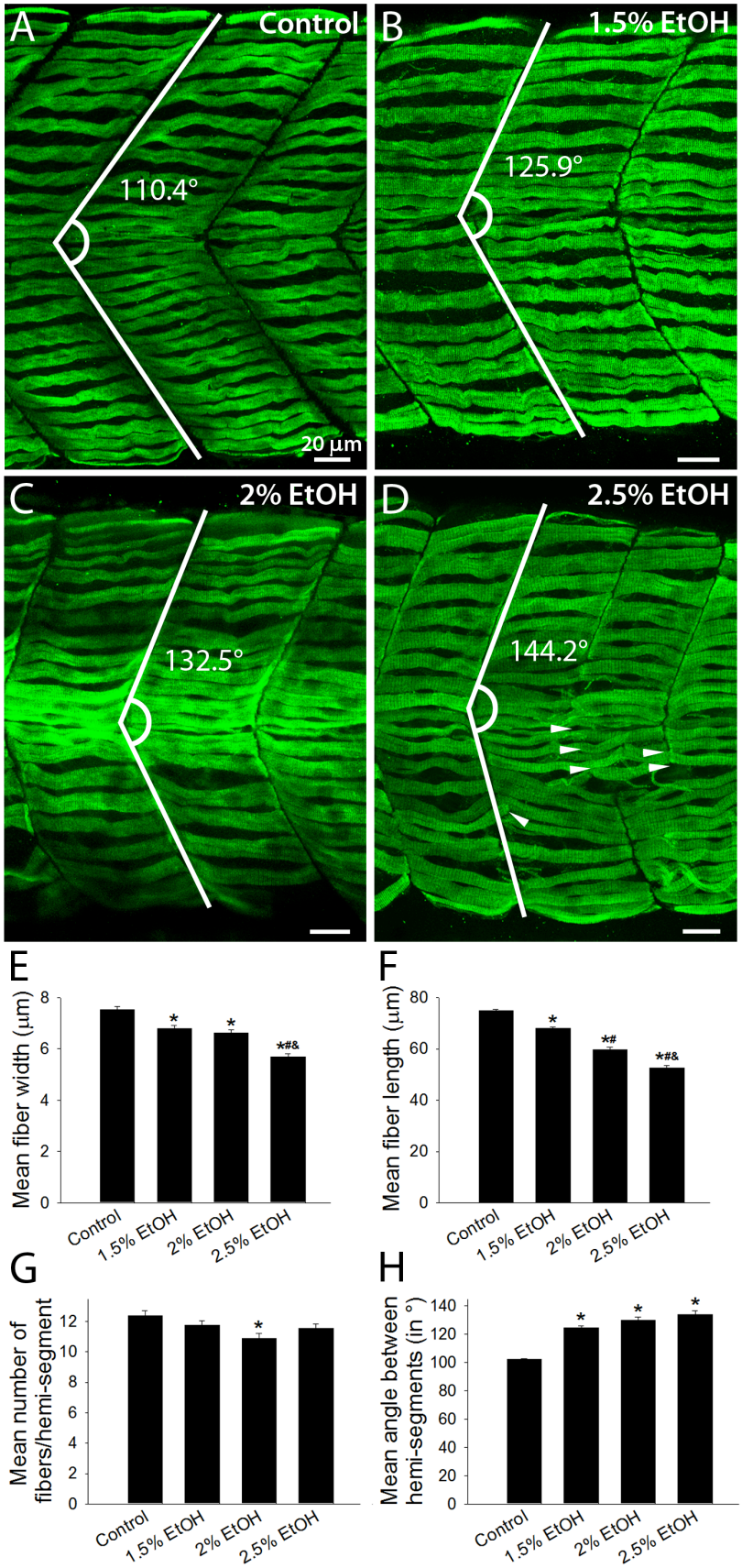
Figure 9). Unfortunately, consistent and reliable counting of the number of primary branches projecting from the secondary motor neuron axons was not feasible.

This data suggested that embryonic exposure to EtOH induced a significant increase in the incidence of abnormal ventral and dorsal motor neurons.

3.3. Muscle fiber morphology

The deficits in hatching and swimming behaviour, as well as motor neuron morphology, prompted me to determine if muscle fibers were affected by early EtOH exposure. Therefore, muscle fibers were labelled with antibodies directed against slow tonic (red) and fast twitch (white) muscle myosin (F59 and F310, respectively; Figure 10,11; Crow and Stockdale, 1986). Red muscle in exposed larvae displayed abnormalities such as a lack of segment division, fibers extending over two segments rather than one, altered angles between dorsal and ventral hemi-segments and smaller muscle fibers (Figure 10A-D). For instance, zebrafish exposed to 2.5% EtOH had significantly shorter (53±1 μm; n=117 fibers) and narrower (5.7±0.2 μm; n=117 fibers) red muscle fibers compared with control larvae (75±1 μm (length) and 7.5±0.1 μm (width); n= 117 fibers; Figure 10A-F; $p<0.05$). Ethanol treatment only affected the number of muscle fibers per segment in fish exposed to 2% EtOH (Figure 10A-D,G; $p<0.05$). In initial observations, the shape of the somites appeared to be affected by EtOH exposure, therefore the angle between the dorsal and ventral hemi-segments was measured at the midline of the fish (see Figure 10). EtOH exposure resulted in a greater angle between dorsal and ventral hemi-segments (134±3º in 2.5% EtOH-treated fish) compared with control larvae (102±1°; Figure 10A-D,H; $p<0.05$).

Figure 10. Red muscle morphology in larvae at 3dpf following exposure to 0% (A, n=117 fibers in 13 fish), 1.5% (B, n=81 fibers in 9 fish), 2% (C, n=90 fibers in 10 fish), 2.5% (D, n=117 fibers in 13 fish) EtOH and labelled with F59. Example fiber abnormalities are shown with arrowheads. (E-H) Bar graphs quantifying fiber width (E), length (F), the number of fibers per segment (G) and the average angle between segments (H). Example measurements of the angle between hemi-segments are shown. * Significantly different from control, $^{\#}$ significantly different from 1.5% EtOH exposed larvae, $^{\&}$ significantly different from 2% EtOH exposed fish, $p<0.05$. Scale bar: 20 μm.


A
Control
110.4°
20 µm
B
1.5% EtOH
125.9°
C
2% EtOH
132.5°
D
2.5% EtOH
144.2°
E
Mean fiber width (µm)
0
2
4
6
8
*
*
*#&
Control
1.5% EtOH
2% EtOH
2.5% EtOH
F
Mean fiber length (µm)
0
20
40
60
80
*
*#
*#&
Control
1.5% EtOH
2% EtOH
2.5% EtOH
G
Mean number of fibers/hemi-segment
0
2
4
6
8
10
12
*
Control
1.5% EtOH
2% EtOH
2.5% EtOH
H
Mean angle between hemi-segments (in °)
0
20
40
60
80
100
120
140
*
*
*
Control
1.5% EtOH
2% EtOH
2.5% EtOH

Since the majority of the trunk musculature is composed of white fast twitch fibers, I wanted to see if they are also affected by EtOH exposure (Buss and Drapeau, 2002; Greer-Walker and Pull, 1975). EtOH-treated (1.5%, 2% and 2.5% EtOH) and untreated fish were labelled with F310 to visualize white muscle fibers (Crow and Stockdale, 1986; Figure 11). Exposed larvae displayed abnormalities such as smaller fibers, a partial absence of segment division and altered angles between dorsal and ventral hemi-segments (Figure 11A-D). Fish exposed to 1.5% EtOH had fibers that were shorter (85±1 μm; n=81) than controls (102±1 μm; n=117; $p<0.001$), whereas 2.5% EtOH-treated fish had fibers that were significantly shorter (55±1 μm; n=99) and narrower (4.9±0.1 μm; n=99) compared to controls (102±1 μm (length) and 6.3±0.2 μm (width); n=117; Figure 11A-F; $p<0.05$). The number of muscle fibers per segment was not significantly different between treatments (Figure 11A-D,G; $p>0.05$). Measuring the angle between the dorsal and ventral hemi-segments demonstrated that EtOH-exposed larvae had a significantly larger angle between hemi-segments (155±3° in 2.5% EtOH-treated fish; n=33) compared to control fish (105±1°; n=39; Figure 11A-D,H; $p<0.05$).

This data suggested that embryonic exposure to EtOH significantly affected muscle fiber size and morphology.

3.4. The motor neuron and their targets

Since motor neuron morphology and muscle morphology were both affected, I wanted to see if the synapses were forming at their usual locations or if they were forming where the altered axon projected. I co-labelled exposed fish with the 3A10 antibody and α-bungarotoxin in order to visualize motor neuron axons and post-synaptic

.

Figure 11. White muscle morphology in larvae aged 3 dpf exposed to 0% (A, n=117 fibers in 13 fish), 1.5% (B, n=81 fibers in 9 fish), 2% (C, n=90 fibers in 10 fish), 2.5% (D, n=99 fibers in 11 fish) EtOH and labelled with F310. Images shown are of one slice taken midway through the white muscle. Example fiber abnormalities are shown with arrowheads. Example measurements of the angle between hemi-segments are shown. (E-H) Bar graphs quantifying fiber width (E), length (F), the number of fibers per segment (G) and the average angle between segments (H). * Significantly different from control, $^{\#}$ significantly different from 1.5% EtOH exposed larvae, $^{\&}$ significantly different from 2% EtOH exposed fish, $p<0.05$. Scale bar: 20 μm.

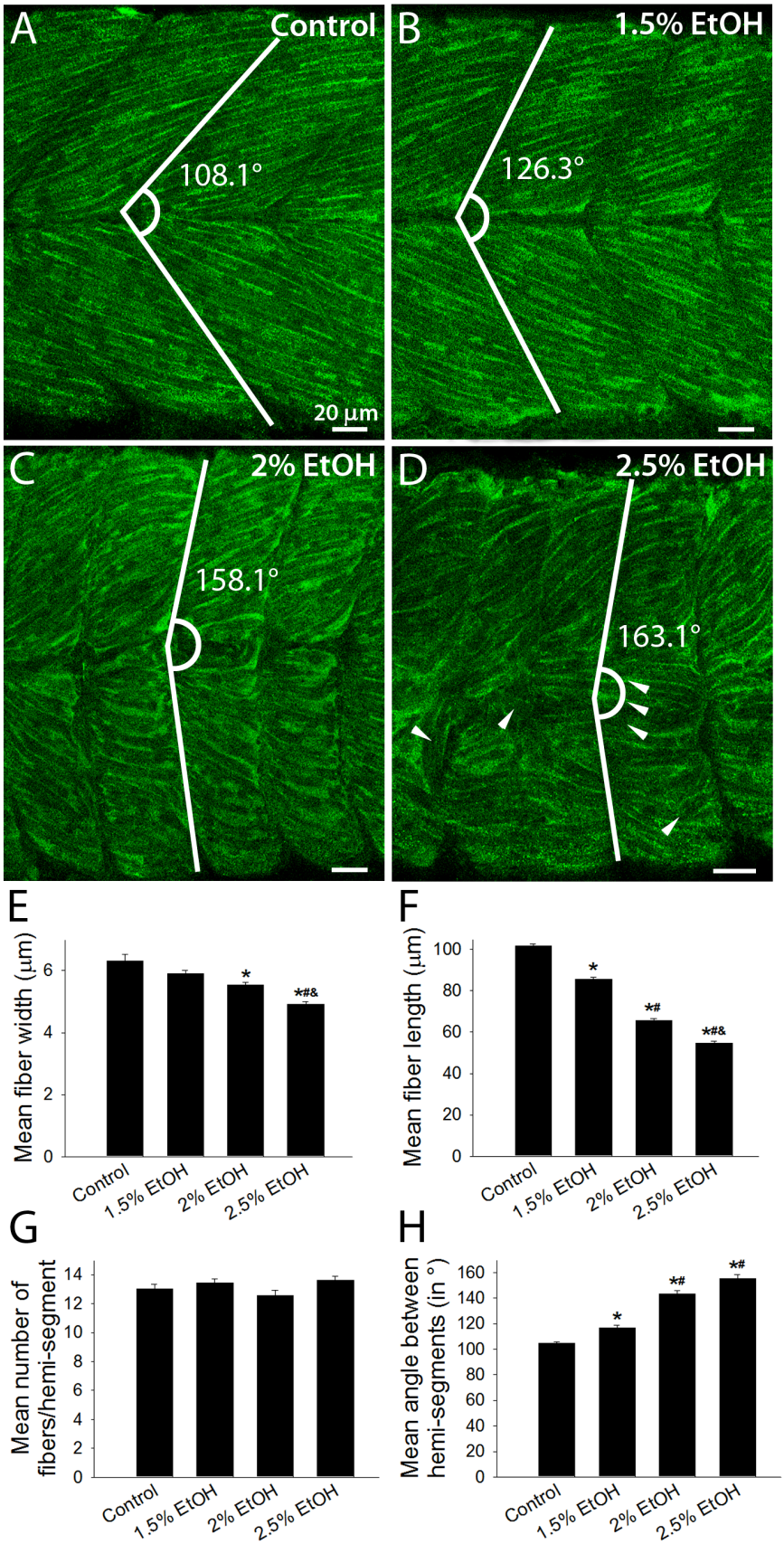
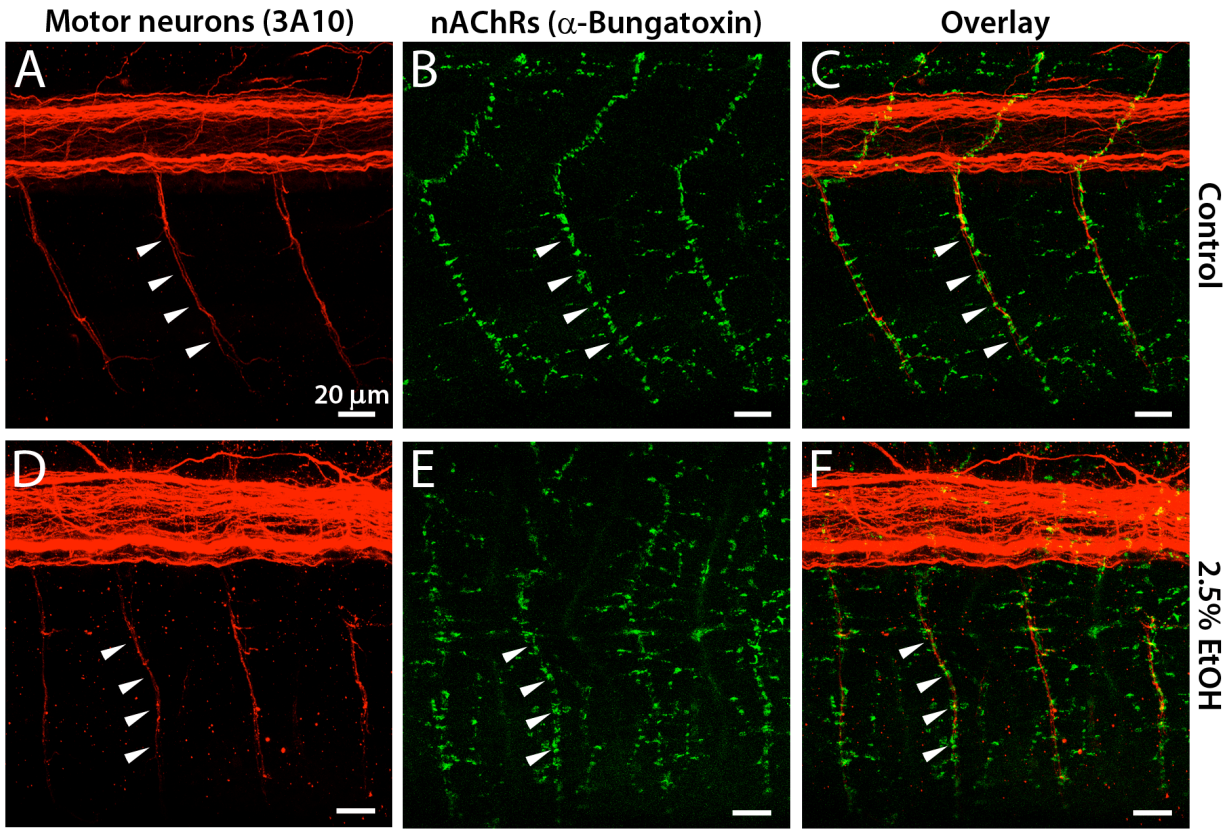

