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Associative learning performance is impaired in zebrafish (*Danio rerio*) by the NMDA-R antagonist MK-801

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Abstract

The zebrafish is gaining popularity in behavioral neuroscience perhaps because of a promise of efficient large scale mutagenesis and drug screens that could identify a substantial number of yet undiscovered molecular players involved in complex traits. Learning and memory are complex functions of the brain and the analysis of their mechanisms may benefit from such large scale zebrafish screens. One bottleneck in this research is the paucity of appropriate behavioral screening paradigms, which may be due to the relatively uncharacterized nature of the behavior of this species. Here we show that zebrafish exhibit good learning performance in a task adapted from the mammalian literature, a plus maze in which zebrafish are required to associate a neutral visual stimulus with the presence of conspecifics, the rewarding unconditioned stimulus. Furthermore, we show that MK-801, a non-competitive NMDA-R antagonist, impairs memory performance in this maze when administered right after training or just before recall but not when given before training at a dose that does not impair motor function, perception or motivation. These results suggest that the plus maze associative learning paradigm has face and construct validity and that zebrafish may become an appropriate and translationally relevant study species for the analysis of the mechanisms of vertebrate, including mammalian, learning and memory.

Keywords

learning and memory; acquisition; consolidation; recall; visual discrimination; NMDA-R; MK-801 dizocilpine; zebrafish

INTRODUCTION

The neurobiological mechanisms of learning and memory have been thoroughly investigated and hundreds of molecular players involved have already been identified (Sweatt, 2010). A large proportion of these studies have been conducted with mammalian model organisms, mainly the house mouse (Sweatt, 2010). Could analysis of zebrafish, a newcomer in this field, add to the wealth of this knowledge?

According to conservative estimates, a vertebrate genome (including mammalian and fish) may have about 30,000 genes. Recent DNA microarray studies have shown that at least 50% of all the genes of the genome are expressed in the brain of vertebrates (see e.g. Pan et al.,

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2010 and references therein). Protein products of a large proportion of these expressed genes may play roles in neuronal plasticity, i.e. in mechanisms of learning and memory. Briefly, there may be thousands of genes and neurobiological processes involved in learning and memory that have remained undiscovered as of today. How can one tackle this complexity?

There may be a number of ways one could systematically and comprehensively assess a large number of molecular players involved. One of these approaches is large scale high throughput mutation screens. Such screens have been attempted with the house mouse with some success (e.g. Reijmers et al., 2006). However, given the large number of animals one has to phenotype, these screens have been prohibitively expensive and thus are performed very rarely. Zebrafish may offer a feasible alternative. This species is particularly amenable to high throughput drug and mutation screens (Patton & Zon, 2001). It is small (4 cm long) and highly prolific (a female can produce 200 eggs per spawning multiple times a week) and is easy to maintain in the laboratory. Numerous successful forward genetic (mutagenesis) screens have been conducted (e.g. Patton & Zon, 2001) and most recently, comprehensive drugs screens have also been performed with zebrafish for behavioral brain research related phenotypes (e.g. sleep, see Rihel et al., 2010). But such screens have not been attempted for phenotypes associated with learning and memory. There are two main issues one needs to resolve before screening for mutation or drug induced changes in learning and memory could take place with zebrafish. First, one has to develop appropriate screening tools, and second, one has to demonstrate that these tools, the behavioral paradigms, have potential construct validity. The current paper is a step towards these goals.

The first step towards the development of appropriate behavioral screening tools is the characterization of the behavior of the species studied. The number of behavioral studies conducted with zebrafish is orders of magnitude less compared to those performed with mice or rats (Sison & Gerlai, 2010; Sison et al., 2006). Recently, however, several papers focusing on zebrafish learning have been published. For example, zebrafish have been found to perform well in a one trial avoidance learning paradigm (Blank et al., 2009), olfactory conditioning (Braubach et al., 2009), shuttle box active appetitive conditioning (Pather & Gerlai, 2009), place conditioning (Eddins et al., 2009), appetitive choice discrimination (Bilotta et al., 2005), visual discrimination learning (Colwill et al., 2005), active avoidance conditioning (Xu et al., 2007), spatial alternation based memory task (Williams et al., 2002), and even an automated learning paradigm has been proposed (Hicks, et al., 2006).

