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利用腦側化反轉斑馬魚探討端腦的功能

Study of telencephalon function using cerebral lateralization inversed zebrafish

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Abstracts in Chinese (中文摘要)

腦側化(cerebral lateralization)是指左右邊大腦半球(cerebral hemisphere)各會偏重執行(dominate)某些特定功能，為一種脊椎動物常見的現象。這種功能性的腦側化(functional lateralization)又可追溯至左右腦結構，或是特定分子於左右腦分佈的不對稱性(asymmetric)。許多文獻指出上丘腦(epithalamus)是探討斑馬魚腦部結構的不對稱(structural asymmetry)之重要標的。副松果體位於(parapineal)上丘腦中，正常情況下，該核團約有98%的機率會位於左腦中，僅少部分「腦反轉」個體(brain inversed)其副松果體會位於右腦。本計劃比較一般個體及腦反轉個體間，外顯行為及兩側端腦功能差異，結果將有助於了解斑馬魚腦結構不對稱性對，於腦功能側化的影響。

目前已知功能性腦側化會體現於個體的認知(cognition)、情緒(emotion) 和學習與記憶(learning and memory)等面向。本研究利用腦側化反轉斑馬魚，探討整合結構不對稱性對情緒與認知的影響。本研究分別使用野生型(wild-type, WT)及 *foxd3:GFP* 品系的基因轉殖(transgenic, TG) 斑馬魚作為實驗對象，該TG品系斑馬魚的副松果體會表現外源性的綠色螢光蛋白(exogenic green fluorescence protein, GFP)，故可藉此判別副松果體位置，以篩選出正常(無反轉)的左側副松果體個體(left-side parapineal, Lpp)及腦反轉的右側副松果體個體(right-side parapineal, Rpp)個體。研究包括了四階段的行為實驗(behavioral experiment)，第一階段是基礎運動能力測試，用以確認各實驗動物的自發性游動(locomotor activity test)是否正常，基礎運動功能正常的個體，方能進入下一階段實驗。第二階段實驗為類焦慮行為

(anxiety-like behavior)測試，本研究選用新穎性水箱測試(novel tank test)，交叉比對不同組別實驗動物的類焦慮行為表現。第三階段則為抑制性逃避學習測試(inhibitory avoidance test)，檢核腦反轉對恐懼記憶(fear memory)的建立是否有差異。第四階段則是利用腦反轉的 TG 斑馬魚，探討隨著腦構造反轉後，其功能性腦側化及偏重執行的外顯行為是否同樣出現反轉的現象。

實驗結果顯示，腦反轉個體(Rpp)的運動功能與學習能力未受影響，但類焦慮行為明顯增加。在認知功能方面，對無反轉斑馬魚(Lpp)施以右側端腦破壞，會干擾空間及恐懼學習能力，故推測其與野生型斑馬魚相同，空間及恐懼的學習主要由右側端腦所主導，而腦反轉的斑馬魚(Rpp)則轉變為左側端腦主導。因而可推論斑馬魚的功能性腦側化將隨腦部發育反轉，而發生左右顛倒的情形。我們相信端腦功能性側化確實存在於斑馬魚的學習和記憶過程中，但該現象是來自於先天形生(innated)的神經迴路？或是後天經驗學習後(acquired)才逐步建立？仍有賴進一步探討。本研究也觀察到即使對端腦進行較小面績的破壞，但倘若破壞到較關鍵的部位，仍會對空間和恐懼學習造成顯著的損害。

關鍵字: 斑馬魚、腦側化、類焦慮行為、學習與記憶、副松果體

Abstract in English

Cerebral lateralization is widely found in vertebrates. It is defined as a hemispheric preference in performing certain functions. Numerous studies reported that the parapineal gland, a nucleus of the epithalamus, is an essential target for exploring the structural and functional asymmetry of zebrafish's brain. Normally, approx. 98% of the zebrafishes, its parapineal glands appear in the left hemisphere. Previous studies also showed the cerebral lateralization is involved in sensory perception, emotion, learning, and memory. Our previous studies showed the spatial learning and memory was impaired after unilateral hemisphere lesion. These results evidence the functional dominance existed in the cerebral hemisphere of the zebrafishes.