A
Control
108.1°
20 µm
B
1.5% EtOH
126.3°
C
2% EtOH
158.1°
D
2.5% EtOH
163.1°
E
Mean fiber width (µm)
0
2
4
6
*
*#&
Control
1.5% EtOH
2% EtOH
2.5% EtOH
F
Mean fiber length (µm)
0
20
40
60
80
100
*
*#
*#&
Control
1.5% EtOH
2% EtOH
2.5% EtOH
G
Mean number of fibers/hemi-segment
0
2
4
6
8
10
12
14
Control
1.5% EtOH
2% EtOH
2.5% EtOH
H
Mean angle between hemi-segments (in °)
0
20
40
60
80
100
120
140
160
*
*#
*#
Control
1.5% EtOH
2% EtOH
2.5% EtOH

Figure 12. Co-localization of motor neurons and nAChRs. Motor neurons (in red, labelled with 3A10) and post-synaptic acetylcholine receptors (nAChRs; in green, labelled with α-bungarotoxin) co-localize in fish exposed to 0% (A-C) and 2.5% (D-F) EtOH at 3 dpf (n=9 fish for each treatment). Notice how the post-synaptic nACh receptors and the motor neuron axons overlap in both treatments (arrowheads). Scale bar, 20 μm.


Motor neurons (3A10)
nAChRs (α-Bungatoxin)
Overlay
A
B
C
D
E
F
20 µm
Control
2.5% EtOH

acetylcholine receptors, respectively (Serafini *et al*., 1996; McCann and Lichtman, 2008; Figure 12). In EtOH-treated fish, the motor neuron axons (in red) displayed similar abnormalities as seen with the acetylated tubulin, znp1 and zn8 staining (Figures 7-9,12; data not shown for 1.5% and 2% EtOH-treated fish). Post-synaptic acetylcholine receptors (AChRs; in green) co-localize with the axons, even when the motor neurons exhibit abnormal trajectories (Figure 12).

To further confirm that pre- and post-synaptic sites were aligned after EtOH treatment, exposed fish were co-labelled with a pre-synaptic vesicle marker, SV2, and a post-synaptic nAChR marker, α-bungarotoxin (Buckley and Kelly, 1985; McCann and Lichtman, 2008; Figure 13). In exposed fish, pre-synaptic vesicles (in red) co-localized with post-synaptic nAChRs, together exhibiting a pattern similar to the axon trajectory revealed with the axon markers (Figures 7-9,13; data not shown for 1.5% and 2% EtOH-treated fish). These results indicate that pre- and post-synaptic terminals at the NMJ align in EtOH-treated zebrafish, although synapse location seems to follow the axon trajectory, which is affected by EtOH exposure.

3.5. Red muscle mEPCs

Since immunohistochemical studies revealed that motor neuron and muscle fiber morphology were affected by early EtOH exposure, I determined if muscle fibers in treated fish were receiving the same electrical information from the motor neurons as seen in control fish. More specifically, it was necessary to determine if pre- and post-synaptic components exhibited properties that were similar to age-matched controls. mEPCs were recorded from muscle fibers to examine if there were changes in the

Figure 13. Co-localization of pre-synaptic vesicles and nAChRs. Pre-synaptic vesicles (in red, labelled with SV2) and post-synaptic nicotinic acetylcholine receptors (nAChRs; in green, labelled with α-bungarotoxin) co-localize in fish exposed to 0% (A-C) and 2.5% (D-F) EtOH at 3 dpf (n=9 fish for each treatment). Notice how the post-synaptic Ach receptors and the pre-synaptic vesicles overlap in all treatments (arrowheads). Scale bar, 20 μm.

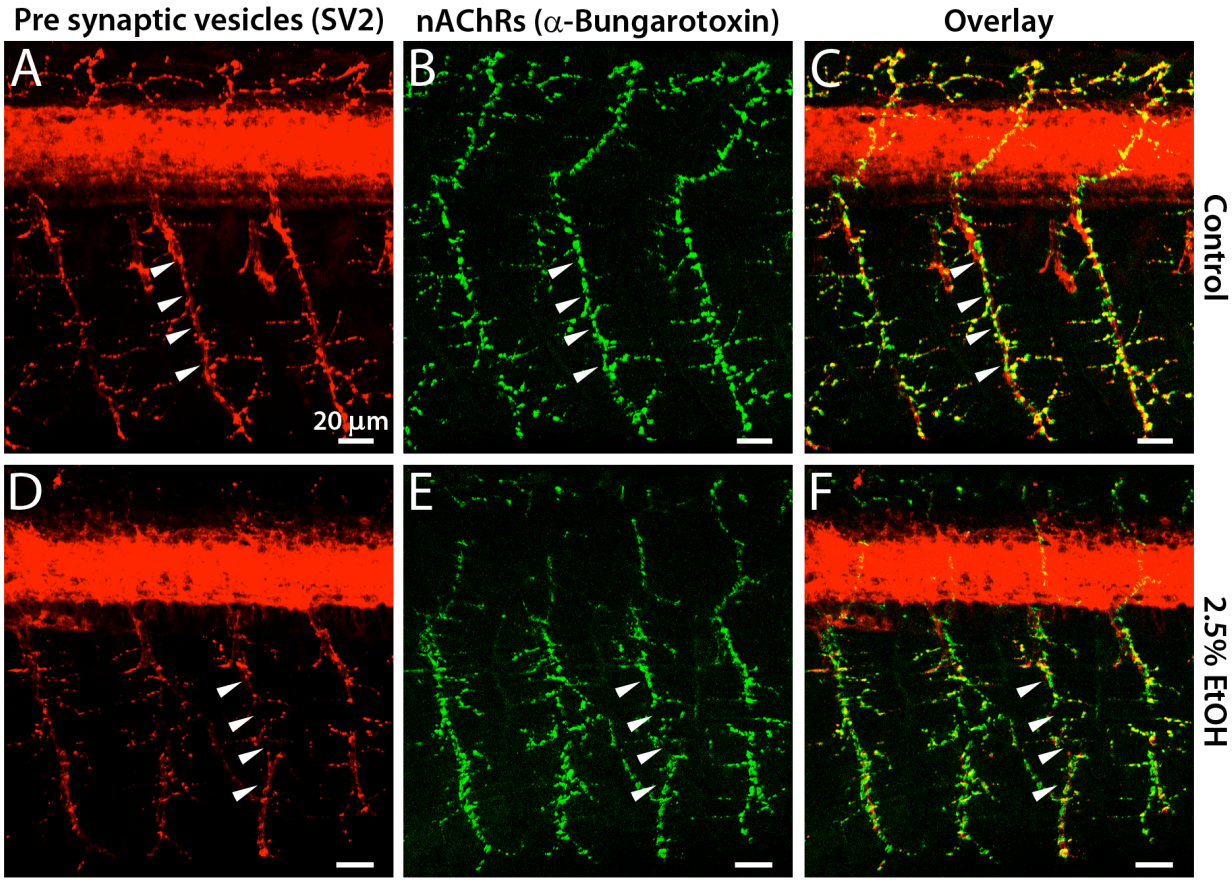
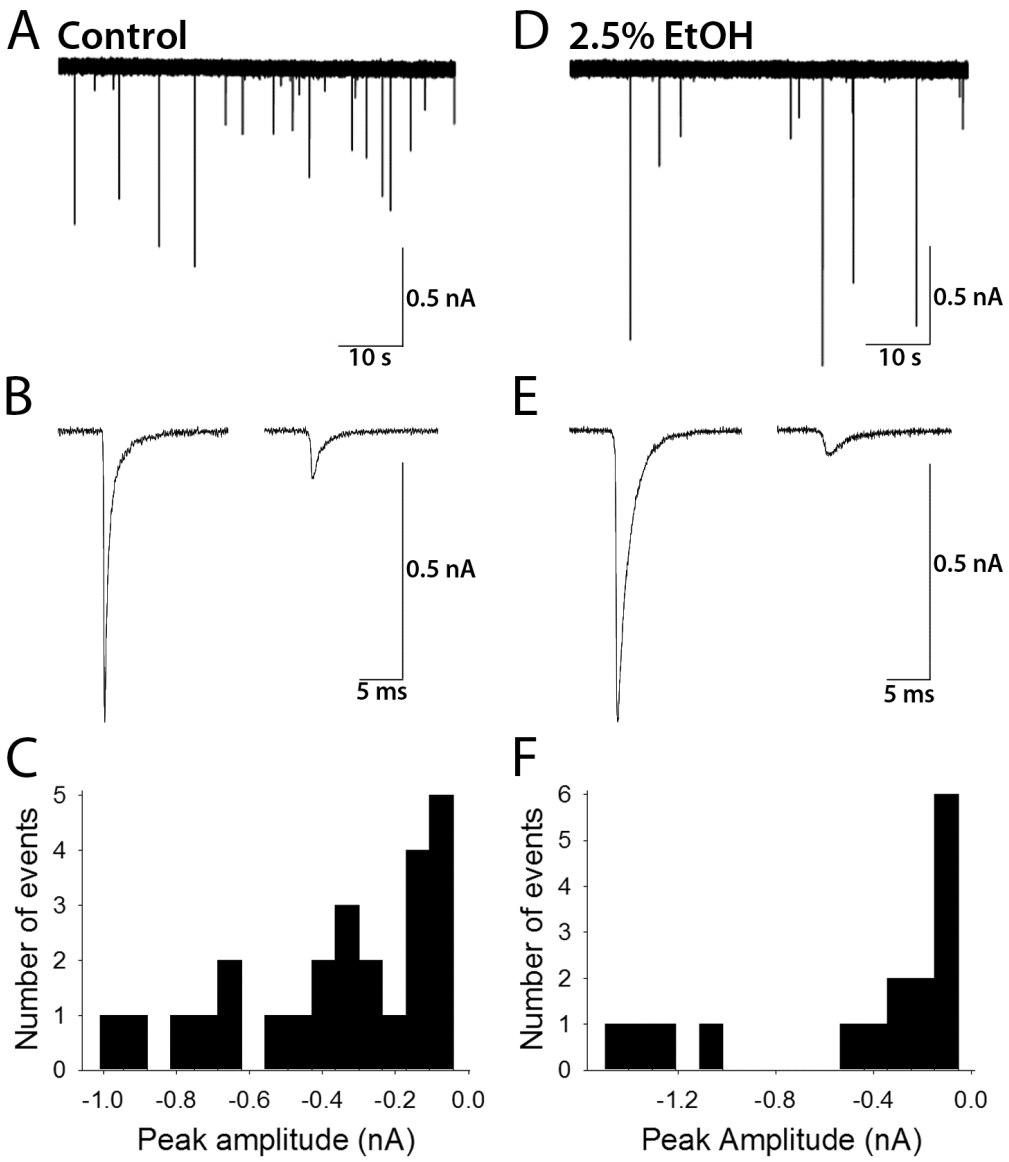

Pre synaptic vesicles (SV2)
nAChRs (α-Bungarotoxin)
Overlay
A
B
C
D
E
F
20 µm
Control
2.5% EtOH

Figure 14. Representative red muscle mEPC recordings from 3 dpf fish exposed to 0% (A) and 2.5% (D) EtOH. (B) Averaged fast and slow events from control larvae. (C) Histogram illustrating the presence of both types of events in an individual control recording. (E) Averaged fast and slow events from 2.5% EtOH-exposed larvae. (F) Histogram showing the presence of two types of events in an individual recording from fish exposed to 2.5% EtOH. Recordings were captured in the presence of TTX and carbonoxolone.


