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Acute Intoxication Affects Pavlovian Conditioning in Zebrafish (*Danio rerio*)

A thesis submitted in partial fulfillment of the requirement
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by

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Abstract

The study explored the effects of alcohol on short-term memory in zebrafish. Memory impairments are pervasive in several conditions resulting from long-term alcohol exposure, for example Fetal Alcohol Syndrome (FAS) and Wernicke-Korsakoff Syndrome. Most of the memory effects observed link to abnormal functioning of the hippocampus and frontal lobes. The effects of alcohol on memory are that it acts as a general central nervous system depressant, but it also affects some areas of the brain more than others. In our laboratory we use two variations of Pavlovian conditioning: Delay and Trace, to examine learning and memory with acute alcohol intoxication. The hypothesis was that zebrafish would respond to alcohol in a way similar to humans and other animal models. I used 60 adult zebrafish, half male and half female. The conditioned stimulus (CS) was a red square, while the unconditioned stimulus (US) consisted of zebrafish images that moved around the screen. In Delay conditioning the US immediately followed the offset of the CS. In Trace conditioning a 30-s interval separated CS offset from US onset. The highest concentration of ethanol, 1%, disrupted conditioned approach behavior in both training conditions, but the 0.5% concentration disrupted conditioned approach behavior only in the Trace procedure. This indicates that in the zebrafish, as in mammals, Trace conditioning is more sensitive to alcohol. Although it is unclear what effect of alcohol produces these learning and memory impairments, it is likely that alcohol disrupted activity in the hippocampus. This could occur via several routes—directly, through effects on hippocampal circuitry, or indirectly, by interfering with interactions between the hippocampus and other brain regions. Based on my findings, the zebrafish can be an important model to study both acute and chronic effects of alcohol on memory.

Keywords: zebrafish, Pavlovian conditioning, Trace conditioning, conditioned approach, appetitive, declarative, ethanol
Introduction

The current study explored the effects of alcohol on short-term/working memory in zebrafish. Alcohol (its chemical name ethanol) alters the basic functions of our regulatory systems and with prolonged exposure can result in permanent damage to certain parts of the brain in humans that can impair our thinking. Approximately 45 to 70 percent of alcoholics have specific deficits in problem solving, abstract thinking, concept shifting, psychomotor performance and difficult memory tasks (Eckardt & Martin 1986; Parsons & Leber 1981; Tabakoff & Petersen 1988). Additional effects of both acute and chronic alcohol exposure include impairments and alterations in the cerebellum. This affects motor function and coordination. Alcohol causes inhibitory effects on neurons of the cerebral cortex, affecting and altering thought processes that decrease inhibition and increase pain thresholds. Alcohol also decreases sexual performance by depressing nerve centers in the hypothalamus. Lastly, it depresses breathing and heart rate by inhibiting neuronal functioning of the medulla. The awareness of alcohol's effects on cognition can better help general health care providers identify alcoholics and refer them to proper treatment.

The consequences of harmful and underage young adult drinking are death, assault, sexual assault, academic problems, alcohol use disorder (AUD) and poisoning. Blackout, which is a drug-related amnesia, is most often associated with GABAergic drugs and is described as having effects similar to that of anterograde amnesia. Memory disruptions by alcohol leading to blackout link to inhibition of long-term potentiation (LTP) in the hippocampus by affecting Gamma-amino-butyric acid (GABA) and N-methyl-D-aspartate (NMDA) neurotransmission. Self-harm and suicide are much more common in people with alcohol problems. This can produce a vicious cycle of behavior: regularly drinking too much makes people feel depressed, and people drink to relieve
anxiety and depression. Alcohol affects the chemistry of the brain, increasing the risk of depression. Hangovers can create a cycle of waking up and feeling ill, anxious, jittery and guilty. Life gets depressing – arguments arise with family or friends, trouble at work, memory and sexual problems. Alcohol not broken down by the liver goes to the rest of the body, including the brain. Alcohol can affect parts of the brain that control movement, speech, judgment, and memory. These effects lead to the familiar signs of drunkenness such as difficulty walking, slurred speech, memory lapses, and impulsive behavior. Long-term heavy drinking can shrink the frontal lobes of the brain, which impairs thinking skills and such exposure results in permanent brain damage.