We have designed an associative learning task, adapted from the mammalian (rodent) literature, which was made deliberately to resemble classical radial arm maze paradigms (e.g. Schwegler & Crusio, 1995). In this task zebrafish are required to swim in a plus shaped maze and have to locate a reward, which is paired with a visual cue or the particular location where the reward is presented (Sison & Gerlai, 2010). Previously, the reward (US) we and others employed was food (Sison & Gerlai, 2010; Williams et al., 2002; Colwill et al., 2005), but in the current paradigm a temporally more stable motivator (which does not satiate as the food rewards), the sight of conspecifics was used. Here we study whether zebrafish can learn the association between a neutral visual stimulus (conditioned stimulus or CS) paired with the presence of conspecifics (the unconditioned stimulus or US). The task resembles a non-spatial version of the radial arm maze of rodents and is in principle a visual discrimination task (e.g. Colwill et al., 2005). Importantly, we explore whether this learning task has construct validity, i.e. whether disruption of a molecular mechanism known to be involved in learning and memory processes in mammals, would impair performance of zebrafish in this task.

The N-Methyl-D-Aspartate receptor (NMDA-R) has been shown to play fundamental roles in learning and memory and in underlying synaptic processes including long-term

potentiation and long-term depression (for a comprehensive review see Sweatt, 2010). This glutamate and voltage-gated calcium channel is a coincidence detector that opens only when glutamate is released from the presynaptic terminal (signal 1) and at the same time the postsynaptic membrane is depolarized (signal 2, resulting in the removal of a magnesium “plug” from the channel). The role of NMDA-R in learning and memory has been extensively investigated with numerous methods including molecular and pharmacological tools (for a comprehensive review see Sweatt, 2010). To manipulate NMDA-R function in the current study, we administer MK-801 (Dizocilpine, i.e. (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate) to zebrafish. MK-801 is a selective non-competitive antagonist of NMDA-R, a drug frequently used in preclinical and basic animal research (e.g. Takahashi et al., 2010; Venable & Kelly, 1990). MK-801 has been shown to impair learning and memory in a variety of species from snails (Solntseva & Nikitin, 2009) through fish (Blank et al., 2009) to mammals including mice (Venable & Kelly, 1990) and monkeys (Harder et al., 1998), suggesting an evolutionarily conserved role of NMDA-R in these processes. NMDA-R has been found in zebrafish and it is highly similar to the mammalian receptor in terms of the nucleotide sequence of the corresponding genes (Cox et al., 2005). Using quantitative RT-PCR and DNA Microarray, we have also confirmed expression of genes corresponding to subunits of NMDA-R in the brain of zebrafish (Pan et al., 2010) in a population similar to the one studied in the current paper. Furthermore, MK-801 has been shown to induce significant behavioral effects in zebrafish (e.g. Blank et al., 2009).

Despite the wealth of knowledge, the particular roles NMDA-R may play in different processes of learning and memory and the effects of MK-801 on these processes have been controversial (for reviews see Castellano et al., 2001; Dix et al., 2010). For example, MK-801 has been shown to impair acquisition (Gould et al., 2002; Nilsson et al., 2007; de Lima et al., 2005) or consolidation (e.g. Liu et al., 2009; Blank et al., 2009; de Lima et al., 2005) or retention (Tomilenko & Dubrinova, 2007), but Nilsson et al. (2007) showed a lack of effect on consolidation, Gould et al. (2002) showed enhancement of consolidation, and de Lima et al. (2005) showed impairing effects both on acquisition and consolidation.

In the current paper, we investigate whether MK-801 affects temporally distinct processes of learning and memory, i.e. whether it can disrupt acquisition, consolidation, and/or recall of memory. We employ a concentration of MK-801 (20 μ M) and dosing procedure that we have shown not to alter performance characteristics important in our learning task, including motor function, visual perception and motivation to shoal (Sison & Gerlai, 2011). Our goal with the current paper is, one, to show that zebrafish can perform well in an associative visual discrimination learning paradigm similar to those employed with rodents, and two, to demonstrate that the paradigm has translational relevance, i.e. can detect learning and memory impairing effects of MK-801.