A Control
0.5 nA
10 s
D 2.5% EtOH
0.5 nA
10 s
B
0.5 nA
5 ms
E
0.5 nA
5 ms
C
Number of events
0
1
2
3
4
5
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
Peak amplitude (nA)
F
Number of events
0
1
2
3
4
5
6
-1.2
-0.8
-0.4
0.0
Peak Amplitude (nA)

properties of spontaneous events.

There were two distinct types of mEPCs present in all the recordings taken from EtOH-treated and untreated fish (Figure 14). Figures 14C,F show the histogram distribution of the events detected in the representative trace shown for each treatment, demonstrating that there are two types of events in these recordings: one type with amplitudes between -0.6 nA and -1.2 nA and the other between -0.5 nA and 0.05 nA (Figure 14). Some events were characterized by a high amplitude, a fast rise time and decay time constant (Figure 14,15,16), while others events had a low amplitude, slow rise time and slow decay time constant (Figure 14,16,17). I therefore dubbed these two types of events fast events and slow events, respectively. I decided to analyse these events separately for both red and white muscle recordings.

Figure 14A,D depicts representative traces of mEPCs recordings from red muscle in 3 dpf control larvae, as well as from exposed larvae, with the averaged fast and slow events from each trace illustrated in Figure 14B,E (representative traces from fish exposed to 1.5% and 2% EtOH not shown). When the fast events were averaged, there was no significant difference between control fast events and fast events from treated fish for the mean event amplitude (Figure 15A), mean event rise time (Figure 15B) and mean event half width time (Figure 15C; $p>0.05$). Fitting the averaged fast events with an exponential function revealed that all averaged fast events from control and treated fish fit better with a double exponential function (Figure 16A). There was no significant difference for the decay time constants, τ_1 and τ_2, between controls and treated fish (Figure 16B,C; $p>0.05$). The mean frequency was calculated for fast events in red fibers, revealing that fish treated with 2.5% EtOH had significantly fewer fast

Figure 15. Summary of fast event properties in red fibers recorded from 3 dpf control fish (n=7) and fish exposed to 1.5% EtOH (n=8), 2% EtOH (n=10) and 2.5% EtOH (n=11). (A-C) Bar graphs showing mean event peak amplitude (A), mean event rise time (B) and mean event half width (C). (D) Mean frequency of fast events in red muscle mEPC recordings. * Significantly different from control, $p<0.05$.

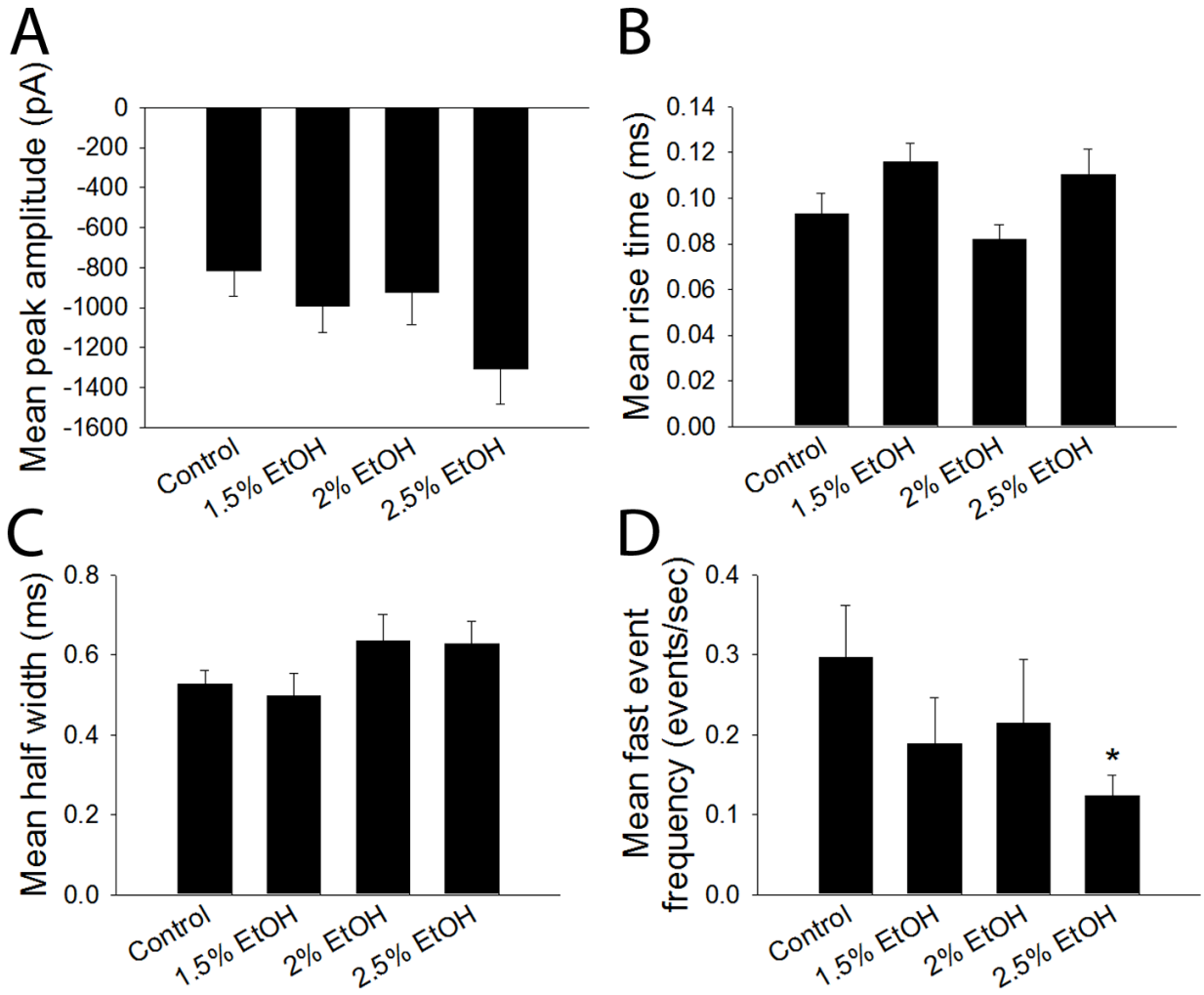
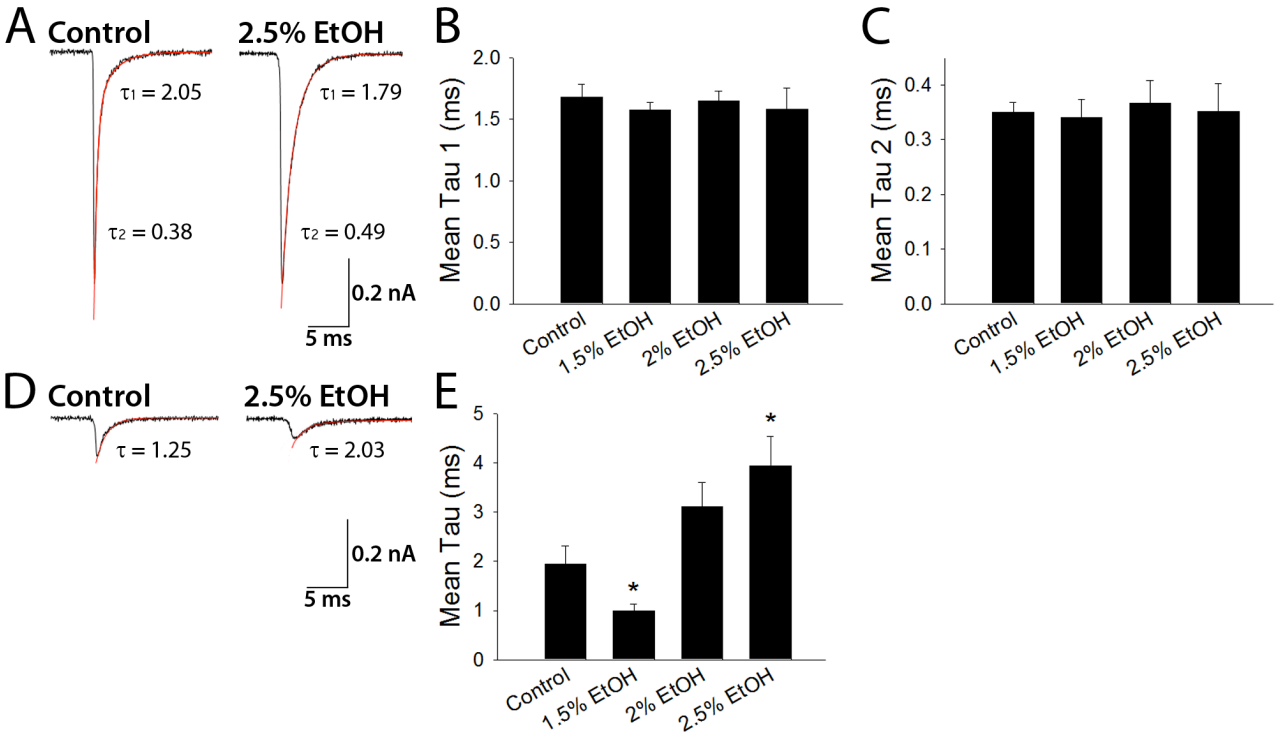

A
Mean peak amplitude (pA)
0
-200
-400
-600
-800
-1000
-1200
-1400
-1600
Control
1.5% EtOH
2% EtOH
2.5% EtOH
B
Mean rise time (ms)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Control
1.5% EtOH
2% EtOH
2.5% EtOH
C
Mean half width (ms)
0.8
0.6
0.4
0.2
0.0
Control
1.5% EtOH
2% EtOH
2.5% EtOH
D
Mean fast event frequency (events/sec)
0.4
0.3
0.2
0.1
0.0
*
Control
1.5% EtOH
2% EtOH
2.5% EtOH

Figure 16. Fast and slow event decay time constants in red muscle. (A) Representative fast events in red muscle from control fish and fish exposed to 2.5% EtOH fitted with a double exponential function. (B-C) Bar graph showing the decay time constants of fast events, τ_1 (B) and τ_2 (C). (D) Representative slow events in red muscle from control fish and fish exposed to 2.5% EtOH fitted with a single exponential function. (E) Bar graph showing the time decay constant of slow events, τ. (control n=7; 1.5% EtOH n=8; 2% EtOH n=10; 2.5% EtOH n=11) * Significantly different from control, $p<0.05$.


A
Control
2.5% EtOH
τ1 = 2.05
τ2 = 0.38
τ1 = 1.79
τ2 = 0.49
0.2 nA
5 ms
B
Mean Tau 1 (ms)
2.0
1.5
1.0
0.5
0.0
Control
1.5% EtOH
2% EtOH
2.5% EtOH
C
Mean Tau 2 (ms)
0.4
0.3
0.2
0.1
0.0
Control
1.5% EtOH
2% EtOH
2.5% EtOH
D
Control
2.5% EtOH
τ = 1.25
τ = 2.03
0.2 nA
5 ms
E
Mean Tau (ms)
5
4
3
2
1
0
*
*
Control
1.5% EtOH
2% EtOH
2.5% EtOH

events per second compared to controls (Figure 15D; $p<0.05$). Therefore, red muscle fast event kinetics were similar between control and treated fish, although the frequency of the fast events was affected in fish treated with a concentration of 2.5% EtOH. The slow events from the red muscle recordings were then averaged (Figure 14B,E). There was a significant difference in mean peak amplitude between control slow events and slow events from fish treated with 2.5% EtOH (Figure 17A; $p<0.05$). There was no significant difference between control and treated fish for the mean event rise time (Figure 17B; $p>0.05$). The mean event half width was significantly different between controls and fish treated with 2% and 2.5% EtOH, where the half width was wider in treated fish (Figure 17C; $p<0.05$). The averaged events were fit with a single exponential function (Figure 16D). There was a significant difference for the decay time constant, τ, between control and fish treated with 1.5% and 2.5% EtOH, where fish treated with 1.5% EtOH had a shorter τ and fish treated with 2.5% EtOH had a longer τ compared to controls (Figure 16E; $p<0.05$). The mean slow event frequency in red fibers showed no significant difference between controls and treated fish (Figure 17D; $p>0.05$). These results indicate that EtOH exposure resulted in red muscle slow events that were smaller in amplitude, longer in length and had altered decay time constants, although the frequency was not affected.