Research on the effects of alcohol on memory has come from three distinct areas, which will be briefly described below: Fetal Alcohol Syndrome, Wernicke-Korsakoff syndrome, and the memory-impairing effects of acute intoxication.

**Fetal Alcohol Syndrome**

Fetal Alcohol Syndrome (FAS) is the most severe collection of alcohol-related birth defects, and is diagnosed by pre and post-natal growth retardation, minor facial abnormalities, and deficiencies in the central nervous system (Jones and Smith, 1973). The effects of alcohol on prenatal development can include much more than those defining criteria and prenatal exposure to alcohol can potentially impact normal development at almost any point in the pregnancy, from embryonic through fetal development. Fetal alcohol exposure occurs when a woman drinks while pregnant. No amount of alcohol is safe for pregnant women to drink. Data from prenatal clinics and postnatal studies suggest that 20 to 30 percent of women do drink at some time during pregnancy. Alcohol can disrupt fetal development at any stage during a pregnancy, including at the earliest stages and before a woman knows that she is pregnant. Research shows that binge
drinking, which means consuming four or more drinks per occasion and regular heavy drinking puts a fetus at the greatest risk for severe problems.

Many of the cognitive problems in people with FAS center around memory impairments. Several studies have demonstrated that FAS participants show impaired habituation (Streissguth et al. 1983), spatial memory (Uecker and Nadel, 1996, 1998), poor recall and recognition memory (Mattson and Riley, 1999) and impaired working memory (Olsen et al, 1998). However, memory impairments are not global in their character, and some types of memory, such as procedural and implicit recall, seem normal (Coles et al., 2010; Lewis et al., 2015; Mattson and Riley, 1999; Olsen et al., 1998). Most of the memory effects observed link to abnormal functioning of the hippocampus and frontal lobes.

Within the fetus, embryonic cells destined to become brain neurons grow in number, move to their final locations and mature into a variety of functionally distinct neuronal cell types, eventually forming connections with other brain cells in a predetermined pattern. Animal experiments suggest that the third trimester may represent a particularly sensitive period for brain cell damage associated with FAS. The brains of alcohol-exposed individuals may have less volume (i.e., microcephaly) and they may have fewer numbers of brain cells or fewer neurons that are able to work correctly, which leads to long–term problems in learning and behavior. Alcohol or its metabolic breakdown products can also interfere with brain development by altering the production or function of natural regulatory substances that help promote the orderly growth and differentiation of neurons. Research using animals or cell cultures show that many of alcohol's dangerous effects on brain cells may be prevented by treatments for restoring the balance of regulatory substances upset by alcohol. Promising results have also been obtained in similar experiments by administering substances that help protect cells against free radical cell damage.
This is only one of several potential mechanisms that may give rise to alcohol-related fetal injury. Further research to decide if such an approach might prove both effective and safe in humans during pregnancy is essential. Studying related changes in brain structure and function may lead to a clearer understanding of how best to treat the devastating consequences of alcohol exposure. Studies with animals show that high doses of alcohol lead to a disruption in the growth of new brain cells. Scientists believe that it maybe the lack of newly generated cells that results in the long–term functional deficits found in some key areas of the brain.

Scientists are investigating the use of complex motor training and medications to prevent or reverse the alcohol–related brain damage found in people prenatally exposed to alcohol. In a study using rats, Klintsova and colleagues (2004) used an obstacle course to teach complex motor skills, and these skills in training led to a re–organization in the adult rats’ brains, enabling them to overcome some of the effects of the prenatal alcohol exposure. These new cells originate from stem cells, which are cells that can divide indefinitely, renew themselves and arise to a variety of cell types. These findings have important therapeutic implications, suggesting that complex rehabilitative motor training can improve motor performance of children, or even adults, with FAS.

