

SHORT COMMUNICATION

Spontaneous alternation behavior in larval zebrafish

Stefan Yu Bögli¹ and Melody Ying-Yu Huang^{1,2,3,*}**ABSTRACT**

Spontaneous alternation behavior (SAB) describes the tendency of animals to alternate their turn direction in consecutive turns. SAB, unlike other mnemonic tasks, does not require any prior training or reinforcement. Because of its close correlation with the development and function of the hippocampus in mice, it is thought to reflect a type of memory. Adult zebrafish possess a hippocampus-like structure utilizing the same neurotransmitters as in human brains, and have thus been used to study memory. In the current study, we established SAB in zebrafish larvae at 6 days post-fertilization using a custom-made forced-turn maze with a rate of 57%. Our demonstration of the presence of SAB in larval zebrafish at a very early developmental stage not only provides evidence for early cognition in this species but also suggests its future usefulness as a high-throughput model for mnemonic studies.

KEY WORDS: Zebrafish, SAB, Maze, Memory, Cognition**INTRODUCTION**

Mazes have been extensively used in a variety of animal models to test and assess a broad range of cognitive functions and behaviors such as spatial memory (Morris, 1984), associative learning (Sison and Gerlai, 2010), innate preference (Avdesh et al., 2012) and anxiety (Walf and Frye, 2007). Among them, spontaneous alternation behavior (SAB) describes the tendency of animals to alternate consecutive choices (Lalonde, 2002). Each choice statistically depends on the previous one, evidencing its mnemonic origin. SAB has most commonly been investigated in rodents using T-mazes. Rodents were repeatedly placed inside the starting arm determining their choice of goal arm. The change of consecutive choices was defined as SAB (Dember and Richman, 1989). Forced-turn trials were performed by closing one of the two goal arms in the first trial, forcing the animal to move into a predetermined arm. This led to an increased rate of SAB (Dember and Richman, 1989). Besides rodents, SAB has also been described in humans (Vecera et al., 1991), marmosets (Izumi et al., 2013), rabbits (Hughes, 1973), cats (Frederickson and Frederickson, 1979), cockroaches (Wilson and Fowler, 1976), fruit flies (Bicker and Spatz, 1976), goldfish (Aderman and Dawson, 1970; Fidura and Leberer, 1974) and coral reef fish (Bate and Kirkby, 1977), among many other species.

The origin of SAB remains largely unknown. Many theories have been proposed attempting to explain the alternation. Among those, curiosity or foraging describes the need for exploration to locate food or shelter (Estes and Schoeffler, 1955). Also commonly cited

are Clark Hull's 1943 behavior theory, stimulus satiation and action decrement (Dember and Fowler, 1958; Hughes, 2004). Hull proposed learning to occur if a stimulus–response pair is followed by reinforcement; simultaneously, reactive inhibition is generated as a result of effort consumed during the response. In the case of missing reinforcement, reactive inhibition alone leads to an aversion towards the previously made choice (Solomon, 1948). Later, turn directions were shown to be susceptible to manipulation by stimulus satiation (Glanzer, 1953). In this way, animals re-entered the same arm consecutively after inversion of certain external sensory stimuli of the goal arms between consecutive trials (Douglas, 1966). Lastly, action decrement was proposed (Walker, 1958). Unlike other theories, Walker (1958) suggested stimulus satiation and reactive inhibition to be beneficial for memory as they delay consolidation of habit learning. Although exploration prolongs the learning process, it leads to enhanced memory. None of the theories above have conclusively been proved to be the sole mechanism underlying SAB.

Nevertheless, all of these theories imply engagement of some sort of memory in SAB. Furthermore, SAB was undetectable during early development in rats (Egger et al., 1973), cats (Frederickson and Frederickson, 1979) and human infants (Vecera et al., 1991) until 30 days, 5 weeks and 49 months after birth, respectively. Thus, the first occurrences of SAB corresponded timewise to hippocampal development in these animals. Several other studies showed decreased rates of SAB due to hippocampal lesions (Isseroff, 1979; Lalonde, 2002), or altered rates due to pharmacological interventions of hippocampal function through modification of acetylcholine efficacy (Egger et al., 1973; Lalonde, 2002). Likewise, mild hippocampal dysfunction has been detected in mice using the SAB paradigm (Gerlai, 1998).