3.6. White muscle mEPCs

mEPCs from white muscle fibers were recorded (Buss and Drapeau, 2002; Greer-Walker and Pull, 1975). Similar to red fiber recordings, two types of events, fast and slow, were also present in white fiber recordings (Figure 18), and they were

Figure 17. Summary of slow event properties in red fibers recorded from 3 dpf control fish (n=7) and fish exposed to 1.5% EtOH (n=8), 2% EtOH (n=10) and 2.5% EtOH (n=11). (A-C) Bar graphs showing mean event peak amplitude (A), mean event rise time (B) and mean event half width (C). (D) Mean frequency of slow events in red muscle mEPC recordings. * Significantly different from control, $p<0.05$.

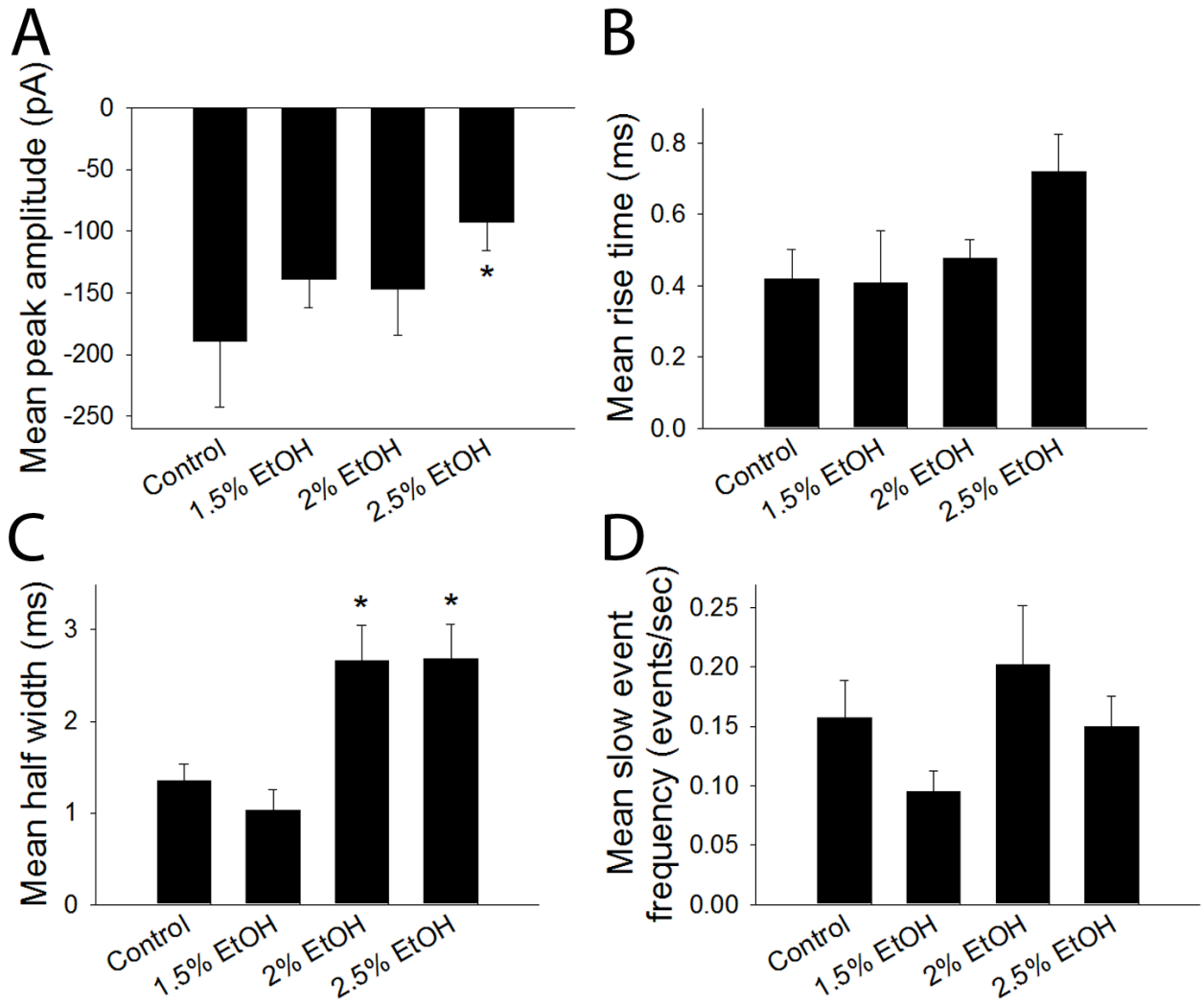
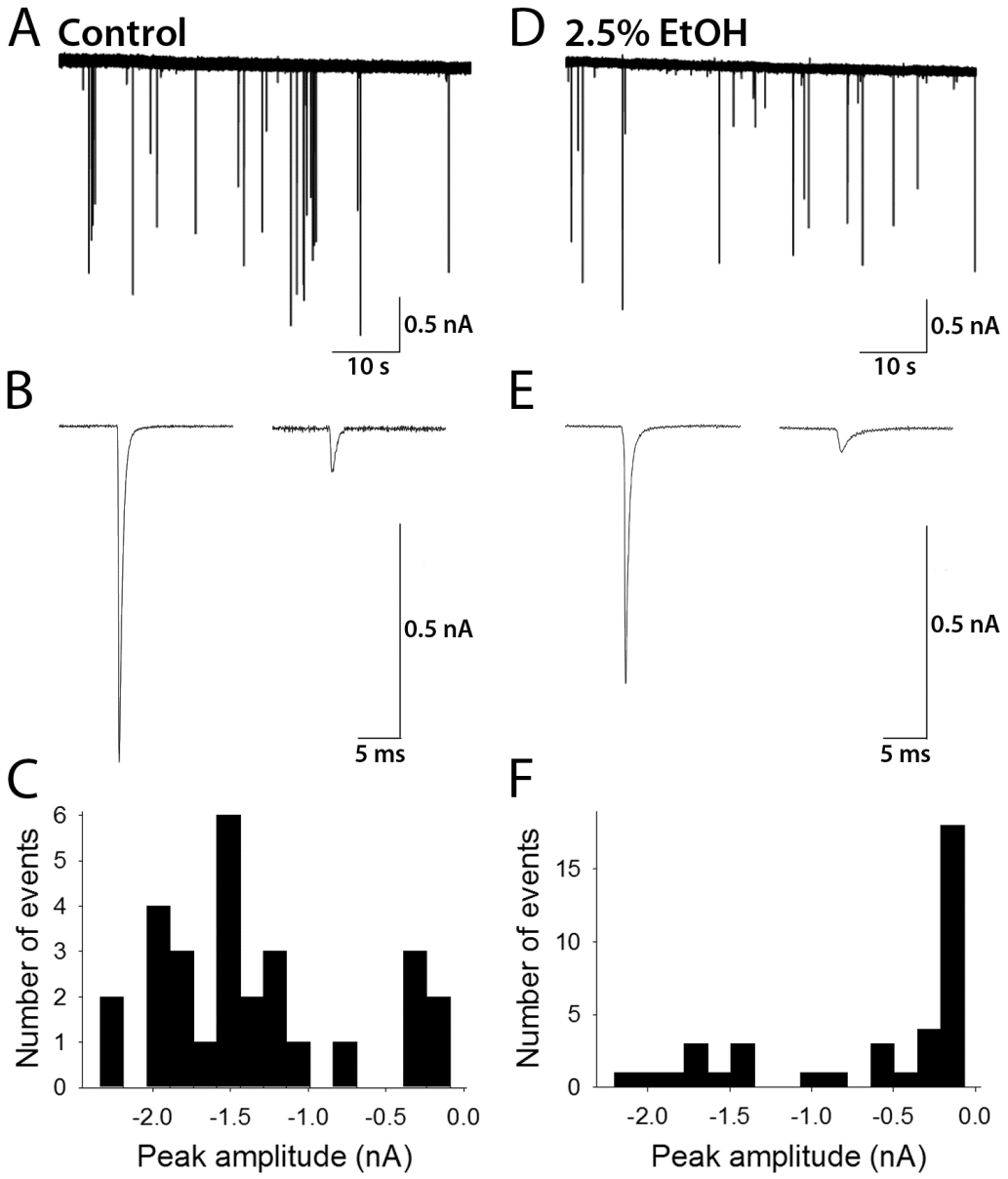

A
Mean peak amplitude (pA)
0
-50
-100
-150
-200
-250
*
Control
1.5% EtOH
2% EtOH
2.5% EtOH
B
Mean rise time (ms)
0.8
0.6
0.4
0.2
0.0
Control
1.5% EtOH
2% EtOH
2.5% EtOH
C
Mean half width (ms)
3
2
1
0
*
*
Control
1.5% EtOH
2% EtOH
2.5% EtOH
D
Mean slow event frequency (events/sec)
0.25
0.20
0.15
0.10
0.05
0.00
Control
1.5% EtOH
2% EtOH
2.5% EtOH

Figure 18. Representative white muscle mEPC recordings from 3 dpf fish exposed to 0% (A) and 2.5% (D) EtOH. (B) Averaged fast and slow events from control larvae. (C) Histogram illustrating the presence of both types of events in an individual control recording. (E) Averaged fast and slow events from 2.5% EtOH-exposed larvae. (F) Histogram showing the presence of two types of events in an individual recording from fish exposed to 2.5% EtOH. Recordings were captured in the presence of TTX and carbonoxolone.


A
Control
0.5 nA
10 s
B
0.5 nA
5 ms
C
Number of events
Peak amplitude (nA)
D
2.5% EtOH
0.5 nA
10 s
E
0.5 nA
5 ms
F
Number of events
Peak amplitude (nA)

distinguished from one another based on amplitude, rise time, half width and the decay time constant (Figures 18-21). Figure 18A,D depicts representative traces of mEPCs recordings from white fibers of control and treated fish at 3 dpf, with the averaged fast and slow events from each trace in Figure 18B,E and the histogram of the event distribution in Figure 18C,F (representative traces from fish exposed to 1.5% and 2% EtOH not shown). There was a significant difference between controls and fish treated with 1.5% EtOH for the mean event amplitude (Figure 19A; $p<0.05$). The mean event rise time was significantly different between controls and fish treated with 1.5% EtOH and 2% EtOH (Figure 19B; $p<0.05$). The mean event half width was not significantly between controls and EtOH-treated fish (Figure 19C; $p>0.05$). The averaged fast events were fitted with a single exponential function (Figure 20A). All the averaged fast events from treated fish were better fit with a double exponential function, as well as about half the control fast events. Because some control fast events fit only with a single exponential function, single decay time constants were compared between treatments (Figure 20B). Events from fish treated with 2.5% EtOH had a significantly longer decay time constant compared to control fast events (Figure 20B; $p<0.05$). I also compared the decay time constants of the events that could be fitted with a double exponential by excluding the events that could only be fit with a single exponential function (Figure 21). There was no significant difference between controls and EtOH-exposed fish for τ_1 and τ_2 (Figure 21; $p>0.05$). I then calculated the mean frequency of fast events in white fibers and found that there was no significant difference between control recordings and recordings from treated fish (Figure 19D; $p>0.05$). In summary, white fiber fast events were affected by EtOH exposure in terms of the amplitude, the rise time and the decay

Figure 19. Summary of fast event properties in white fibers recorded from 3 dpf control fish (n=9) and fish exposed to 1.5% EtOH (n=8), 2% EtOH (n=12) and 2.5% EtOH (n=8). (A-C) Bar graphs showing mean event peak amplitude (A), mean event rise time (B) and mean event half width (C). (D) Mean frequency of fast events in white muscle mEPC recordings. * Significantly different from control, $p<0.05$.