Wernicke-Korsakoff syndrome

Chronic alcohol consumption can lead to Wernicke-Korsakoff syndrome. The symptoms of Wernicke’s syndrome include mental confusion, paralysis of the nerves that move the eyes and difficulty in muscle coordination. Approximately 80 to 90 percent of alcoholics with Wernicke’s syndrome also develop Korsakoff’s psychosis, a chronic and debilitating condition characterized by persistent learning and memory problems. Some of the changes in the brain of alcoholics are reduced cerebral blood flow (Ishikawa et al. 1986) and altered electrical activity (Porjesz &
Begleiter 1981), but there is not yet any clear evidence implicating these changes as the cause of observed cognitive deficits (Ron 1979; Wilkinson 1987).

Wernicke-Korsakoff syndrome is largely diagnosed on the basis of memory impairments coupled with known long-term alcohol use (Scalzo et al., 2015) Memory deficits are initially relatively specific for declarative memories, while procedural memories are not as adversely affected (Verfaelli and Keane, 2014). The cause of Wernicke-Korsakoff syndrome is not alcohol per se, but is rather the result of long-term thiamine deficiency (Vedder et al., 2015). Alcohol impairs the brain’s ability to use thiamine and a chronic deficiency of this nutrient can lead to cell death within the hippocampal region (Sullivan & Pfefferbum, 2009). Researchers have not found conclusive evidence that any one variable is solely responsible for the brain deficits found in alcoholics. Trying to characterize what makes some alcoholics vulnerable to brain damage where others are not remains the subject of active research. Promising new medications are in the early stages of development, as researchers strive to design therapies that can help prevent alcohol’s harmful effects and promote the growth of new brain cells to take the place of those that are damaged by alcohol.

*Acute alcohol intoxication and memory*

Alcohol acts as a general central nervous system depressant, but it also affects some areas of the brain more than others. Some of the regions that are particularly affected by acute alcohol are those that are affected by long-term exposure, and these include the cerebellum, frontal lobes and hippocampus. Alcohol inhibits neuronal activity in the hippocampus, which impairs memory encoding since the hippocampus plays an important role in forming new declarative memories (Milner, 1970). Alcohol primarily interferes with the ability to form new long–term memories,
leaving previously established memories intact. As the amount of alcohol consumed increases, so
does the size of the memory impairment. High doses of alcohol, particularly if consumed rapidly,
can produce partial or complete blackouts, which are periods of memory loss for events
experienced while a person is intoxicated. Blackouts are much more common among social
drinkers—including college students—than was previously assumed. Mechanisms underlying
alcohol-induced memory impairments are not completely understood, although alcohol’s
disruption of activity in the hippocampus has been well documented (White et al., 2000).

Alcohol severely disrupts the ability that neurons can set up long–lasting, heightened
responses to signals from other cells (Bliss and Collinridge 1993), a phenomenon known as long–
term potentiation (LTP). Researchers have theorized that something like LTP occurs naturally in
the brain during learning and many investigators have used LTP as a model to study the
neurobiology that underlies the effects of drugs on memory. Manipulations that disrupt the theta
rhythm also disrupt the ability to do tasks that depend on the hippocampus (Givens et al. 2000).
Alcohol-induced disruptions in the theta rhythm result, in large part, by the suppression of output
signals from the medial septal neurons to the hippocampus (Steffensen et al. 1993; Givens et al.
2000). The powerful influence that the medial septum has on information processing in the
hippocampus, and the impact of alcohol on cellular activity in the medial septum, likely plays an
important role in the effects of alcohol on memory.

In addition to the hippocampus, recent research with humans has yielded compelling
evidence that key areas of the frontal lobes play important roles in short–term memory and the
formation and retrieval of long–term explicit memories (e.g., Shastri 2002; Curtis and D’Esposito
2003; Ranganath et al. 2003). Damage to the frontal lobes leads to profound cognitive
impairments, which creates difficulty in forming new memories (anterograde amnesia). Recent
evidence suggests that memory processes in the frontal lobes and the hippocampus are coordinated via reciprocal connections (Wall and Messier 2001; Shastri 2002), raising the possibility that dysfunction in one structure could have deteriorating effects on the functioning of the other. Considerable evidence suggests that chronic alcohol use damages the frontal lobes and leads to impaired performance of tasks that rely on frontal lobe functioning (Kril and Halliday 1999; Moselhy et al. 2001). “Shrinkage” in brain volume, changes in gene expression and disruptions in how performing certain tasks affect blood flow in the brain are observed in the frontal lobes of alcohol–dependent subjects.