METHODS

Animals and Housing

Short-finned wild type (SF) zebrafish (*Danio rerio*) were used in the experiments. This zebrafish population was the second filial generation that originated from breeders purchased from a local pet store (Big Al's Aquarium Services, Mississauga, Ontario, Canada). The SF population is a genetically uncharacterized heterogeneous stock. Their advantage over genetically well defined standard zebrafish strains is that SF fish are expected not to possess strain specific idiosyncratic features (this population has not gone through inbreeding induced genetic drift, i.e. random fixation of alleles). As a result, SF fish breed and grow well and most importantly are expected to show behavioral responses typical of wild type zebrafish. Eighty-four fish were used for the analysis of the effects of

MK-801 on learning and memory performance (we had seven treatment groups as explained below and the sample size, n , equaled 12 in each group). All fish were between the ages of 6–8 months and in all experiments the sex ratio was approximately 50-50%. Animals were bred, raised and maintained in house (University of Toronto Mississauga Vivarium). Adults were kept in 1 liter acrylic tanks (one fish per tank) that were part of a high density rack system (Aquaneering Inc., San Diego, CA, USA). This individual housing did not lead to visual or olfactory isolation of the experimental fish as the tanks were transparent and were placed side by side next to each other and the tank water recirculated among the tanks. This system provided multistage filtration including a mechanical filter, a fluidized glass bed biological filter, an activated carbon chemical filter, and a fluorescent UV light sterilizing unit. 10% of the water was replaced daily with deionized water supplemented with 60mg/l Instant Ocean Sea Salt. The water temperature was maintained at $26 \pm 2^\circ\text{C}$. Illumination was provided by fluorescent light tubes from the ceiling of the room with lights turned on at 08:00 h and off at 20:00 h. Fish were fed a mixture of ground flake food (4 parts, Tetraamin Tropical Flakes, Tetra, USA) and powdered spirulina (1 part, Jelmco Inc., Lambertville, NJ, USA).

MK-801 dosing procedure

Zebrafish were randomly assigned to seven experimental groups to analyze the effect of MK-801 on learning and memory performance. Six of these groups represented a 2×3 between subject experimental design with two MK-801 concentration levels (0 μM control and 20 μM) and 3 exposure times (immediately before the first training trial of the day [acquisition], immediately after the last training trial of the day [consolidation], and immediately before probe [recall]) were investigated (Figure 1). The seventh group was a control group in which fish received 0 μM MK801 and were trained with an unconditioned stimulus (US = sight of conspecifics) and conditioned stimulus (CS = colored plastic sheet) presented separately (unpaired control group). The method of drug delivery was identical to what we used before (Sison & Gerlai, 2011) for the analysis of potential performance altering effects of MK-801. Briefly, MK-801 hydrogen maleate (M107, Sigma-Aldrich) was dissolved in system water. Fish were placed in the corresponding concentration for 30 min in a 2 L rectangular treatment tank, one fish at a time, where the subject absorbed the drug (via the skin, gills and orally) while swimming in the drug solution (Sison & Gerlai, 2011). The timing of exposure employed in the current study was similar to the one employed in mice (Nilsson et al., 2007) and an exposure period and method were also similar to what were used in fish before (Swain et al., 2004).

The rationale for the timing of MK-801 delivery was as follows. We (Sison & Gerlai, 2011) and others (Swain et al., 2004) have found that immersion for 30 min in the drug solution had a significant behavioral effect for at least 30 min after the cessation of drug delivery but not as long as for 24 hours. We argued that a 30 min drug exposure immediately before the first trial should affect mainly acquisition related processes during the four trials of each day of training (as these trials complete within 26 min, see Figure 1). Furthermore, administering the drug immediately after the completion of the last trial of the day should affect consolidation of memory because consolidation related processes have been found sensitive to intervention within 90–120 min after training (for a comprehensive review see Sweatt, 2010). Last, administering MK-801 immediately before the probe trial was intended to test if MK-801 affects recall of already stored information.

The associative learning task

The apparatus of the learning paradigm was essentially a plus maze that was first employed in zebrafish by Al-Imari & Gerlai (2008) and subsequently by Sison & Gerlai (2010). The current apparatus was a significantly modified version of this maze. One important change