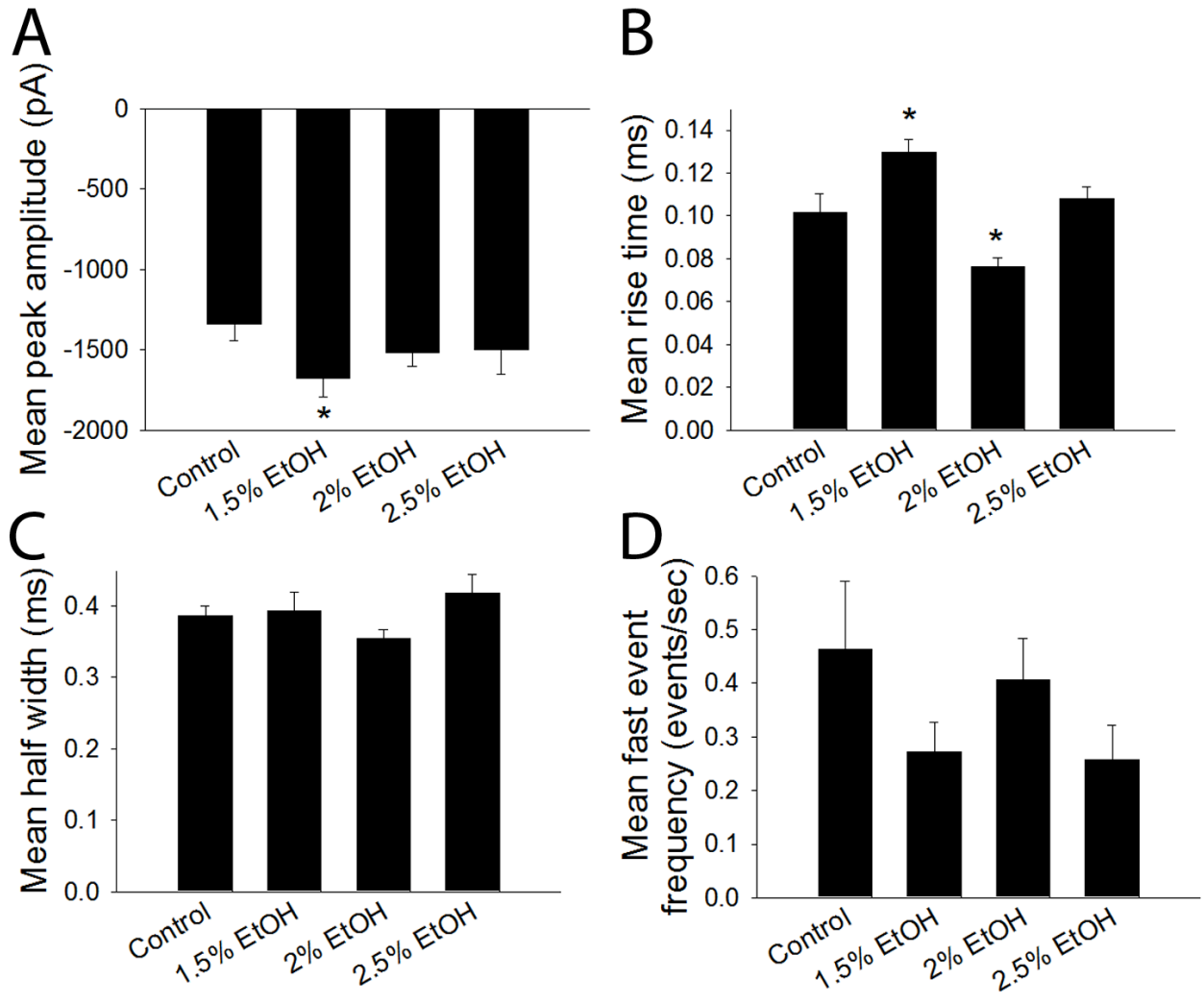

A
Mean peak amplitude (pA)
0
-500
-1000
-1500
-2000
*
Control
1.5% EtOH
2% EtOH
2.5% EtOH
B
Mean rise time (ms)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
*
Control
1.5% EtOH
2% EtOH
2.5% EtOH
C
Mean half width (ms)
0.4
0.3
0.2
0.1
0.0
Control
1.5% EtOH
2% EtOH
2.5% EtOH
D
Mean fast event frequency (events/sec)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
1.5% EtOH
2% EtOH
2.5% EtOH

Figure 20. Fast and slow event decay time constants in white fibers. (A) Representative white muscle fast events from control fish and fish exposed to 2.5% EtOH fitted with a single exponential function. (B) Bar graph showing the decay time constant for fast events, τ. (C) Representative slow events in white fibers from control fish and fish exposed to 2.5% EtOH fitted with a single exponential function. (E) Bar graph showing the decay time constant for slow events, τ. (control n=9; 1.5% EtOH n=8; 2% EtOH n=12; 2.5% EtOH n=8) * Significantly different from control, $p<0.05$.

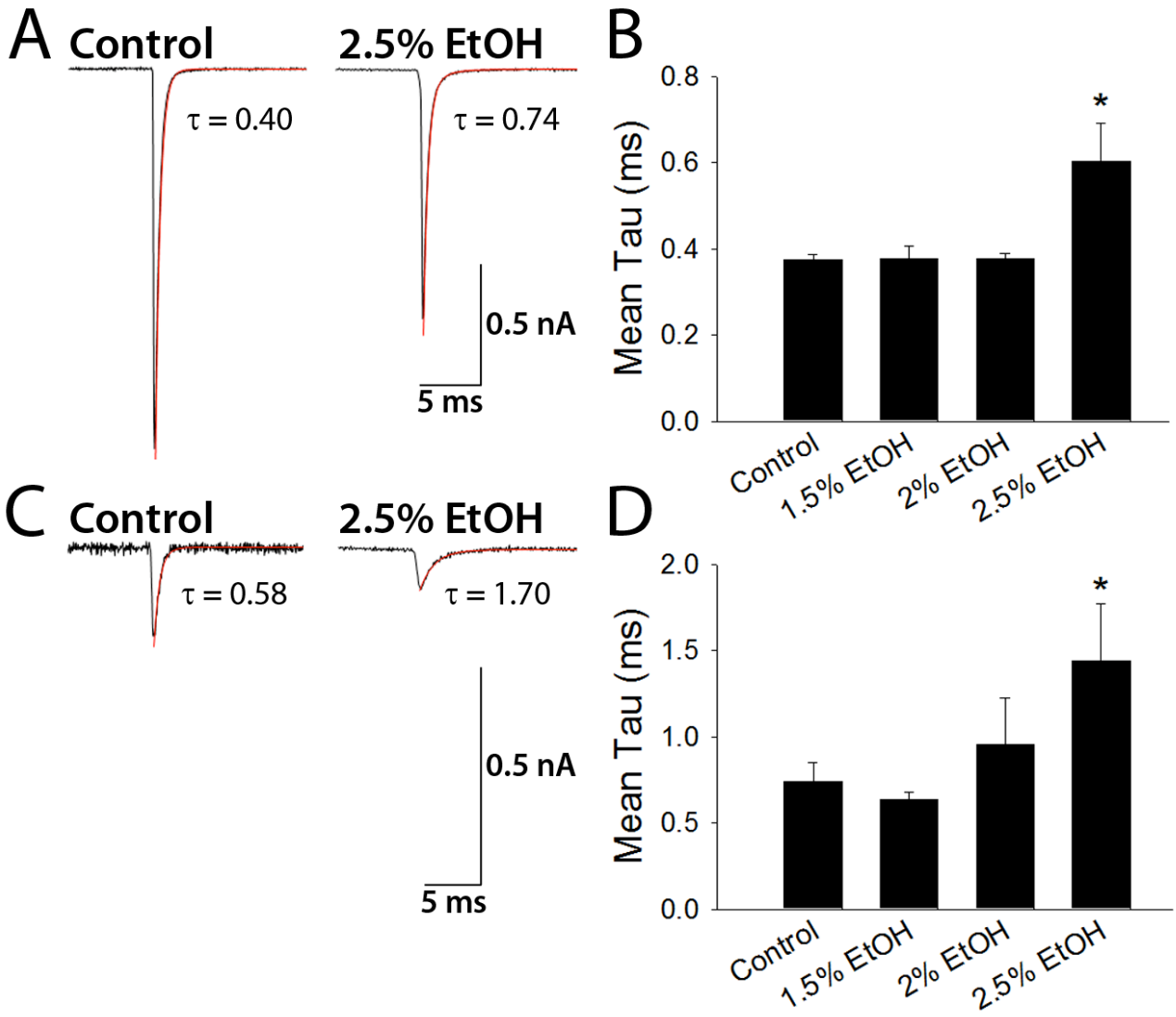

A
Control
2.5% EtOH
τ = 0.40
τ = 0.74
0.5 nA
5 ms
B
Mean Tau (ms)
0.0
0.2
0.4
0.6
0.8
*
Control
1.5% EtOH
2% EtOH
2.5% EtOH
C
Control
2.5% EtOH
τ = 0.58
τ = 1.70
0.5 nA
5 ms
D
Mean Tau (ms)
0.0
0.5
1.0
1.5
2.0
*
Control
1.5% EtOH
2% EtOH
2.5% EtOH

Figure 21. Mean decay time constants for fast events in white muscle, τ_1 (A) and τ_2 (B), when the averaged fast events are fitted with a double exponential function (not shown). Averaged events that could only be fitted with a single exponential function were omitted. No significant difference in decay time constants were observed for any of the treatments ($p>0.05$; control n=5, 1.5% EtOH n=8, 2% EtOH n=12, 2.5% EtOH n=8).

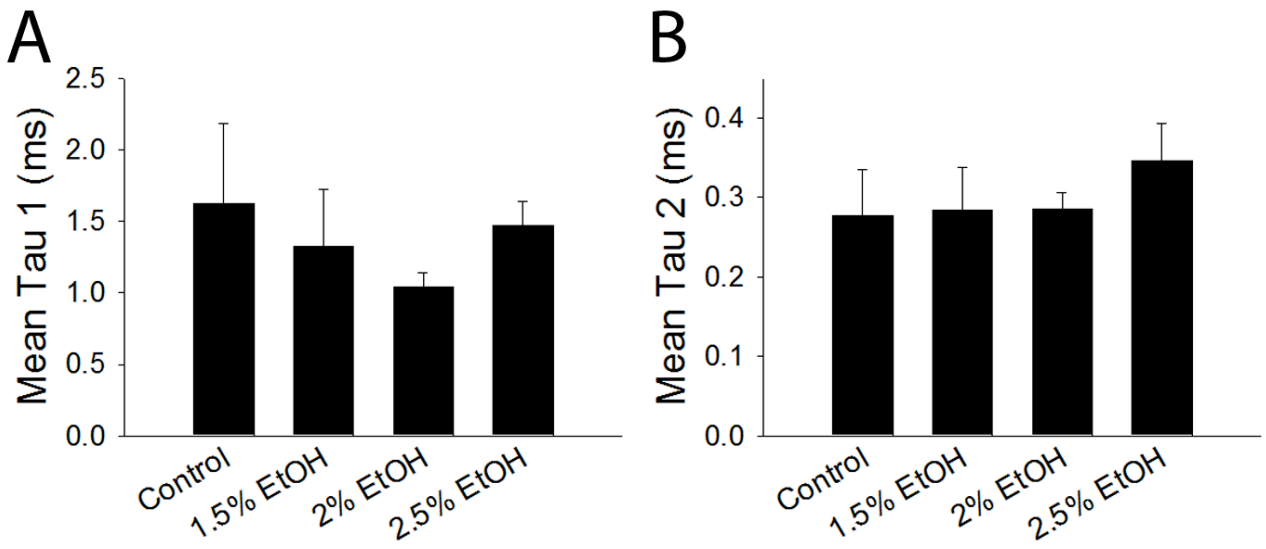
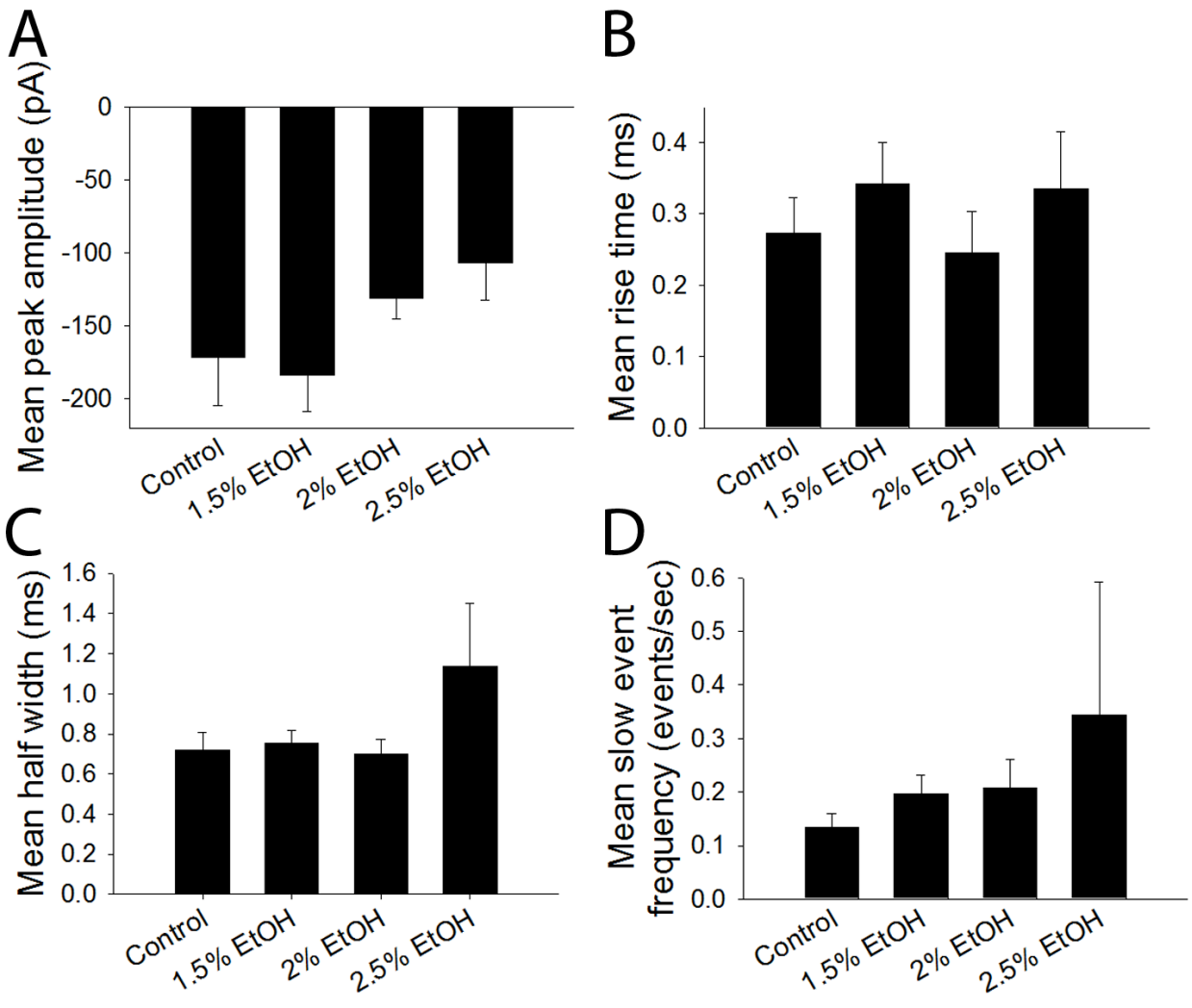

A
Mean Tau 1 (ms)
2.5
2.0
1.5
1.0
0.5
0.0
Control
1.5% EtOH
2% EtOH
2.5% EtOH
B
Mean Tau 2 (ms)
0.4
0.3
0.2
0.1
0.0
Control
1.5% EtOH
2% EtOH
2.5% EtOH

Figure 22. Summary of slow event properties in white fibers recorded from 3 dpf control fish (n=9) and fish exposed to 1.5% EtOH (n=8), 2% EtOH (n=11) and 2.5% EtOH (n=8). (A-C) Bar graphs showing mean event peak amplitude (A), mean event rise time (B) and mean event half width (C). (D) Mean frequency of slow events in white muscle mEPC recordings. $p>0.05$.