A transgenic foxd3: GFP line was applied in the current study. It expresses an exogenic green fluorescence protein in the parapineal. Therefore, the location of the parapineal could be visualized and confirmed via fluorescence microscopy. The transgenic zebrafishes were then cataloged by its parapineal location to the left side parapineal group (Lpp) and the right side parapineal group (Rpp). A modified aspiration ablation paradigm was applied. Animals were subjected to the unilateral ablation then subjected to behavioral experiments five days after the ablation. There are four stages in the present study. The first stage was aimed to evaluate the motor function and spontaneous activities using locomotor activity monitoring. Abnormal zebrafishes were then excluded from the subsequent experiments. In the second stage, novel tank dive test was used to examine anxiety-like behavior exhibited in the wild type and foxd3: GFP zebrafishes. The cerebral lateralization effect on fear learning was studied via an inhibitory avoidance test in the third stage. The fourth stage was aimed to explore whether the functional brain lateralization of zebrafish with inversion of the brain structure also reverses in explicit behavior.

Results showed that neither motor function nor learning ability were impaired in the brain inverted zebrafishes (Rpp), but there was a significant increase in anxiety-like behavior compared with the control group. Which showed consistency to the previous studies. Besides, both non-inverted and wild-type zebrafishes showed a functional dominant on the right-side telencephalon. In contrast, the functional dominant changed to the left-side telencephalon in the brain inverted zebrafish (Rpp). Therefore, it could be inferred that the functional lateralization of the zebrafish would be reversed as the brain inversion.

In conclusion, we suggest the functional lateralization of the telencephalon in the learning and memory does exist in the zebrafish. Further experiments will be required to determine whether the functional lateralization is natural borned (innated) or required the exposure of postnatal experiences (acquired). We also found that even a tiny ablation was performed may have a tremendous impairment effect on both the spatial and emotional learning.

Keywords: zebrafish, brain lateralization, anxiety-like behavior, learning and memory, parapineal.

List of abbreviations

fMRI:	functional Magnetic Resonance Imaging
foxd3_L:	foxd3 strain zebrafish with parapineal on the left
foxd3_R:	foxd3 strain zebrafish with parapineal on the right
fsi:	frequent-situs-inversus
GFP:	green fluorescent protein
hpf:	hours post-fertilization
IA:	Inhibitory avoidance test
L-lesion:	left side telencephalon lesion
Lpp:	left side parapineal
LTM:	long-term memory
MS-222:	tricaine methanesulfonate
ppm:	parts per million
R-lesion:	right side telencephalon lesion
Rpp:	right side parapineal
SEM:	standard error of the mean
TG:	transgenic
TZCAS:	Taiwan Zebrafish Core Facility at Academic Sinica
ZFIN:	The Zebrafish Information Network

Introduction

1. Cerebral lateralization

Cerebral lateralization, also known as hemispheric specialization, is referred to the lateralization of brain function. This phenomenon also refers to the brain's tendency to perform neurological functions or specific processes of cognitive tasks in one particular cerebral hemisphere (Ocklenburg, 2014). Early studies in this field mostly used brain injury patients as research subjects and connected specific brain regions with physiological functions by confirming the location of the patient's brain injury and the patient's obstacles. For example, aphasia or relation speech disorder occurred after a specific area of the left brain is damaged (Manning & Thomas, 2011). A significant decrease in the emotional perception and expression in the traumatic brain injury patient with right hemisphere damage was also reported (Demaree et al., 2005). With the advent of fMRI, the phenomenon of brain lateralization can be observed and evaluated more conveniently. The fMRI results showed that the left hemisphere of the subject is more active when performing language-related functions. In contrast, the right hemisphere became more active when performing spatial processing or facial recognition tasks. (Badzakova et al., 2010). In summary, the human studies on cerebral lateralization concluded that the left hemisphere largely dominates several functions such as language, logical interpretation, and viewing details; the right hemisphere is dominated on the global viewing, processing visual-spatial tasks, and drawing geometrical diagram or illustration (Gazzaniga, 2005).