was that in the new maze stimulus tanks (each measuring $20 \times 10 \times 10$ cm, length $\times$ width $\times$ depth) were placed inside the target compartments at the end of each arm (Figure 1). These stimulus tanks could hold conspecific stimulus fish. The rationale behind this was as follows. In a previous plus maze task we were able to demonstrate acquisition of a CS-US association as well as spatial learning in zebrafish (Sison & Gerlai, 2010). However, this paradigm suffered from a drawback. We found the food reward (US) employed in the paradigm to be problematic. Even small amounts of the food reward we dispensed satiated our experimental fish quickly and thus training became ineffective after repeated trials. In the current paper, we utilize a different reward, the sight of conspecifics (representing the US), which avoids satiation due to the highly social nature of this species (Al-Imari & Gerlai, 2008). The stimulus tanks now allowed us to present this non-satiating reinforcement, the sight of conspecifics. Furthermore, the stimulus tanks were covered on three sides by red or white plastic sheets (Figure 1). This served two purposes. One, the experimental fish exploring the maze could view the contents of the stimulus tank only when it has entered the target compartment and swum behind the stimulus tanks but not from other parts of the maze. Two, the color of the plastic cover sheets served as the CS, i.e. predicted the content of the stimulus tank (red = conspecifics present, white = conspecifics absent). This prediction was consistent for 6 experimental groups (the paired groups) but for the seventh (unpaired control), the white vs. red stimulus was random with respect to the presence of stimulus fish in the stimulus tank (see e.g. Figure 2). Furthermore, the location of the target compartment containing the stimulus fish varied from trial to trial in a random manner, i.e., the only predictive cue (for the paired groups) was the presence of the red vs. white color (CS).

The current maze was fitted with a removable start box in its central compartment that could be lifted up using nylon strings and a pulley system from a remote location and thus the experimental fish could be released without disturbance. The learning task was preceded by an extensive habituation procedure. As most learning tasks, our paradigm also required testing single subjects. Zebrafish are a highly social species and being placed in a novel tank alone may be stressful for them (Gerlai et al., 2000). To minimize this negative aspect of the task and to facilitate active exploration of the maze all experimental fish were subjected to a 2 hour long habituation session every day for four consecutive days. During the first habituation session 20 fish were exposed to the maze at a time, and during subsequent habituation sessions progressively smaller number of fish, i.e. 10, 5 and finally 1 fish, were/ was exposed to the maze at a time as described previously (Sison & Gerlai, 2010). During habituation sessions the CS or US was not present in the maze. After the completion of habituation, fish were housed individually in 1 liter holding tanks, permitting visual (transparent tanks) and olfactory (shared water on the rack system) access to their shoal mates while allowing individual identification of the experimental subjects.

For training, the experimental subject was netted singly into the release box positioned in the central compartment of the maze and after a 30 sec acclimation period it was released into the maze by lifting the box up using a nylon string from a remote location. The fish was allowed to explore the maze for 5 min. Each fish had 4 consecutive trials a day with an inter-trial interval of 2 min. Training was conducted for 4 consecutive days, i.e. a total of 16 training trials were administered (Figure 1). During training, one of the stimulus tanks contained 5 zebrafish (the rewarding US) and the other three stimulus tanks were empty. The tank that contained the stimulus fish had red plastic sheets around it (CS) so that the red color was visible from any direction in the maze but the stimulus fish could only be viewed once the test fish entered the target compartment and swam behind the stimulus tank (Figure 1). This arrangement was similar for the empty stimulus tanks except that they showed a white color (CS-) instead of red and their content (empty) could also be seen only once the test fish swam into the target compartment.

After the 16 training trials, all experimental zebrafish were tested in a probe trial. The probe trial was identical to the training trials except that no stimulus fish were presented in any tank. Fish that associated the red color cue (CS) with the presence of conspecifics (US) were expected to choose the red colored stimulus tank and spend more time in the target compartment.

All behavioral sessions were video-recorded as described above and the recordings were later replayed. The behavior of the fish was quantified using the event recorder software application, Observer Color Pro 5.0 (Noldus, Wageningen, The Netherlands). Fish of the seven experimental groups received the same experimental procedures (including dosing procedures) and were trained, tested and their behavior quantified in a randomized and blind manner with respect to their group designation.

The percent of time experimental fish spent in different areas of the maze and the number of entries to these areas were quantified throughout training and the probe trial. The following measures were analyzed and are presented here: 1, Daily average of the percent of time in the target compartment during training: $T_d = (\sum t_i)/4$ where 'd' represents the training day (from 1 to 4), and 'i' represents the trial number running from 1 to 4 each day (i.e. we averaged over the four trials per day and present and statistically analyze the daily averages); 2, Daily average of the total number of entries (a measure of exploratory activity) to the end compartments of the maze: $E_d = (\sum e_i)/4$, where 'd' and 'i' are as above (i.e. we averaged over the four trials per day); 3, Percent of time in the target compartment during the probe trial; 4, Average percent of time in the end compartments other than the target compartment during the probe trial: $P = (\sum p_c)/3$ where 'c' represents the compartment (running from 1 to 3, i.e. we calculated the average percent of time per end compartment excluding the target compartment); and finally 5, Total number of entries to the end compartments during probe trial.