A
Mean peak amplitude (pA)
0
-50
-100
-150
-200
Control
1.5% EtOH
2% EtOH
2.5% EtOH
B
Mean rise time (ms)
0.4
0.3
0.2
0.1
0.0
Control
1.5% EtOH
2% EtOH
2.5% EtOH
C
Mean half width (ms)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
1.5% EtOH
2% EtOH
2.5% EtOH
D
Mean slow event frequency (events/sec)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
1.5% EtOH
2% EtOH
2.5% EtOH

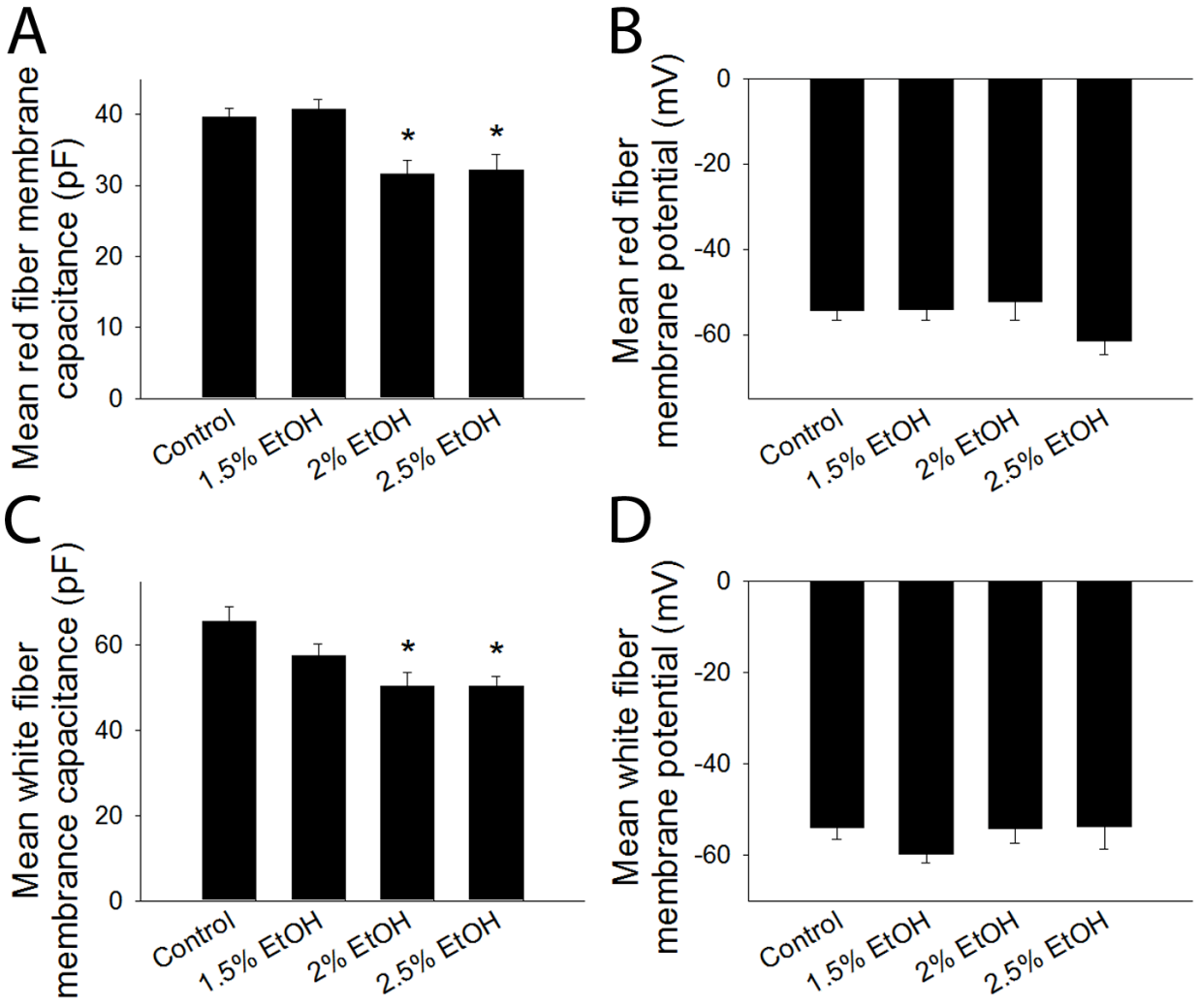
time constant when fit with a single exponential function.

The slow events for the white muscle recordings were subsequently analyzed (Figure 18C,G). There was no significant difference for the mean event amplitude between controls and treated fish (Figure 22A), for the rise time (Figure 22B) or the half width (Figure 22C; $p>0.05$). Fitting the averaged slow events with a single exponential function demonstrated that slow events from fish treated with 2.5% EtOH had significantly longer decay time constants compared to control slow events (Figure 20C,D; $p<0.05$). Calculating the mean slow event frequency from white fibers in control and treated fish demonstrated that there was no significant difference in frequency between control and exposed fish (Figure 22D; $p>0.05$). All in all, EtOH exposure resulted in slow events from white fibers that had longer decay time constants.

3.7. Membrane capacitance and membrane potential

During mEPC acquisition, the membrane capacitance and the membrane potential of red and white fibers were also recorded. For both red and white muscle fibers, membrane capacitance was significantly lower in fish exposed to 2% and 2.5% EtOH (Figure 23A,C; $p<0.05$). The membrane potential of red and white muscle fibers was unaffected by EtOH exposure (Figure 23B,D; $p>0.05$).

Figure 23. Membrane capacitance and membrane potential of red and white muscle fibers. (A) Membrane capacitance of red muscle fibers from control fish (n=17) and fish exposed to 1.5% EtOH (n=19), 2% EtOH (n=13) and 2.5% EtOH (n=15). (B) Membrane potential of red muscle fibers from control fish (n=12) and fish exposed to 1.5% EtOH (n=18), 2% EtOH (n=9) and 2.5% EtOH (n=9). (C) Membrane capacitance of white muscle fibers from control fish (n=16) and fish exposed to 1.5% EtOH (n=12), 2% EtOH (n=15) and 2.5% EtOH (n=15). (D) Membrane potential of red muscle fibers from control fish (n=10) and fish exposed to 1.5% EtOH (n=12), 2% EtOH (n=9) and 2.5% EtOH (n=7). * Significantly different from control, $p<0.05$.


A
Mean red fiber membrane capacitance (pF)
0
10
20
30
40
*
*
Control
1.5% EtOH
2% EtOH
2.5% EtOH
B
Mean red fiber membrane potential (mV)
0
-20
-40
-60
Control
1.5% EtOH
2% EtOH
2.5% EtOH
C
Mean white fiber membrance capacitance (pF)
0
20
40
60
*
*
Control
1.5% EtOH
2% EtOH
2.5% EtOH
D
Mean white fiber membrane potential (mV)
0
-20
-40
-60
Control
1.5% EtOH
2% EtOH
2.5% EtOH

4. Discussion

In this study, we examined the effect of embryonic EtOH exposure on 3 dpf zebrafish larvae. I specifically focused on cells involved in locomotion because motor deficits and altered reflex responses have been observed in young humans, rodents and zebrafish that were exposed to EtOH during development (Kalberg *et al.*, 2006; Staisey and Fried, 1983; Driscoll *et al.*, 1990; Carvan *et al.*, 2004). To this end, following an initial evaluation of the effects of EtOH exposure on gross morphological features, I have concentrated on examining treatment effects on muscle morphology, motor neuron development and innervation patterns, and development and properties of the neuromuscular junction.

4.1. Gross morphology

The results presented in this study indicate that EtOH exposure resulted in shorter trunks, pericardial edema, cyclopia, axial malformations and a curved body axis. In humans, FAS is characterized by pre- and post-natal growth deficiencies, ocular deficits, cranial and facial abnormities, as well as joint and cardiac anomalies (Jones and Smith, 1973; Jones *et al.*, 1973). When exposed to EtOH pre-natally, rodent models display very similar neurobehavioral and physiological effects compared to children with FAS, including altered brain development and poor motor coordination (Driscoll *et al.*, 1990). Fish exposed to 1% EtOH did not display a significant increase in physical deformities, whereas fish exposed to 1.5% EtOH showed an increase in pericardial edema. Furthermore, 1.5% EtOH treatment resulted in only a few, mild deficits in motor neuron and muscle fiber morphology, indicating that 1.5% EtOH might be the threshold

for toxicological effects over this 16 hour exposure window. This is mirrored in the hatching rate and swimming behaviour of larvae exposed to 1.5% EtOH when compared to larvae exposed to 2% and 2.5% EtOH. Other studies have reported comparable effects in zebrafish morphology when using similar EtOH concentrations over slightly different exposure windows (Arenzana *et al*., 2006; Bilotta *et al*., 2004). Another study reported mortality rates of 50% or more at 5 dpf when zebrafish were exposed from 3 to 24 hpf to EtOH concentrations over 380 mM (approximately 2.2%; Reimers *et al*., 2004). In this study, none of the ethanol concentration resulted in a mortality rate of 50% at 5 dpf when fish were exposed to EtOH concentrations between 1% and 3% from 8 to 24 hpf. However, by 7 dpf, more than half of fish exposed to 2.5% and 3% EtOH had died. The discrepancy between our results may be due to the different exposure windows used in the two studies. Exposing the embryos to EtOH earlier in development, when developmental abnormalities are more lethal to the embryos, could result in an increased mortality rate.

It is important to note that although zebrafish have been used in several studies as a model for FAS, the timing, the length and the ethanol concentration used in the studies vary greatly, making it difficult to compare results between studies. For example, Aranzana *et al*. (2006) exposed embryos to 1.5% and 2.4% EtOH from 4.7 hpf to 24 hpf, while Dlugos and Rabin (2007) exposed embryos to concentration between 0.1% and 1.0% v/v from 0 hpf to 72 hpf. Interestingly, although zebrafish display defects that are similar to other species as a result of early ethanol exposure, the doses required to produce these defects in zebrafish embryos are remarkably higher. One possible explanation is that the chorion impedes the flow of ethanol from the surrounding

medium to the embryo. In my preliminary studies, I exposed zebrafish to EtOH in the presence and absence of the chorion (data not shown), and found no difference between treatments, similar to a number of other studies (Blader and Stähle, 1998; Li *et al.*, 2007; Dlugos and Rabin, 2007). A second explanation could be that placental fetuses may be exposed to alcohol concentrations that are higher than the mother's blood alcohol level due to the lack of alcohol dehydrogenase in the embryo prior to liver differentiation (Yelin *et al*., 2005), resulting in embryonic blood alcohol levels that are higher than originally thought. Alternatively, zebrafish embryos, like all cold-blooded vertebrates, absorb nutrients and oxygen through the skin (Matsui *et al*., 2006). It is possible they are also absorbing ethanol in the same fashion, resulting in an embryonic blood alcohol level that is lower and therefore similar to the one attained in mammalian models (Matsui *et al*., 2006). Reimers and colleagues (2004) measured internal ethanol concentrations that were around 30% that of the exposure concentration after 4 h of exposure, when the chorion was intact. Li and colleagues (2007) have found that after an exposure to 2% EtOH for 6 h (4.25-10.25 hpf), with the chorion intact, zebrafish blood alcohol content only reached 0.034 gdL^{-1}, which is lower than the legal blood alcohol content limit for operating a motor vehicle in Canada (0.08 gdL^{-1}). In my study, I exposed zebrafish to 1% to 3% EtOH for 16 h (8-14hpf); therefore, based on the findings of Li and co-workers (2007), it is possible that the blood alcohol content in zebrafish was comparable to physiological levels attained in humans. However, Bradfield and colleagues (2006) reported that when the zebrafish chorion is removed, the ethanol concentrations within the water compartments of the embryos equilibrate with the exposure concentration after 3 h of exposure. Thus, there is still some question

regarding the ability of the chorion to allow ethanol equilibration. The blood alcohol levels attained in children with FASD and in zebrafish embryos are unclear, and uncertainties remain when assessing the physiological relevance of a particular dose (Fernandes and Gerlai, 2009).

4.2. Locomotion

Children exposed to alcohol *in utero* display delays in motor and reflex development (Barr *et al.,* 1990; Kalberg *et al*., 2006; Staisey and Fried, 1983; Simmons *et al*., 2002). Rats exposed pre-natally to EtOH also show reflex development delays, as well as deficits during tasks that require balance and motor coordination (Shaywitz *et al*., 1979; Lee *et al*., 1980; Driscoll *et al*., 1990). In addition, walking patterns and gait in rats have been shown to be affected as a result of early EtOH exposure, where the animals displayed shorter strides and a lack of symmetry in their gait compared to controls (Hannigan and Riley, 1989). Both central and peripheral mechanisms are believed to be affected by pre-natal exposure to alcohol (Simmons *et al*., 2002).