Functional lateralization of the brain is not unique to the human, but a phenomenon that exists universally in the animal kingdom. Whether it is an invertebrate or a vertebrate, the brain's anatomical and functional asymmetry was widely reported (Concha & Wilson, 2001; Rogers, 2014). In terms of invertebrates, some literature points out that most drosophila has a small nucleus named asymmetric body (AB) is involved in forming long-term memory (LTM). Individuals with AB only on the right side showed better LTM formation than the fellows with bilateral AB (Pascual et al., 2004). In the vertebrates, studies have shown that if IV mice have hippocampal asymmetry defects, they will lead to spatial learning and working memory impairment (Goto et al., 2010).

Besides learning and memory, lateralization phenomena are also manifested in other explicit behaviors, such as using dominant hands or the eye using certain animals' preferences. Previous studies reported zebrafish trends to use different eyes on observing the environment in different situations. For example, zebrafish preferred to use their left eye for exploring unfamiliar environments or identify and learn new things; the right eye was often used for decision-making to assess whether to bite the food (Andrew, 1999; Miklósi et al., 1999; Lu et al., 2013).

The evolutionary conservation of brain lateralization shows that lateralization has a certain degree of advantage for the survival of species. Brain asymmetry is believed to bring cognitive benefits to individuals because when the individual is in a complex environment, specific brain hemispheres can handle parallel tasks. This idea was supported by related experiments. For example, visual lateralization could improve chicks' performance in dual tasks to distinguish between seeds (food) and small pebbles while monitoring predators (Rogers et al., 2004). Dadda & Bisazza (2006) used *Girardinus falcatus* as an experimental model to study the eye preference phenomenon when encountering a predator. Since eye preference is highly hereditary in this species, three different lines were screened, including the NL line, which showed lower laterality (no preference on turning left or right), the LD line that preference turning left, and RD line has a preference of turning right. In predators' presence, the NL line took more time to catch the same amount of prey than the other two lines. They evidenced the first time that cerebral lateralization did exist in the bony fish.

2. Cerebral lateralization study using zebrafish

Recently, the brain imaging research technique became a powerful tool for studying brain structural and functional asymmetry (Reynolds, 2019). However, it is currently unable to elucidate particular genes' contribution to the human brain's functional asymmetry. To gain insight into the relevance between neurodevelopment and functional brain asymmetry. Scientists established transgenic animal models for studying structural asymmetry and its effects on cerebral lateralization (Miletto et al., 2020a). In this regard, the zebrafish proved as an ideal animal model because it shows neuroanatomical and behavioral asymmetry and is feasible for genetic screening and *in vivo* neuronal networks imaging (Miletto et al., 2020b).

The most widely studied asymmetry brain structure in zebrafish is the epithalamus (Concha & Wilson, 2001; Concha et al., 2009). It contains the habenula and the pineal complex. The structural asymmetry in this area is widely found in the vertebrate. Its asymmetry appeared in both the size, neuronal organization, neurochemistry, and neural connections (Concha et al., 2001). For example, the expression of the kctd8 gene is different in the left and right brains of zebrafish. Besides, it is known that the unequal size between the left and right habenula nucleus is related to the asymmetry of nerve tissue. The pineal gland projecting to the left habenula nucleus is also asymmetrical (figure I). The left and right habenula receive signals from the parapineal and the olfactory bulb, and deliver them to the IPN from the dorsal and ventral subnucleus, respectively.