Statistical analysis

The results were analyzed using SPSS (version 14.1). To investigate the effect of treatment (7 levels) across days (4 levels, the repeated measure factor) during training, repeated measure univariate variance analyses (ANOVAs) were performed. Probe trial performance was also analyzed using ANOVA with a single factor (treatment with 7 levels). In case a significant main effect or interaction term was found, post hoc Tukey Honestly Significant Difference (HSD) test was conducted to establish which group(s) differed from each other. The null hypotheses of "no treatment effect" or "no difference across daily performance during training" were rejected when their probability was less than 0.05.

RESULTS

First we consider the behavioral results obtained during training, i.e. the acquisition trials. Figure 2 shows how much time experimental fish spent in the target compartment, the end of one of the arms of the maze that contained the stimulus fish during training (figure 2). ANOVA revealed no significant Treatment effect ($F(6, 77) = 1.948$, $p > 0.05$), no significant Training day effect ($F(3, 231) = 0.972$, $p > 0.40$) and no significant interaction between these factors ($F(18, 231) = 0.563$, $p > 0.90$), suggesting that fish in all treatment groups got exposed to the target compartment and thus the conspecifics during training similarly. It is also important to realize that the apparent, and nonsignificant, difference between the MK-801 treated and control groups cannot be due to the drug treatment because, for example, fish of the second and third graphs (of figure 2) did not even receive the drug until after the training trial was completed.

The total number of entries to the end compartments during training, a measure of exploratory activity or motor function, however, appeared to differ among some treatment groups (Figure 3). ANOVA confirmed this observation and found a significant Treatment effect ($F(6, 77) = 4.068, p < 0.01$). The effect of Training day ($F(3, 231) = 3.169, p < 0.05$) as well as the interaction between Treatment and Training day ($F(18, 231) = 3.399, p < 0.001$) also turned out to be significant. Figure 3 suggests that these significant effects may be due to the higher values and the apparently more robust decrease of these values across days obtained for the group of fish that received MK-801 before training (the black squares on the first line graph of Figure 3). Tukey HSD post hoc multiple comparison analysis indeed confirmed this observation and showed that on the first three days fish of this group entered the end compartments significantly ($p < 0.05$) more frequently as compared to fish of all other groups but by day 4 this difference became non-significant.

Perhaps the most important finding of this study concerns how much time experimental fish spent in the target compartment during the probe trial that followed training, i.e. when the compartment contained no fish but only the conditioned stimulus (Figure 4). ANOVA revealed a significant Treatment effect ($F(6, 77) = 3.45, p < 0.01$) demonstrating that some group(s) differed from the other. Tukey HSD showed that fish exposed to MK-801 after training or before the probe as well as those fish that did not receive the paired presentation of CS and US (unpaired control) spent significantly ($p < 0.05$) less time in the target compartment compared to all other groups of fish, whereas other group differences were non-significant ($p > 0.05$). Thus, MK-801 administered after training or before probe impaired memory performance to a level that was not distinguishable from the performance of fish that received no CS-US pairing, i.e. baseline performance.

We also analyzed the time experimental fish spent in the end compartments other than the target compartment because we wanted to see whether the above described differences were specific to the target compartment or whether they reflect changes in the way fish visit any end compartments in the maze. Figure 5 shows the percent of time fish spent in the end compartments other than the target averaged for the three end compartments. ANOVA revealed no significant Treatment effect ($F(6, 77) = 1.121, p > 0.35$) confirming that differences found in target compartment dwell time among the treatment groups are specific to that compartment.

Last we quantified how many times fish visited the end compartments (Figure 6). ANOVA revealed no significant Treatment effect ($F(6, 77) = 1.706, p > 0.10$) suggesting that general exploratory activity of the experimental fish of the 7 treatment groups was not different from each other during the probe trial.

DISCUSSION

The above results demonstrate that zebrafish are capable of acquiring and remembering the association between a neutral visual stimulus (CS) and the sight of conspecifics (US). Figure 4 shows that all fish that received the CS-US pairing but no MK-801 (grey bars on figure 4), spent about 25–30% of their time in the target compartment. This performance is significantly above the level of the unpaired control group, which did not receive the CS-US pairing and spent about 8% of their time in the target compartment.