The effects of acute alcohol on learning and memory formation can be studied in several ways. The first is through human memory experiments, where participants ingest alcohol before performing a task. Nonhuman animal experiments also show alcohol impairment in learning and memory under well-controlled conditions. Animal research is important for identifying the mechanism producing these effects.

Several studies have demonstrated that the hippocampus becomes disrupted by alcohol intoxication and, as a result, hippocampus-dependent memories are typically affected. Acute alcohol impairs recall and recognition memory (Duka et al., 2001; Ryback, 1971) and declarative memory formation (Lister et al., 1991). Other types of memory that do not rely on proper hippocampal function are less severely affected, although high doses of alcohol can have general disruptive effects (Ryback, 1971).

There are many types of hippocampus-dependent learning tasks that are used to study alcohol’s effects in nonhuman animals. In our lab we use two variations of Pavlovian (classical) conditioning that dissociate hippocampus-dependent and –independent forms of learning. These are called Delay and Trace conditioning. In both procedures the animals are presented with a
neutral stimulus, called the conditioned stimulus (CS), that is followed by presentation of a biologically relevant stimulus, called the unconditioned stimulus (US). After several pairings of the CS and US the animals come to respond during the CS with some type of anticipatory response. In the case where the US is aversive (e.g. shock) the animals display defensive or avoidance behaviors to the CS. In a situation where the US is rewarding (e.g. food) the animals engage in approach behavior during the CS.

In Delay conditioning, the CS immediately follows the US, and this type of procedure typically results in the strongest associative learning. Numerous studies have shown that the amygdala is a critical brain region for Delay conditioning (LeDoux, 2000). The amygdala receives sensory afferents that convey information about the CS and US, and cells within this structure integrate this sensory information that orchestrate the response output. Trace conditioning trials are similar, except that the offset of the CS and onset of the US are separated by a stimulus-free period known as the trace interval. As the length of the trace interval increases, learning becomes progressively worse. The hippocampus, in addition to the amygdala, is required for learning in the Trace procedure, presumably because the task requires a memory “trace” of the CS to be associated with the US (McEchron et al., 1998). Studies have shown that hippocampal damage or manipulations that affect the functioning of the hippocampus lead to impaired Trace conditioning while leaving Delay conditioning relatively intact (Hunt and Richardson, 2007; Kaneko and Thompson, 1997).

Research conducted with rodents has verified a relatively selective effect of alcohol on hippocampus-dependent forms of learning. Melia et al. (1996) have shown that acute intoxication disrupts contextual fear conditioning and White et al. (2000) have reported that acute alcohol disrupts spatial learning and memory. Weitemier and Ryabinin (2003) reported that low-to-
moderate doses of alcohol impaired Trace conditioning in mice. Our lab has confirmed alcohol’s effects on hippocampus-dependent learning in rats by using the Delay and Trace conditioning procedures as well. Alcohol intoxication, at low-to-moderate doses, impairs Trace conditioning but spares Delay conditioning (Hunt and Barnet, 2016). More chronic binge-like exposure to alcohol also leads to disrupted Trace, but not Delay, conditioning when several intoxicating doses of alcohol are administered several days before (Yttri et al., 2004) or after (Hunt et al., 2009) the conditioning sessions.

Our lab has recently developed procedures for Delay and Trace conditioning in the zebrafish. The fish are tested in a 10-gallon tank and given visual images via computer monitors. The CS is a red square and the US is a dynamic display of zebrafish images. The zebrafish is a social fish and prefers to be with conspecifics. The experimental fish readily approaches the moving images of the zebrafish when presented on the display. Conditioned approach behavior is seen to the CS trained with either Trace or Delay trials, and the size of the response of the two groups is indistinguishable. This gives us the ability to study alcohol’s effects on Delay and Trace conditioning against an equivalent baseline response level.