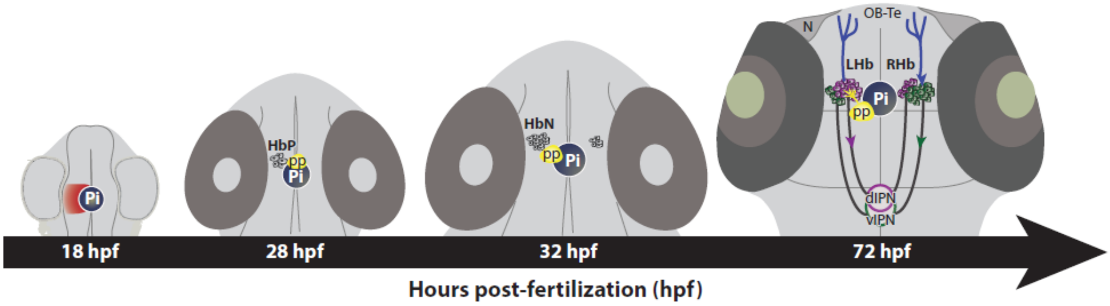


Figure I. Schematic diagram of the asymmetric development of the zebrafish epithalamus (Duboc et al., 2015). The full name of the abbreviations: habenular progenitors (HbP), habenular neurons (HbN), pineal gland (Pi), parapineal (pp), the left (LHb) and right (RHb) dorsal habenula, olfactory bulb (OB).

Previous studies showed that the habenulae are an essential structure that formed a functional connection between the forebrain and midbrain (Bianco and Wilson, 2009). It is involved in modulating many kinds of behavior, including the experience-dependent fear response in zebrafish (Agetsuma et al., 2010). The pineal complex consists of two photosensitive structures (Schredelseker, 2018) At night, the neuroendocrine cells in the pineal gland release melatonin, and in teleost, it is directly photosensitive. The pineal gland located on the midline and the parapineal situated on the midline's left side. It is well-known that the pineal gland's key function is the regulation of circadian rhythms (Ben-Moshe et al., 2014), but the parapineal's exact function is still unclear. According to the definition of ZFIN, the parapineal is an unpaired left-sided accessory organ that plays a role in establishing lateral brain left-right asymmetry (ZFIN).

The epithalamus's asymmetric development in zebrafish can be observed within three days of post-fertilization (please refer to figure I). First, 18 hours after the embryo is fertilized, the nodal-related gene ndr2/Cyclops can be observed on the left side of the epithalamus. The pineal gland is located in the midline of the epithalamus. At 28 hours after fertilization, the parapineal develops in front of the pineal gland and gradually moves to the left. Simultaneously, it can be observed that habenular progenitors develop earlier in the brain's left side than the right.

At 72 hours post-fertilization, the parapineal can be seen ultimately moving to the left rear of the pineal gland, the left and right habenulae have also developed, and the dorsal habenula can be further subdivided into the lateral and the medial sub-nucleus. Those who have nerve bundles projected to the lateral interpeduncular and medial interpeduncular, respectively (Duboc et al., 2015)

The lateralization behavior of zebrafish is easy to be observed. Therefore several behavioral tests in both adults (Miklósi et al., 1997 & 2001; Heuts, 1999; Miklósi & Andrew, 1999; Fontana et al., 2019) and larva (Miklósi et al., 1997 & 2001; Sovrano & Andrew, 2006) have been previously documented. Watkins et al. (2004) described that if an 8-day-old larva experienced ambient light intensity changes, they would show biases in the directionality of turning to change their swimming trajectory. Also, larva tended to monitor the environment with their left eye. If the larva was in a dark environment where it had been previously recognized by the left eye, the individual would be less likely to exhibit avoidance behavior. This finding was consistent with the use of adult zebrafish's left eye to assess whether they had encountered similar experiences in the past (Miklósi et al., 1997; Lucon et al., 2020).

3. The correlation between structural asymmetry and behavior

For examining whether these lateralization behaviors relevant to the asymmetry of the anatomical brain structure. Researchers used gene mutations to interference with the directional asymmetry in zebrafish embryos. A zebrafish strain named frequent-situs-inversus (*fsi*) was generated. It has a higher rate of inversion of the heart, guts, pancreas, and parapineal than wild-type. The neuroanatomical analysis of the epithalamus in *fsi* individuals found a left-right inverse occurred. Subsequent experiments showed that certain lateralized behaviors' directionality was also reversed (Barth et al., 2005). Another group (Facchin et al., 2009) used transgenic progeny from mating between Tg(foxd3:GFP)^{fkg17} and Tg(ela3l:GFP)^{gz2}; Tg(fabp10:dsRed)^{gz4} adults, which had GFP labeling in the parapineal and pancreas, and dsRed in the liver. Therefore the organ inversion was easy to be confirmed. Our results showed that the epithalamus's inversion did not affect the motor response and directional behavior, making it an ideal model for studying telencephalon inversion's behavioral effect.