The apparent preference for the target compartment among fish that received the CS-US pairing (but no MK-801) may be due to the acquisition of CS-US association but it may also reflect alternative strategies not specific to the CS. For example, instead of learning the predictive value of CS, fish may have learned that the US is localized in end compartments and thus preference for all end compartments, vs. the center or the connecting tunnels of the

maze, is the solution of the task. Our results demonstrate that this alternative strategy is unlikely (Figure 6). Fish from all groups spent about 5–8 % of their time on average in an end compartment that was not marked by the CS, a value that is similar to how much time fish of the unpaired control group spent in the target compartment (8 %). We regard this value “baseline” (chance performance level) and conclude that fish unimpaired by MK-801 treatment showed specific preference (25 – 30%) for the target compartment and have acquired and remembered the association between CS and US. We conclude that zebrafish are capable of performing well in the plus maze visual stimulus discrimination associative learning task reinforced only by the sight of conspecifics. Access to conspecifics has been previously shown to have rewarding properties in zebrafish (Al-Imari & Gerlai, 2008) and in other fish species (Gerlai & Hogan, 1992) and the current results support the notion that this natural stimulus will be appropriate as a reward for future zebrafish learning tasks.

Importantly, we found MK-801 to reduce the target compartment dwell time to a level indistinguishable from that of the unpaired control when the drug was administered either after training or before the probe trial. That is, when delivered at these time points, MK-801 abolished learning and/or memory. The impairing effects of MK-801 are unlikely to be due to disruption of performance characteristics such as motor function, perception or motivation. The effect of MK-801 on these factors was analyzed previously (Sison & Gerlai, 2011) and when administered at 20 μ M in a manner identical to that used here the drug was not found to alter these performance characteristics. Furthermore, the total number of entries to the end compartments during training analyzed here remained unaffected by MK-801 administered after training or before the probe confirming that motor function and the motivation to explore the maze were unaltered. Interestingly, fish treated with MK-801 just before the training trials did show elevated frequency of entries to the end compartments suggesting hyperactivity induced by the drug. Hyperactivity inducing effects of MK-801 have been demonstrated in mammals (Martin et al., 1997) as well as zebrafish (Seibt et al., 2010) before. Notably, however, these hyperactive fish performed well in the probe trial and showed no learning performance deficit. Last, the level of activity of zebrafish of any experimental group did not differ during the probe trial. Thus we conclude that motor performance or motivation are unlikely to explain the MK-801 induced abolishment of learning or memory in fish that received the drug either after training or just before the probe trial.

Perhaps the most interesting question of this paper concerns what temporal phase of the process(es) underlying learning and memory may have been affected by MK-801 administration. Our results suggest that consolidation and recall were both affected. When we administered MK-801 for the 30 min period immediately preceding the training trials (once every day for the four days of training), probe trial performance remained unaltered. This is despite that MK-801 did induce an apparent hyperactivity during training. From a previous study we conducted (Sison & Gerlai, 2011) as well as from other papers (e.g. Nilsson et al., 2007; Swain et al., 2004) we know that a 30 min MK-801 immersion period is sufficient for this drug to reach the brain in zebrafish. Thus, the lack of alteration of probe trial performance found we interpret as indicative of lack of effect on acquisition.

On the other hand, the 30 min immersion in the MK-801 solution after training was aimed at targeting consolidation. The consolidation window, within which manipulation of brain function is expected to impair that process, is usually found to be the 90 min interval immediately following acquisition, i.e. the training trial. We employed the 30 min long MK-801 immersion immediately after the last trial of the four-trial training every day, i.e. fish were immersed from the 21st to the 51st min, from the 14th to the 44th min, from the 7th to the 37th min and from the 0 to the 30th min after the end of the first, second, third, and fourth consecutive training trial of the day respectively (see Figure 1). Thus we suggest that

our MK-801 administration procedure targeted the consolidation window. This administration abolished memory, i.e. brought the target compartment dwell time down to a level that was indistinguishable from the performance of fish that were not trained to associate the CS with the US (the unpaired control). The disruption is unlikely to be due to performance factors such as motor function, perception or motivation because such effects were excluded by a previous study at the 20 μ M concentration of MK-801 (Sison & Gerlai, 2011) and also by our current work as discussed above. Therefore, we conclude that MK-801 disrupted memory consolidation of zebrafish in the conspecific reinforced visual discrimination plus maze associative learning paradigm.