The present experiment used the zebrafish (*Danio rerio*) as the experimental subject. The hypothesis was that zebrafish would respond to alcohol in a way similar to humans and other animal models (e.g. rodents). The zebrafish is becoming an important model organism in behavioral neuroscience, but information about its learning and memory abilities, reactions to drugs of abuse including alcohol, and the neurobiological mechanisms underlying learning and memory are still poorly understood. It is an important step to confirm some of the basic memory processes and their responsivity to alcohol so that further research using this important animal can move forward.
Methods

Subjects.

The subjects were 60 adult zebrafish, half male and half female. Fish were randomly assigned to one of the six groups (n = 10/group), designated by the ethanol concentration used during training and testing (0%, 0.5% or 1.0%) and the conditioning treatment used for training (Delay or Trace). All subjects were bred and reared in the lab, with breeders obtained from a fish distributor (www.petsolutions.com). Fish were 6-8 months of age at the time of testing. Fish were maintained in 3-l tanks in a Thoren Aquatics double-sided rack (Model P/N 90305). The Thoren system provided both biological and mechanical filtration, and a UV sterilizer. System water was created by adding 60 mg/l Instant Ocean sea salts to deionized water, and was maintained at 25-27°C. The room was maintained on a 14:10 h light:dark cycle with light onset at 0600h. Fish were fed two times per day with Tetramin flakes. Feedings were supplemented twice per week with frozen brine shrimp.

Apparatus.

The testing apparatus is shown in Figure 1. Fish were tested individually in a 36-l (10-gal) commercially available tank (50.8 cm x 27.9 cm x 33.0 cm). The floor and back wall of the tank was covered with white plastic sheeting to increase contrast and reduce visual distractions. The tank was filled with 33 l of system water. Ethanol (100%, Sigma-Aldrich) was added to create the 0.5% and 1.0% solutions. Training sessions were recorded using a JVC Everio hard disk camcorder (Model GZ-MG27U). Two 17” LCD monitors (Acer Model V173), connected to two laptop computers (Acer Aspire Model E1-531-2686), were used to present the visual displays. The monitors were positioned flush against the two shorter walls of the test tank. An overhead full-
spectrum bulb provided added illumination to the test tank. A custom-made software program (Saverino and Gerlai, 2008) controlled the stimulus presentations.

**Figure 1.** The 10-gallon tank used for training and testing. Stimuli were presented to the fish via two monitors placed flush against the two shorter walls of the tank.

**Procedure.**

Fish were removed from their home tank and placed, individually, into the test tank for a 10 min period of adaptation prior to the first training trial. The conditioned stimulus (CS) consisted of a red square (Figure 2a) that was presented for 60 s. The US was a dynamic display of 5 zebrafish that moved back and forth across the monitor and lasted for 120 s (Figure 2b). All fish were given 5 training trials in which the CS and US were presented on the same side of the tank. Trials were pseudorandomly on both sides of the tank in the following order: L-R-R-L-L. Inter-trial intervals
ranged from 4-6 min. The test trial consisted of a 5 min presentation of the CS on the right side of the tank. The test trial occurred 5 min after the last training trial. Sessions lasted 51 minutes.

**Figure 2.** Stimuli used for training. *Left:* the conditioned stimulus (CS) was a red square that lasted 60 s. *Right:* the unconditioned stimulus (US) consisted of zebrafish images that moved around the screen, simulating “swimming” behavior. US duration was 120 s.

A schematic depicting the stimulus presentation procedure for a single trial of Delay and Trace conditioning is shown in **Figure 3.** For animals in the Delay groups the US was presented immediately after the offset of the CS. For those in the Trace groups the US was presented 30 s after the offset of the CS on each training trial. Intertrial intervals were adjusted to accommodate the 30 s trace interval, such that the schedule of CSs and the test trial were the same for both groups.
**Figure 3.** Schematic showing the manner of stimulus presentations for one training trial for Delay and Trace conditioning groups. For both groups the CS was presented for 60 s and the US was presented for 120 s. For Trace groups, the interval separating CS offset from US onset (trace interval) was 30 s.