The results in this study show that EtOH-exposed zebrafish larvae take longer to hatch out of the chorion, if they hatch at all, and exhibit fewer bouts of swimming after touching the trunk. Although it is probable that multiple sensory systems are affected by EtOH exposure, I focused on whether the larvae kept swimming after responding to touch in order to determine if EtOH exposure specifically affected swimming behaviour. One could argue that the behaviour I examined occurred only after touch and therefore was related to sensory phenomena that might also be affected by EtOH exposure.

However, I also noticed an overall reduced amount of swimming in normal egg water, suggesting a reluctance or reduced ability to move.

4.3. Motor neuron morphology

My results suggest that the axons of primary and secondary motor neurons in EtOH-exposed fish were adversely affected. In general, they exhibited a disordered pattern of muscle innervations, early truncation, aberrant curvatures and reduced branching. Primary motor neurons undergo the final round of division between 9 and 16 hpf and secondary motor neurons are born 5-6 h after primary motor neurons. However, secondary motor neurons send growth cones into the periphery about 6h after primary motor neurons have sent their growth cones, so on average at 26 hpf. Therefore, the majority of secondary motor neurons extend their axons into the periphery after the EtOH has been removed in this study (at 24 hpf), while the latest observed primary motor neuron to send its growth cone was at 23 hpf. Since primary and secondary motor neuron morphology was affected in a similar fashion, this suggests that factors controlling growth cone guidance and development, such as sonic hedgehog (Shh) were affected by EtOH. The effects of EtOH last long after it has been removed, possible because guidance cues that are normally influenced by Shh are altered, resulting in abnormal motor neuron guidance after EtOH is removed, or because secondary motor neurons follow the path of primary motor neurons and guidance cues were altered by EtOH during primary neurons axon elongation (Myers *et al*., 1986). Shh is expressed in the notochord and the floor plate of the neural tube during early embryogenesis (Li *et al.*, 2007) and regulates the development of neural components,

such as progenitors of motor neurons (Roelink *et al*., 1994; Ericson *et al*., 1996). Li and colleagues (2007) found that exposing Zebrafish embryos to EtOH concentrations higher than 1% from 4.25 hpf-10.25 hpf blocks covalent modification of Shh by cholesterol which then impairs Hedgehog transduction and causes defects similar to those observed with FASD. Loucks and Ahlgren (2009) recently suggested suppression of Shh signalling is one of the major effects of EtOH exposure. Altered Shh expression in *Xenopus* and mouse embryos has been reported as a result of EtOH exposure (Yelin *et al*., 2007a; Yamada *et al*., 2005). Therefore, it is possible that the abnormalities in motor neuron morphology that I detected are a result of the effects of EtOH on Shh, but this remains to be resolved.

Alternatively, retinoic acid (RA) acts on Shh during development and RA deficiencies have been shown to affects dorsal and ventral patterning (Ribes *et al*., 2006). A current model attempting to explain the teratogenic effects of EtOH on embryos suggests that EtOH might function as a competitive inhibitor for the metabolism pathway of vitamin A (retinol; ROL) to RA (Duester, 1991; Deltour *et al*., 1996). The biosynthesis of RA from ROL involves two oxidation steps and these two steps also convert EtOH to acetic acid (Clagett-Dame and DeLuca, 2002; Marill *et al*., 2003). Yelin and colleagues (2005) have shown that EtOH exposure during gestation in mice causes lower RA levels and combined EtOH and ROL exposure rescues the teratogenic effects of EtOH exposure alone. EtOH acting as a competitive inhibitor would lead to reduced RA levels during development when EtOH is present, causing several abnormalities, which may be in part mediated by altered Shh signalling as a

result of altered RA levels (Clagett-Dame and DeLuca, 2002; Marill *et al*., 2003; Yelin *et al*., 2007a).

A previous study examined the effects of EtOH exposure on motor neuron morphology in zebrafish (Parng *et al*., 2007), but their exposure window differed greatly from the one presented here. In that study, an exposure of 2.5% EtOH from 5 hpf to 6 hpf, resulted in a complete loss of primary motor neurons by 2 dpf (Parng *et al*., 2007). When I exposed embryos to 2.5% EtOH from 8 hpf to 24 hpf, and then left them to develop normally, the motor neuron axons were still present in most exposed larvae at 3 dpf, but displayed numerous abnormalities. The discrepancy between our results may be due to the different exposure windows used in the two studies. Although primary neurons are born between 10 and 12 hpf, involution happens around 5 hpf (Kimmel *et al*., 1991; Kimmel and Westerfield, 1990; Kimmel *et al*., 1995). It is possible that EtOH exposure between 5 and 6 hpf is affecting motor neuron progenitors, resulting in the loss of motor neurons, while an exposure between 8 and 24 hpf affects motor neuron axon outgrowth. Interestingly, EtOH has been shown to block migration of glial cells and growth of spinal motor neurons in chick embryos cultures (Museridze, 2005), and studies on rats indicate that early EtOH exposure causes a significant loss of motor neurons and a reduction of motor neuron diameter (Barrow Heaton *et al*., 1999).

4.4. Muscle morphology

I examined the muscle morphology of larvae exposed to 1.5%, 2% and 2.5% EtOH and found that muscle fiber width, length and angle are affected. Similarly, humans and rats exposed to EtOH during pregnancy had significantly smaller body

weights and muscle weights compared to controls pre- and post-natally (Abel, 1981, 1982; Nwaogu and Ihemelandu, 1999). Guinea pigs exposed to EtOH pre-natally were also found to have structural malformation of skeletal muscle (Nyquist-Battie *et al*., 1987).

This suggests that the muscle pioneers may be affected by EtOH exposure, resulting in abnormal muscle fiber morphology. Since I exposed the embryos to EtOH from 8 hpf to 24 hpf, and muscle fiber formation starts around 11.5 hpf, 1-2 h after segmentation (Devoto *et al*., 1996; Stickney *et al*., 2000), perhaps EtOH exposure disrupts somite and muscle fiber formation, which results in abnormal muscle segment division and muscle fiber morphology. The Notch signalling pathway is thought to be important in the segmentation patterns in the Zebrafish embryo (Stickney *et al*., 2000). Disruption of the Notch pathway has been shown to cause muscle fiber disorganization and an absence of somite borders (Takke and Campos-Ortega, 1999).

Alternatively, proper Shh signalling is necessary for patterning of the ventral neural tube, the anterior-posterior limb axis, and the ventral somites (Echelard *et al*., 1993; Roelink *et al*., 1994; Riddle *et al*. 1993; Johnson *et al*., 1994). Li and colleagues (2007) have shown that zebrafish exposed to EtOH (0.25%-2%) from 4.25 hpf to 10.25 hpf displayed abnormal red muscle fiber morphology and segment shape at 2 dpf, similar to what is reported in this study. Interestingly, the incidence of this phenotype was decreased by cholesterol supplementation (Li *et al*., 2007). Shh modification by cholesterol was shown to be decreased in EtOH-exposed fish, while Shh protein expression was unchanged and cholesterol content in exposed fish decreased in a dose-dependent fashion (Li *et al*., 2007).

Therefore, it is possible that EtOH exposure disrupted the Notch signalling pathway or other pathways involved in segmentation in the zebrafish embryo.

4.5. The motor neurons and their targets

In order for the neural circuitry to be properly wired during development, certain mechanisms that direct the axons towards their appropriate post-synaptic targets need to take place (Panzer *et al*., 2006). Since the motor neurons and muscle fibers show an abnormal morphology, I wanted to know if the synapses were forming properly. The formation of the neuromuscular synapse can be separated into two phases: (1) the establishment of the nAChR pre-pattern at prospective synaptic sites and (2) the pre-synaptic vesicle clustering and the increase of post-synaptic nAChRs densities after the motor axon makes contact with the muscle fiber. First, nAChRs cluster in the center of the muscle fiber where the motor neuron growth cone will make contact (Lin *et al*., 2001; Jing *et al*., 2009). Pre-synaptic derived agrin was originally thought to be responsible for pre-innervation nAChR clustering (Arber *et al*., 2002), although this process has been shown to be independent of nerve contact or agrin (Lin *et al*., 2001; Yang *et al*., 2001). However, this pre-patterned nAChR clustering and gene expression does requires the muscle-specific receptor tyrosine kinase (MuSK), which is a component of agrin signalling (DeChiara *et al*., 1996; Glass *et al*., 1996; Kim *et al*., 2008; Zhang *et al*., 2008). Activated MuSK recruits and clusters MuSK, as well as nAChRs (Jones *et al*., 1999). Once the growth cone makes contact with muscle fibers, the original nAChR clusters become incorporated into prospective neuromuscular synapses (Flanagan-Steet *et al*., 2005; Panzer *et al*., 2006; Lin *et al*., 2001). This is

followed by the clustering of pre-synaptic vesicles around the site of release and the insertion of additional nAChRs post-synaptically (Panzer *et al*., 2006). Panzer and colleagues (2006) have shown that motor growth cones preferentially contact muscle sites with nAChR clusters already present and form synapses at those sites.

Since motor neurons are showing an abnormal morphology, perhaps the nAChR post-synaptic pre-pattern is altered in EtOH-exposed fish, resulting in synapses forming at unusual sites, giving the motor neurons and overall abnormal trajectory. Conversely, if motor neurons are unable to target nAChR clusters on the muscle fiber, perhaps due to altered Shh signalling, synapses are forming at abnormal sites. However, since the pre-synaptic region is present at the same site as the post-synaptic density at 3 dpf, as shown by Figures 12 and 13, these synapses might be capable of normal synaptic transmission even though synapse location is affected.

4.6. Muscle mEPCs recordings

Two distinct event types are present in all of the recordings, from red and white muscle fibers alike. The slow events are thought to be events from immature synapses where there appears to be a lower density of acetylcholine receptors compared to mature synapses (Drapeau *et al.*, 2001). There are multiple changes that occur between 1 and 3 dpf that all contribute to the amplitude and the time course seen in older fish (Drapeau *et al.*, 2001). When comparing event kinetics from control recordings in this study to events recorded by Nguyen *et al*. (1999), fast events had decay time constants relatively close to those reported by Nguyen and colleagues (1999) at 3 dpf while slow control events had decay time constants closer to values

recorded around 27 hpf. However, Nguyen *et al*. (1999) pooled all of the detected events and did not specify whether the recordings were from red or white muscle fibers (or both). Todd and colleagues (2004) also discussed the possibility of different types of events being present in their white fiber recordings from zebrafish aged 4 to 6 dpf, where small, slow events had a mean peak amplitude of 200 pA and had rise times between 0.1-0.4 ms whereas the fast, larger events had amplitudes over 400 pA and rise times between 0.05-0.09 ms. The lower magnitude of peak amplitude and longer rise time in both fast and slow events observed in control larvae in this study as compared to Todd and colleagues (2004) may be due to the difference in developmental stages of fish used (3 dpf vs 4-6 dpf). Therefore, it is possible I am recording from immature and mature synapses. It is also possible that the slow events are actually fast events coming from adjacent muscle fibers through electrical coupling, which after being filtered, are recorded as slow, low amplitude events; however, this is unlikely. Carbonoxolone, which blocks gap junctions (Luna *et al*., 2004), was present during all recordings. Furthermore, visual confirmation with Lucifer yellow of the muscle fiber type showed a lack of coupling after individual recordings (data not shown).

mEPC recordings from red muscle indicated that there was no significant difference between control larvae and treated fish for fast event kinetics, although slow events from treated fish were smaller in amplitude and had a longer half width and decay time constants. Recordings from white muscle fibers indicated that EtOH exposure resulted in longer decay time constants for both fast and slow events, and fast event rise times and amplitudes were also affected in some treatments. A change in the amplitude could indicate changes in the amount of ACh released from the pre-synaptic

terminal or a difference in the number of channels opening during each release of ACh. The latter is linked to the number of active nAChRs, since they might be present but not non-functional, and the size of the synapse, since the ACh released may not be getting to all the receptors. A lengthening of the rise time indicates a slowing in the opening kinetics of individual nAChRs or an increase in the synaptic size, as ACh takes longer to get to receptors that are further away. Event half width is a measure of the open time of the receptors, whereas, the decay time constant is a measure of the closing kinetics of the nAChRs. Changes in either could possibly indicate a change in the closing kinetics of the receptors. Since a longer decay time constant was observed in slow red muscle events, slow white muscle events and fast white muscle events, perhaps an effect of treatment with 2.5% EtOH is altered receptor closing kinetics. One could examine this by studying the single channel kinetics of nAChRS.