The third time point of MK-801 manipulation was aimed at targeting recall of memory. For this we administered MK-801 right before the probe trial (Figure 1). Analysis of neural plasticity at the behavioral as well as at the synaptic function level has shown that recall is an active process (for a comprehensive review see Sweatt 2010). It is therefore expected that interfering with such processes may have memory performance altering effects. Alternatively, one could argue that pre-probe trial MK-801 administration may affect the manifestation of memory, e.g. by altering performance characteristics. However, as discussed above, alterations in these performance characteristics are not supported by a previous study (Sison & Gerlai, 2011) or by the current results and therefore we conclude that the impaired probe trial performance induced by pre-probe trial MK-801 administration likely be the result of disruption of recall of memory.

NMDA-R has been implicated in the mechanisms underlying acquisition, consolidation, as well as recall (for a comprehensive review see Sweatt, 2010). However, the findings have not always been confirmatory (Castellano et al., 2001; Dix et al, 2010). The review of this exciting but vast literature is beyond the scope of the current paper but, briefly, there may be several reasons for the controversies, including the method of genetic manipulation or the type of pharmacological tools employed, or the timing of the manipulations, or the type of learning task utilized, or the species studied to mention but a few experimental factors. Interestingly, it is notable that even with the same pharmacological tool employed on the same species such controversies exist. For example, MK-801 was found to disrupt learning and memory performance when administered right after training in an inhibitory avoidance task in zebrafish, suggesting MK-801 exerted a significant effect on memory consolidation (Blank et al., 2009), but in an active avoidance conditioning task post-training administration of MK-801 was found ineffective in zebrafish (Xu et al., 2007). Our current results confirm those of Blank et al (2009) and contradict those of Xu et al. (2007). Furthermore, our work extends these findings by showing MK-801 having no effect on acquisition and having a significant effect not only on consolidation but also on recall, results that are consistent with a large body of work using a variety of psychopharmacological agents affecting NMDA-R function in mammals (for review see e.g. Castellano et al., 2001).

Last, we discuss whether our results have any relevance for high throughput mutation or drug screening, the ultimate purpose behind our work. The plus maze paradigm as ran here is clearly not appropriate for high throughput screening. The task is slow and labor intensive. However, the results obtained with it demonstrate good associative learning capabilities of zebrafish and thus set the stage for future, more efficient paradigms. It is also notable that the CS and US utilized in this task are both simple visual stimuli and may be presented in a computer controlled automated manner. For example, we have already shown that animated (moving) images of zebrafish elicit strong shoaling responses (Gerlai et al., 2009; Fernandes & Gerlai, 2009) and can also be utilized in a simple shuttle box learning task (Pather & Gerlai, 2009). A conditioned stimulus may also be easily presented in a paired or unpaired manner on the computer screen for experimental subjects and thus

associative learning tasks principally similar to the current one may be designed. Given that the behavioral output quantified, i.e. the location of the fish, is also easily measurable using automated techniques, e.g. video-tracking (Blaser & Gerlai, 2006), it is likely that automated associative learning tasks could be run in parallel and thus their throughput increased sufficiently for large scale screening.

In conclusion, although zebrafish is quite novel in the analysis of learning and memory several studies have shown good promise for this species. Our current work demonstrates that zebrafish can learn the plus maze visual discrimination associative task as well as rodents learn certain CS-US associations in the radial arm or plus maze (e.g. Schwegler & Crusio, 1995 and references therein). This suggests good face validity of the paradigm. It also appears that MK-801, a pharmacological agent shown to be an antagonist of the mammalian NMDA-R, does disrupt memory processes in zebrafish, suggesting construct validity of the zebrafish learning paradigm. Last, the features of the paradigm make it amenable to high throughput screening. Taken together these points suggest that zebrafish will be a good laboratory tool with which several molecular mechanisms of learning and memory may be revealed in the future.

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RESEARCH Highlights

1. Zebrafish performed well in a plus maze associative learning task.
2. MK-801 disrupted memory performance when administered after training.
3. MK-801 disrupted memory performance when administered before memoryprobe trial.
4. MK-801 did not disrupt memory performance when administered before training.
5. The results suggest good face and construct validity of the zebrafish learning task

Figure 1.

Panel A, The design of the plus maze. The plus maze consisted of 4 end compartments and one center compartment (linear dimensions of the maze are indicated by the numbers in cm, the depth of the maze was 10 cm). In the middle of the center compartment the start box is shown. Each end compartment contained a stimulus tank. During training, one of the stimulus tanks contained stimulus fish (5 zebrafish, indicated by the grey fish shaped pattern in the right tank) the others were empty (indicated by the grey fill). The end compartment with the stimulus fish is designated as the target compartment (gray dashed background). Note that the empty stimulus tanks are surrounded on three sides by white cover sheets (CS-, indicated by the black solid line) so as to prevent visual access to their content from all directions except from behind the tank. Also note that the stimulus tank with the stimulus fish also had cover sheets on three sides but the color of the sheets was red (the CS+, indicated by the gray and black patterned line). Other walls of the maze were transparent.