**Video scoring and data analysis.**

Recordings of the test sessions were scored manually. The test tank was divided into three vertical sections of equal width. The amount of time (s) that the fish was in the area closest to the stimulus was recorded for each minute of the 5-min CS test. Data from the test trial were analyzed separately for Delay and Trace groups using 3 (ethanol concentration) x 5 (minute of test) mixed-factor Analyses of Variance (ANOVA). Where appropriate, post-hoc comparisons made used the Fisher test (Keppel, 1982) with $\alpha = .05$. 
Results

**Delay conditioning.** The cumulative amount of time (s) that each ethanol concentration group spent in proximity to the CS during the test is shown in Figure 4. The 3 x 5 ANOVA revealed significant main effects of ethanol concentration \[ F (2, 27) = 5.02, p < .05 \] and minute of test \[ F (4, 108) = 50.99, p < .001 \]. The Ethanol Concentration x Minute of Test interaction was also significant, \[ F (8, 108) = 3.35, p < .05 \]. Post hoc comparisons showed that the animals tested in the 0% and 0.5% concentrations displayed conditioned approach behavior, exhibiting increasing time near the CS as the test trial progressed. In contrast, animals tested with the 1% concentration failed to show approach behavior.

**Trace conditioning.** The cumulative amount of time (s) that each ethanol concentration group spent in proximity to the CS during the test is shown in Figure 5. The 3 x 5 ANOVA revealed significant main effects of ethanol concentration \[ F (2, 23) = 4.21, p < .05 \] and minute of test \[ F (4, 92) = 37.24, p < .01 \] as well as an Ethanol Concentration x Minute of Test interaction \[ F (8, 92) = 3.48, p < .05 \]. Both concentrations of ethanol disrupted conditioned approach behavior in the zebrafish to an equal extent. The control fish (0%) spent increasing amounts of time in proximity to the CS during the test, but the animals tested in 0.5% or 1.0% ethanol solutions spent much less time near the CS. The two ethanol groups did not differ from each other but both differed from the control group by minute 2 of the test.
Figure 4. Test data obtained from the groups trained with Delay conditioning trials. Data are the time (s) spent near the CS during each minute of the 5-min test trial. Time is cumulative. As seen, the highest concentration of ethanol (1%) disrupted conditioned approach behavior, but the 0.5% concentration did not.
Figure 5. Cumulative time (s) that the fish spent near the CS during the test trial following Trace conditioning trials. Both concentrations of ethanol (0.5 and 1%) disrupted conditioned approach behavior.
Discussion

The purpose of this experiment was to examine the effects of alcohol intoxication on learning and memory in the zebrafish. Two tasks were used: Delay and Trace conditioning. Studies with rodents show that Trace conditioning is more sensitive to the disrupting effects of alcohol than is Delay conditioning (e.g. Hunt & Bannet, 2016) and the present results show the same thing in zebrafish. The highest concentration of alcohol (1%) impaired learning in both tasks, but only Trace conditioning became impaired by the lower concentration (0.5%). This indicates that in the zebrafish, as in mammals, Trace conditioning is more sensitive to alcohol.

In vitro research has demonstrated that alcohol suppresses cell activity in some brain regions, while increasing it in other regions (e.g., Verback et al., 1990). In vivo research supports the claim that alcohol decreases cellular activity in the medial septum but not in the lateral septum, and elevates responses in the ventral tegmental area (Gwens, 1996). The differential distribution of NMDA, glutamate and GABA receptors, coupled with ethanol’s effects on certain classes of ion channel function, gives rise to regional differences in brain sensitivity. The suppressing effects of alcohol on cell activity is seen in the hippocampus. In vitro studies show that alcohol reduces spontaneous activity of hippocampal pyramidal cells as well as induction of LTP (Pyapali et al., 1999; White et al., 2000).

While the lower alcohol concentration (0.5%) selectively impaired Trace conditioning, the higher concentration (1%) resulted in deficits in conditioned approach in both Delay and Trace groups. It is unclear why both tasks were affected. One possible reason for this effect is that alcohol impaired visual processing of the stimuli, either the CS or the US. Alcohol could also reduce motivation for the US. Relevant to this is a study by Varlinskaya and Spear (2004) showing
that acute alcohol intoxication inhibits social behavior in adolescent rats. Another possibility is that sedation resulting from intoxication impaired general locomotor ability. During the test trial, many fish were observed to be immobile on the bottom of the tank. Finally, alcohol may have affected associative learning processes more generally, even in the absence of alterations in responding or motivation. High doses of alcohol have been reported to interfere with Delay conditioning in rodent subjects (McKinzie et al., 1994; Melia et al., 1996). Further study is needed to find the reasons for the deficit in Delay conditioned responding with this high alcohol concentration.