The observed changes in mEPC kinetics may also reflect the effects of EtOH treatment on synapse maturation. Since synaptic maturity is characterized by fast, high amplitude events (Drapeau *et al.*, 2001), perhaps a general effect of EtOH treatment is to delay synapse maturity. By having slower event kinetics and smaller amplitudes, treated fish appear to exhibit event properties that are closer to younger fish compared to 3 dpf fish (Nguyen *et al*., 1999). In addition, red muscle exhibit fewer fast events per second, reinforcing the idea that these synapses are displaying a delay in maturation, as fast events display properties that are closer to events recorded from mature synapses (Drapeau *et al.*, 2001). During development, the fast event frequency of mEPC increases with age, along with the shift towards higher, faster events (Drapeau *et al*., 2001). Since the overall mEPC frequency was not affected by EtOH exposure when

compared to age-matched controls (data not shown), we can conclude ACh was being spontaneously released at the same rate in exposed fish, although the type of mEPC elicited was altered. This indicates that EtOH may be delaying post-synaptic maturation at the NJM, although pre-synaptic vesicles appear to display age-appropriate release kinetics. Delayed development of synaptic transmission at the NMJ may contribute to the impairment of swimming behaviour observed and the delayed/absent hatching ability in treated fish, since changes in synaptic properties are thought to be mirrored in the changing swimming ability of the fish (Nguyen *et al*., 1999; Saint-Amant and Drapeau, 1998; Buss and Drapeau, 2001). However, because the recordings were only done at one age, this needs to be verified by recording from exposed larvae at various age points before and after 3 dpf. A developmental delay induced by ethanol exposure is not uncommon. For instance, children with FASD display growth retardation as well as delayed development of motor skills, although these children usually catch up with age matched children later in childhood (Jones and Smith, 1973; Jones *et al*., 1973; Driscoll *et al*., 1990). Rats also display similar delays in coordination and motor control as a result of early ethanol exposure (Shaywitz *et al*., 1979; Abel and Dintcheff, 1978; Hannigan and Riley, 1989). In addition, cortical neurons in rats exposed pre-natally to ethanol displayed delayed migration, possibly leading to abnormal cortical circuitry (Miller, 1993). Developmental arrest was observed in 27-hpf zebrafish embryos after ethanol exposure (1.4% and 2.8% v/v) from 3 hpf to 27 hpf (Laale, 1971) and significant developmental delays were observed in 5-dpf zebrafish exposed to concentrations above 150 mM (approximately 0.9%) from 3 to 24 hpf (Reimer *et al*., 2004). Keeping track of the developmental stage of fish exposed to ethanol from the time of exposure

up until 7 dpf would allow one to determine if the fish display gross developmental delays at 3 dpf (when the recordings were taken in this study), and if the fish catch up with aged-matched controls by the end of the first week of development.

The conversion of nascent synapses to mature synapses is dependent, in part, on two programs: (1) the control of agrin over nAChR expression at nascent synapses (in order to maintain nAChR clustering) and (2) the turning off of nAChR expression throughout the rest of the muscle, which may be activity dependent (Arber *et al*., 2002). Since muscle fiber and motor neuron morphology, as well as synapse location, are affected in EtOH-exposed fish, perhaps agrin or MuSK expression is altered, resulting in abnormal nAChR clustering and therefore altered synapse maturation. nAChR gross distribution, as revealed by α-bungarotoxin, appeared similar between controls and exposed fish in terms of the amount of nAChRs present in the clusters and the cluster locations relative to the motor neuron axons. However, I did not have the optical resolution to examine synaptic size and the distribution of nAChRs within the synaptic clefts. It is possible that EtOH exposure is affecting the size of the synapse (i.e. the density of nAChRs), this would explain the reduced amplitude, longer rise time and increased half width and decay time constants. The released ACh would take longer to get to nAChRs that are further away from the release site, resulting in a longer rise time, and possibly wouldn't get to all the receptors, resulting in a reduced amplitude, and therefore the channels that opened last would also close last, resulting in a wider half width and longer decay time constants. In addition, mammalian studies have revealed that there is a subunit switch in muscle nAChR composition, where the fetal nAChRs are composed of $\alpha_1\beta_1\gamma\delta$ subunits, which switch to $\alpha_1\beta_1\varepsilon\delta$ shortly after birth (Goldman *et*

al., 1988; Fagerlund and Eriksson, 2009). The adult nAChR is more resistant to degradation and has a quicker response to agonists of AChRs (Missias *et al*., 1996). This subunit switch is thought to mediate in part the increase seen in synaptic efficiency between mature and immature synapses (Fagerlund and Eriksson, 2009). Although a subunit switch has not been shown in zebrafish, it is possible that a similar switch occurs around 3 dpf. Since my EtOH fish show slower mEPC kinetics, perhaps this subunit switch is delayed, making 3-dpf mEPC recordings from exposed fish look more like recordings from younger fish.

Gross morphological and immunohistochemical results observed in this study clearly display an overall linear and dose-dependent increase in defect occurrence and severity with increased concentrations of EtOH. Unexpectedly, some mEPC properties, such as the decay time constants for slow events in red muscle (Figure 16D) and the rise times for fast events in white muscle (Figure 19B), were not affected by EtOH in a linear, dose-dependent manner. The reason for this is unknown but a possible explanation can be considered. Effects of EtOH on an observed endpoint may be manifested via multiple mechanisms. Activation of these mechanisms may have different effects on the observed endpoint and the threshold for EtOH activation of each of these mechanisms may not be identical. Thus a suppression of an observed response parameter may be seen with one dose of EtOH while an enhancement may be observed with another dose. Whether this actually occurs or not requires future investigation.

Regarding the membrane capacitance, we found a significant difference between controls and EtOH-exposed fish for both red and white fibers, where fibers from

exposed fish had a significantly smaller membrane capacitance. Since membrane capacitance is directly proportional to the surface area of cell membranes, and is therefore indirectly a measure of the cell size, this supports my immunohistochemistry findings with F59 and F310, which revealed that EtOH exposure resulted in smaller red and white muscle fibers (Figure 10 and 11).

As a test of the health of the muscle fibers, I recorded their membrane potential and found that there was no significant difference in the membrane potential between groups of cells, suggesting that the cells I recorded from were healthy.

4.7. Physiology, function and future studies

What effect might these morphological and physiological changes have on locomotion? First of all, a shorter trunk length could be the result of smaller muscle fibers. EtOH-exposed fish did not appear to have fewer muscle segments (data not shown), but simply smaller segments, both in width and length, with an angle between hemi-segments closer to a 180°, as opposed to 100°. Improper orientation and length of the muscle fibers could result in altered muscle contractions and an inability to produce enough force to allow the fish break out of their chorion, for example. It is possible the sarcomere lengths and numbers are also affected by EtOH, although further studies need to be done to confirm this. In addition, the motor neurons might be innervating the muscle fibers at abnormal locations along their lengths, due to either an abnormal nAChRs pre-pattern prior to synaptogenesis or incorrect motor axon growth cone pathfinding during synaptogenesis. As a result, the contractions elicited by motor neuron action potentials could be uncoordinated, perhaps contributing in the locomotion difficulties seen, since the timing of the muscle contractions during swimming is key for

efficient swimming. *In vivo* studies of the muscle contractions elicited during swimming would indicate whether the muscles are contracting at the proper time. Examining *in vivo* the contractile force and length of sarcomere from skeletal muscle in fish exposed to EtOH, using the methods described by Dou and colleagues (2008), would also tell us if EtOH exposure affects the ability of the muscle fibers to contract, in addition to their morphology.

Since zebrafish skeletal muscle morphology is affected by early EtOH exposure, it is possible other types of muscle are also affected, such as the heart muscle. A lower heart rate has been observed in Zebrafish exposed to EtOH (Bilotta *et al*., 2004) and approximately 54% of children with FAS display heart defects (Abel, 1990). Forouhar and colleagues (2004) have explored the use of recording pipettes to acquire electrocardiogram (ECG) recordings from Zebrafish larvae. In addition, Yelin and collegues (2007b) have explored the use of optical imaging to study the heart microstructure. They found that *Xenopus* embryos exposed to EtOH displayed compromised heart looping and loss of ventricular trabecular mass (Yelin *et al*., 2007b). By using these two techniques, one could quantify the effects of early EtOH exposure on the heart structure and its functioning *in vivo* in zebrafish larvae. In addition, EtOH has been shown to stimulate the expression of Notch receptors and downstream target genes, resulting in increased angiogenesis in adult human endothelial cell cultures (Morrow *et al*., 2008). Therefore, it is possible that disruption of Notch as a result of EtOH exposure results not only in muscle fiber malformations but also abnormal angiogenesis, where the latter could be verified using optical imaging.

Pericardial edema was observed in 3-dpf fish exposed to EtOH concentrations as low as 1% EtOH in this study (Figure 6C). Edema can be a sign of kidney malfunction, since kidneys are key players in water elimination. Sheep exposed *in utero* to EtOH displayed a reduction in nephron endowment, which is thought to have important implication on cardiovascular health during post-natal health. Alternatively, cardiovascular function is essential for kidney development (Serluca *et al.*, 2002) as well as gill and kidney functions. Since zebrafish exposed to EtOH display an altered heart rate (Bilotta *et al*., 2004) and approximately 54% of children with FAS display heart defects (Abel, 1990), perhaps compromised cardiovascular function is affecting kidney development and function, resulting in the edema observed. Since gross morphology was observed at 3 dpf in this study, it is possible embryos were displaying signs of edema prior to this stage. Zebrafish embryos must maintain osmotic balance without the aid of the kidneys during the first two days of development (Kimmel *et al*., 1995). It is possible EtOH exposure is affecting the water permeability barrier that surrounds the embryo before the kidneys become functional. However, I noticed that, in fish that displaying pericardial edema, fluid accumulation was more pronounced as the fish aged (up until 7 dpf, when the studies ended; data not shown). This indicates that kidney function was most likely affected as opposed to the water permeability barrier, although it is possible both are affected by EtOH exposure. The effects of early EtOH exposure on kidney morphology and function still need to be examined.

It is likely that multiple sensory systems are affected by EtOH exposure in zebrafish. When assessing the swimming ability of control and EtOH-exposed fish in this study, I noticed some control larvae would respond to the presence of the fishing

line in the solution surrounding the fish before I touched the fishing line to the fish, indicating they perceived the fishing line either through water movement using their lateral line, or visual cues, etc. Since none of the exposed larvae responded to the fishing line before they were touched, it is likely one or more sensory systems were affected by EtOH exposure. Preliminary data with tubulin staining in fish exposed to 2% EtOH showed reduced olfactortory pits, otoliths, eye diameter and overall head size (data not shown). The effects of early EtOH exposure on the eye development of the zebrafish has been extensively studied (Arenzana *et al*., 2006; Matsui *et al*., 2006; Dlugos and Rabin, 2007; Bilotta *et al*., 2002; Stromland 1985, 1987) and hearing apparatus defects have been reported in children with FAS and in EtOH-exposed zebrafish (Church and Kaltenback, 1997 for review; Reimer *et al*., 2004). In addition, Carvan and collegues (2004) reported an altered startle response. Rats exposed to ethanol *in utero* demonstrated a reduced taste aversion to ethanol and its odour, indicating chemosensory systems are affected by pre-natal alcohol exposure (Youngentob and Glendinning, 2009). The effects of EtOH exposure on the zebrafish lateral line, as well as the olfactory sensory cells, still need to be examined.

Another explanation of the effects of EtOH exposure observed at the NMJ could be related to the effect of EtOH on NMDA receptors and $GABA_A$ receptors. Studies found that EtOH exposure during synaptogenesis in rodents leads to apoptotic neurodegeneration via the blockade of NMDA receptors and the hyperactivation of $GABA_A$ receptors, resulting in microcephaly and possibly neurobehavioural deficits (Olney *et al*., 2001; Ikonomidou *et al*., 2000; Olney *et al*., 2002; Olney, 2004). When NMDA antagonists and GABA mimetics were administered during synaptogenesis

instead of EtOH, a similar neurodegenerative response was triggered in the rodent CNS (Olney *et al*., 2001; Ikonomidou *et al*., 2000; Olney *et al*., 2002). Since the Mauthner neuron in zebrafish receives input via glutamate and glycine (Ali *et al*., 2000a; 2000b), blockade of NMDA receptors and the hyperactivation of $GABA_A$ receptors would result in a decreased probability of the Mauthner cells sending action potentials down to the motor neurons. With the help of S.A. Patten, I acquired preliminary mEPC recordings from the Mauthner cells in 3-dpf fish exposed to 2% (n=1) and 2.5% EtOH (n=2) (data not shown). These revealed that the frequency of events was significantly reduced in EtOH-treated fish, which might reflect changes in NMDA and glycine receptor activation. However, this needs to be explored further. In addition, pre-synaptic ionotropic glutamate receptors, NMDA and AMPA receptors, have been shown to co-localize with primary motor neuron axon tracts in the zebrafish and agonists of these receptors resulted in an increased frequency of spontaneous ACh release (Todd *et al*., 2004). Therefore, if EtOH is blocking the NMDA receptors centrally, as shown in rodents (Olney *et al*., 2001), perhaps this effect is also seen peripherally, resulting in altered ACh release at the NMJ. Todd and colleagues (2004) suggested that NMDA and AMPA receptors located pre-synaptically at the NMJ might have a role in a feedback mechanism during fast swimming. Activation of these receptors is thought to increase the release of ACh, which allows a high frequency of muscle contractions to be achieved. If this is the case, a blockade of NMDA receptors as a result of early EtOH exposure could result in a reduced ability to perform fast swimming. This could explain, in part, why the majority of 2.5% EtOH-exposed fish did not exhibit a sustained swimming response after responding to a touch stimulus (Figure 6D).

In conclusion, aberrant innervation, fewer axonal branches and smaller muscle fibers would likely result in weaker, uncoordinated movements during swimming. Red and white fibers are involved in slow swimming and fast bursts of activity respectively, and because both sets of fibers and motor neurons are affected by EtOH, it is probable that both types of movement will be negatively impacted. This may be akin to children with FAS that are weaker than healthy children and that exhibit deficits in fine and gross motor control. In addition, altered synaptic transmission indicates EtOH exposure may be delaying synaptic maturation and/or affecting NMDA receptors centrally and at the NMJ, contributing to the lack of swimming and the lower hatch rate observed in exposed larvae. The goal of this thesis was to study the effects of early EtOH exposure on the cells involved in locomotion in zebrafish. The current results indicate that early EtOH exposure results in an altered ability to swim, altered motor neuron and muscle fiber morphology and slower mEPC event kinetics, as well as numerous gross morphology defects.

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