SAB requires neither prior training nor reinforcement and has only a few simple steps of testing. Therefore, the chances of experimental bias caused by animal handling or slight protocol deviations is much smaller compared with other behavioral memory paradigms (Gerlai, 1998). Hence, SAB has been used for testing and screening for pharmacological substances that may enhance or reduce both short-term and long-term memory (Handelmann et al., 1989; Hughes, 2004; Ukai et al., 1997).

Zebrafish models are used in various biomedical research fields such as toxicology, addiction, neurological disease and aging. Adult zebrafish perform well in multiple memory-related experiments including olfactory conditioning (Braubach et al., 2009), spatial alternation (Cognato et al., 2012), and associative (Sison and Gerlai, 2010; Valente et al., 2012) and non-associative (Tran and Gerlai, 2014) learning. Mnemonic studies using larval zebrafish, in contrast, have only commenced recently (Roberts et al., 2013). Non-associative forms of learning, such as habituation and sensitization, were successfully shown in larval zebrafish (Best et al., 2008). However, associative forms of learning were only shown in a few studies at later developmental stages (Aizenberg and Schuman, 2011). The advantages of using early-stage zebrafish larvae include the large amount of offspring and the translucency of the animals,

¹Department of Neurology, University Hospital Zurich, University of Zurich, Zurich 8091, Switzerland. ²Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich 8057, Switzerland. ³Neuroscience Center Zurich (ZNZ), University of Zurich and ETH Zurich, Zurich 8057, Switzerland.

*Author for correspondence (ying-yu.huang@usz.ch)

 M.Y.-Y.H., 0000-0003-4368-9648

which allows for various *in vivo* imaging techniques visualizing neural activity during the actual behavior. Just a few days after hatching, larval zebrafish possess many major central nervous structures found in adult animals (Strähle and Blader, 1994; Tropepe and Sive, 2003). Even though the zebrafish brain does not have a proper hippocampal structure, the lateral pallium serves as its functional equivalent (Rodríguez et al., 2002) and uses the same neurotransmitters involved in learning as in mammals (Xu et al., 2007).

This study was designed to establish the presence of SAB in zebrafish larvae as a first attempt at developing a novel behavioral paradigm for memory studies.

MATERIALS AND METHODS

Animal maintenance and breeding

We used two wild-type zebrafish, *Danio rerio*, lines *TU* (Tuebingen) and *WIK* (WIK) for the study. Fish were maintained and bred as previously described (Mullins et al., 1994). Embryos were raised at 28°C in E3 medium (5 mmol l⁻¹ NaCl, 0.17 mmol l⁻¹ KCl, 0.33 mmol l⁻¹ CaCl₂ and 0.33 mmol l⁻¹ MgSO₄) under a 14 h light:10 h dark cycle and staged according to development in days post-fertilization (Haffter et al., 1996). All experiments were performed in accordance with the animal welfare guidelines of the Federal Veterinary Office of Switzerland.

Maze apparatus

A custom-made maze consisting of four arms and two pools, as shown in Fig. 1, was used. The starting arm (A) and main arm (B) had a length of 50 mm, while the goal arms (C/D) had a length of 25 mm, all with a width of 5 mm, a depth of 10 mm, and with interlocking intersections of 25 mm². The two goal arms led to separate pools (E/F) with a size of 1950 mm². The maze was both heated and illuminated from below.

Measurement of SAB

For each trial, 20 larvae were tested simultaneously. The maze was pre-filled with 28°C E3 medium. The intersection between the starting and the main arm was closed using a translucent tube; the larvae were subsequently transferred into the starting arm. After

10 min of adaptation, the tube was removed to start the actual trial. Successful entry was counted when a larva fully (head to tail) entered one of the goal arms within the first 10 min of the trial. In the case of returning to the main arm and/or entering the second goal arm after a prior successful goal arm entry, only the first entry was counted.

Statistical analysis

A binomial test was used to test the null hypothesis of equal probability of entering either goal arm.

RESULTS AND DISCUSSION

In this study, we describe the SAB observed using a custom-made forced-turn maze (Fig. 1). SAB was defined as the alternation of consecutive turn directions. Out of a total 380 tested larvae, 240 successfully entered either goal arm within 10 min of opening of the gate to the main arm. Among these 240 larvae, 137 displayed SAB (57.083%, binomial test, $P=0.0329$).