Nevertheless, Trace conditioning was selectively disrupted by the 0.5% alcohol concentration, a finding in-line with previous studies (e.g. Hunt and Barnet, 2016). It may be informative to assess the consequences of more modest doses of ethanol on trace conditioning, using doses lower than 0.5%. This type of experiment could find whether alcohol’s effects on Trace conditioning conform to a dose-response function (a linear relationship) or need some absolute threshold to be observed (a step-wise function). It is also possible that low doses of alcohol could enhance learning, as has occasionally been reported in studies with humans (Bruce and Pihl, 1997; Parker et al., 1980).

Not only are some types of learning and memory impaired by acute intoxication, but exposure to high doses of alcohol can affect learning that occurs before or after alcohol administration. For example, Yttri et al. (2004) gave rat subjects multiple high doses of alcohol for several days before the animals were trained, and both training and testing occurred in the absence of alcohol. They found that pre-training alcohol administration impaired Trace conditioning but not Delay conditioning. This study showed that binge-like exposure to alcohol has a long-lasting influence on subsequent hippocampus-dependent learning. This research
indicates that patterns of drinking that cause high blood alcohol levels in rats can affect future learning and memory. Hunt et al. (2009) conducted another study in which binge-like alcohol exposure was given for several days after Trace conditioning, but before a test. This procedure also resulted in impaired Trace conditioned responding, even though both training and testing sessions occurred without alcohol. Alcohol therefore interfered with post-acquisition memory processing, likely also dependent upon the hippocampus.

The purpose of the present experiment was to study alcohol-induced disruptions in learning and memory in the zebrafish. The premises of the experiment were the many findings in human participants and mammalian animal models demonstrating the hippocampus is a region that is particularly sensitive to alcohol. Hippocampus-dependent tasks, such as spatial learning, context conditioning, and trace conditioning, are disrupted to a greater extent by alcohol than are non-hippocampus-dependent variants of the tasks (Hunt and Barnet, 2016; Melia et al., 1996; White et al., 2000). The question here was whether zebrafish would show a comparable result and, indeed, they did. However, the fish do not have a hippocampus or amygdala, although areas of the fish brain maybe homologous in function to these areas of the mammalian brain (Portavella and Vargas, 2005). In the goldfish, for example, two regions of the pallium have been shown to act similarly to the mammalian amygdala and hippocampus. The medial pallium appears to serve the same role as the mammalian amygdala, while the lateral pallium serves similar functions to the mammalian hippocampus (Broglio et al., 2005). Studies could assess the role of these two regions (medial and lateral pallium) in the Delay and Trace conditioning tasks employed in the current experiment and, further, to examine alcohol’s effects on cellular function within these regions.
Benefits of the zebrafish model

Zebrafish show a variety of complex behaviors in both social and defensive paradigms, such as shoaling, diving, avoidance, and choice behavior. Larval zebrafish show non-associative learning (habituation) of the startle reflex. Blaser and Vira (2014) describe a C-start startle response, which is a short-latency response, and compare it to an O-bend startle response which is a longer latency reaction. Also, when zebrafish are first placed into a novel tank, they will dive to the bottom of the tank, which is called the diving response. A preference for dark, relative to brightly-illuminated, areas has also been demonstrated. These latter two tasks are used to model anxiety (Maximino et al., 2012) and allow the zebrafish to be used for screening of novel anxiolytic drugs (Maximino et al., 2011).