Panel B. The training and testing schedule. The timeline for three different drug administration periods is shown. The expected target mechanism of these administrations is indicated at the beginning of the time line. The black and gray dashed line indicates the period of MK-801 administration. The solid black lines indicate the period of training (5 min long each) and the thin black lines in between the training periods represent the intertrial intervals (2 min each). Note that the drug administration and training schedule shown is for a single day, and this schedule is repeated for an additional 3 times on consecutive days, i.e. totaling 4 days of training. 24 h after the last training trial, a probe trial is administered (indicated by the black and gray square patterned line).

Figure 2.

The percent of time zebrafish spent in the target compartment during training does not significantly depend upon MK-801 administration or whether the fish were given CS and US in a paired or unpaired manner. Mean $\pm$ Standard Error are shown. $n = 12$ for each group. The timing of MK-801 administration is indicated above the graphs. The gray and black squares represent fish that were administered paired CS-US training: fish represented by the gray squares received 0 μ M MK-801 and fish represented by the black squares received 20 μ M MK-801. Fish represented by the empty squares were administered the CS and the US in a randomized manner (unpaired group) and these fish also received 0 μ M MK-801. Note that all these groups were treated and tested in a fully randomized manner and the way their results are graphed is only for better clarity.

Figure 3.

The total number of entries to the end compartments of the plus maze during training is significantly increased in zebrafish that received MK-801 before each training trial. Mean $\pm$ Standard Error are shown. $n = 12$ for each group. The timing of MK-801 administration is indicated above the graphs. The gray and black squares represent fish that were administered paired CS-US training: fish represented by the gray squares received 0 μ M MK-801 and fish represented by the black squares received 20 μ M MK-801. Fish represented by the empty squares were administered the CS and the US in a randomized manner (unpaired control) and these fish also received 0 μ M MK-801. Note that all these groups were treated and tested in a fully randomized manner and the way their results are graphed is only for better clarity.

Figure 4.

Percent of time in the target compartment during probe trial is significantly reduced by post-training trial and pre-probe trial administration of MK-801. Mean $\pm$ Standard Error are shown. $n = 12$ for each group. The timing of MK-801 administration is indicated above the graphs. The gray and black bars represent fish that were administered paired CS-US during training: fish represented by the gray bars received 0 μ M MK-801 and fish represented by the black bars received 20 μ M MK-801. Fish represented by the white bar were administered the CS and the US in a randomized manner (unpaired control) during training and these fish also received 0 μ M MK-801. Note that all these groups were treated and tested in a fully randomized manner and the way their results are graphed is only for better clarity. Also note that the performance of fish that received MK-801 after training or before probe is indistinguishable from that of the unpaired control, a performance level we consider baseline.

Figure 5.

The average percent of time in the end compartment (other than the target compartment) during the probe trial is not significantly affected by MK-801 treatment or lack of CS-US pairing. Mean $\pm$ Standard Error are shown. $n = 12$ for each group. The timing of MK-801 administration is indicated above the graphs. The gray and black bars represent fish that were administered paired CS-US during training: fish represented by the gray bars received 0 μ M MK-801 and fish represented by the black bars received 20 μ M MK-801. Fish represented by the white bar were administered the CS and the US in a randomized manner (unpaired control) during training and these fish also received 0 μ M MK-801. Note that all these groups were treated and tested in a fully randomized manner and the way their results are graphed is only for better clarity.

Figure 6.

The total number of entries to the end compartments is not significantly affected by MK-801 treatment or lack of CS-US pairing. Mean $\pm$ Standard Error are shown. $n = 12$ for each group. The timing of MK-801 administration is indicated above the graphs. The gray and black bars represent fish that were administered paired CS-US during training: fish represented by the gray bars received 0 μM MK-801 and fish represented by the black bars received 20 μM MK-801. Fish represented by the white bar were administered the CS and the US in a randomized manner (unpaired control) during training and these fish also received 0 μM MK-801. Note that all these groups were treated and tested in a fully randomized manner and the way their results are graphed is only for better clarity.