As a comparison, similar to zebrafish, coral reef fish were reported to alternate after one forced turn (Bate and Kirkby, 1977). Rats (so far the most commonly studied SAB model) (Dember and Richman, 1989) and other animals such as marmosets (Izumi et al., 2013) and rabbits (Hughes, 1973) displayed considerably higher rates of SAB. Apart from species differences, one likely explanation for the lower SAB rate observed in our study lies in the very early developmental stage of our subjects. We used 6 day old larvae, while most studies used adult animals as their subjects. SAB in cats, rats and human infants developed over a similar time period to the hippocampus (Egger et al., 1973; Frederickson and Frederickson, 1979; Vecera et al., 1991). Hence, an increased SAB rate at a later developmental stage in zebrafish is foreseeable. Mammals are nurtured during early post-natal development; therefore, immediate explorative behaviors are unnecessary. In some cases, such behaviors could even be dangerous. In contrast, zebrafish larvae have already depleted their yolk at 5–7 days post-fertilization, making foraging indispensable. Thus, early cognitive functions such as memory and learning, which may help improve navigational skills, are essential for better survival. This is reflected by the early presence/maturation of many underlying brain structures and neural

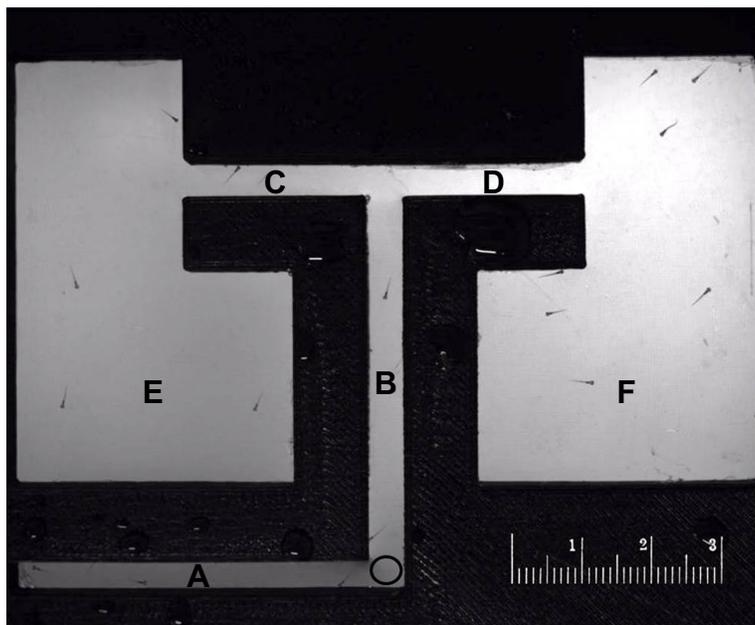


Fig. 1. Forced-turn maze. The experimental set-up. A depicts the starting arm, B shows the main arm, C and D are the goal arms, and E and F show the adjacent pools. The circle indicates the gate, which was closed during adaptation.

circuits engaged in those cognitive behaviors (Strähle and Blader, 1994; Tropepe and Sive, 2003).

Compared with other methods in behavioral mnesic research, one key advantage of testing SAB lies in the reduced study bias caused by investigators or protocol deviations, as it requires neither training nor reinforcement. SAB was shown to be highly sensitive to hippocampal dysfunction in rodents (Gerlai, 1998). Because of the existence of hippocampal-like structures in adult zebrafish brains and the similar neurotransmitters used in both fish and mammals, future investigation of SAB in zebrafish should focus on identifying the ontogeny and neural substrates of this behavior. The use of group mazes to test larval zebrafish will allow for high-throughput screening and evaluation of cognition/memory-altering substances. Emerging technologies for *in vivo* imaging as well as pharmacological and genetic interventions in larval zebrafish increase the possibilities for the use of this potential animal model in SAB-based mnesic research.

In conclusion, we have established the presence of SAB in larval zebrafish suggestive of early memory, which can be further applied as an *en masse* method to test animals' responses to substances known to influence mnesic functions.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.Y.B.: Study design, experiments, data analysis and manuscript preparation.

M.Y.-Y.H.: Study design, data analysis and manuscript preparation.

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