Zebrafish are important animal models because adult zebrafish breed readily (about every 10 days) and can produce as many as 150 eggs at a time. This is different from mice, as they generally produce litters of only a few pups and can only breed about once every two months. Scientific experiments are generally repeated multiple times to prove that the results are accurate, so having an animal that can produce a large number of offspring is helpful. Zebrafish embryos are also fertilized externally, which allows them to be easily manipulated. For example, the fertilized eggs are easily injected with DNA or RNA to permanently change their genetic makeup to generate transgenic or knock-out zebrafish lines. Thus genomic studies are quite easy to join into behavioral neuroscience research on drug addiction. Zebrafish are vertebrates and therefore share a high degree of sequence and functional homology with mammals, including humans. Due to the conservation of cell biological and developmental processes across all vertebrates, studies in fish can give great insight into human disease processes.
Fear conditioning in zebrafish uses visual or olfactory cues paired with shock, touch or alarm pheromone. Meanwhile, operant conditioning uses positive (appetitive stimulus) and negative (aversive stimulus) reinforcements. As this research is relatively new in behavioral psychology more information about the sensory capabilities, nature of motivating stimuli, and choice of responses systems to measure in zebrafish becomes needed. These fish have excellent tetrachromatic vision, chemosensation and vestibular sensation. An investigation into unconditioned stimulus preferences and aversive stimuli used in other techniques might offer clues about extra motivating stimuli for future research. A true skinner-box type apparatus has been successfully used with fish and developing a standard model for zebrafish using operant conditioning paradigms would be extremely useful for drug self-administration experiments. Thus they are well-suited to research on associative learning processes, cognition, genetics and drug addiction.

**Future research**

Classical conditioning is a powerful technique for studying associative learning processes and how they contribute to the development, maintenance and treatment of addictive behaviors. Research on drug addiction has involved classical conditioning in several ways. For example, the use of a conditioned place preference procedure. The conditioned place preference task involves pairing a specific context with a drug, and a different context with no drug. After a few such pairings, animals are given a choice between the two contexts. A preference for the drug-paired context is used as a model for the rewarding properties of the drug. Researchers are using the place-preference task in zebrafish and have found reliable effects with alcohol (Mathur et al., 2011). Food-motivated conditioning techniques have been problematic with zebrafish because
their response to food, or food-paired cues, is variable. Zebrafish stay healthy for several days even with severe food deprivation, and show inconsistent motivation to feed in novel experimental tanks (Blaser and Vira, 2014). The use of the zebrafish images as an US, as in the present experiment, avoids these issues. The zebrafish respond to these visual images in a robust manner, the response does not habituate over time, and there is no need for a preceding period of social deprivation (isolation) in order for the stimuli to be rewarding.

Based on the importance of classical and instrumental conditioning in the development and maintenance of addictive disorders, the results of this experiment have implications for further study into drug addiction and recovery. Many researchers have suggested that cue-exposure-based extinction training of conditioned, drug-related responses is an effective treatment for addiction. Another potential therapeutic intervention would be based on the reconsolidation theory. According to this hypothesis, already-consolidated memories return to a state when reactivated, allowing them to undergo another phase of consolidation-reconsolidation (Forcato et al., 2007), which can be pharmacologically manipulated. These approaches suggest that extinction of drug-related memories may represent a practical treatment strategy in the future treatment of addiction. Studies suggest that primary exposure to stimulants and alcohol may enhance hippocampal function and, therefore, forming augmented drug-context associations that contribute to the developing addiction. In line with the self-medication hypothesis, withdrawal from a drug such as alcohol, cannabis or stimulants can result in hippocampus-dependent learning and memory deficits and further drug self-administration is used to overcome these problems. This may give rise to relapse to drug use and the maintenance of drug addiction.
Conclusion

As reviewed in this thesis, alcohol can have a dramatic impact on memory. Alcohol primarily disrupts the ability to form new long–term memories; it causes less disruption of recall of previously established long–term memories. At low doses, the impairments produced by alcohol are often subtle, although they are detectable in controlled conditions. As the amount of alcohol consumed increases, so does the size of the memory impairments. Tremendous progress has been made toward understanding the mechanisms underlying alcohol–induced memory impairments. Alcohol disrupts activity in the hippocampus via several routes—directly, through effects on hippocampal circuitry, and indirectly, by interfering with interactions between the hippocampus and other brain regions. The impact of alcohol on the frontal lobes remains poorly understood, but probably plays an important role in alcohol–induced memory impairments.
References