4. Learning and memory in zebrafish

Studies have pointed out that the brain's evolution may be more conservative than previously thought (Salas et al., 2006). Data shows that the characteristics and neural basis of learning and memory between teleost fishes and terrestrial vertebrates are incredibly similar. For example, lesion studies have demonstrated that teleost fish's cerebellum is crucial in the classical conditioning of various motor responses (Portavella et al., 2002; Portavella et al., 2004). The teleost fish's lateral telencephalic pallium is homologous to the hippocampus and is involved in spatial learning and memory. On the other hand, the medial pallium is believed to be homologous to the amygdala and is participated in teleost fish's emotional regulation. There is significant parallelism between mammals and teleost fish in their brain regions responsible for processing specific functions.

In recent years, behavioral research on learning and memory in zebrafish has become more popular and diverse, particularly in spatial learning and fear learning (Buatois & Gerlai, 2020). In terms of spatial learning, predecessors have used the T-maze model to conduct in-depth exploration on zebrafish (Wu, 2008). Results showed when zebrafish carried out T-maze spatial memory learning tasks, and the rightward learning group showed a better learning curve than the leftward learning group. Furthermore, a spatial learning and memory deficit was found after the zebrafish's telencephalon was ablated bilaterally (Wu, 2008).

A modified version of the well-known inhibitory avoidance learning task was developed to examine the zebrafish's inhibitory avoidance (IA) learning and memory (Manuel et al., 2014; Gorissen et al., 2015). Zebrafish preference to stay in the deepwater zone for evading the predator’s attack. An aversive stimulus (electrical shock) was delivered to established fear learning (Ng et al., 2011). Blank and colleagues pointed out that IA learning and memory are mediated by the glutamate NMDA receptor and its related signaling pathway (Blank et al., 2009). Conclusively, zebrafish is an ideal and reliable model for exploring neural mechanisms underlying learning and memory.

5. Application and research of Tg(foxd3:GFP) strain

As we mentioned earlier, the zebrafish epithalamus is an essential model for studying the inter-relationship between the anatomical left-right asymmetry and behavior. The Tg(foxd3:GFP) (Gilmour et al., 2002) is widely used in this field's studies. Researchers can determine whether the structural inversion simply based on the location of the GFP labeled parapineal gland. Typically, only about 2% to 5% of the epithalamus's asymmetry directionality would be spontaneously reversed (Concha et al., 2000; Liang et al., 2000). The reversal ratio could be raised to nearly 50% by destroying the nodal signaling pathway (Gamse et al., 2003; Facchin et al., 2009). Another way to increase the percentage of brain reversal was to transfer the embryo from 28℃ to 22℃ at the tailbud stage for 3 to 4 hours (Roussigne´et al., 2009)

For the related research of the Tg(foxd3:GFP) strain, a research team (Facchin et al., 2015) placed the fish in a behavior box to detect its exploratory behavior. The results showed that L-R reversal in epithalamic asymmetry reduced exploratory behavior in adult zebrafish, and the degree of reduction was likely to be affected by visual stimuli. If there was a mirror in the behavior box to provide visual stimuli, the decrease in exploratory behavior would be more significant. Besides, individuals with brain inversion would spend more time in the lower half of the tank when subjected to the novel tank test, which meant they had a higher degree of anxiety (Facchin et al., 2015). The cortisone level in the body would also be significantly higher than the control group. This abnormal behavior could be suppressed by treatment with anxiolytic buspirone (Facchin et al., 2015). In addition to the developmental brain inversion, the researchers also found that the individual's exploratory behavior was significantly reduced after the normal zebrafish's parapineal lesion. It could be seen that whether after the developmental or surgical manipulation, change of the brain asymmetry would cause the individual's behavior and physiological response.

It is generally accepted that the telencephalon of teleost fish plays a vital role in learning and memory. It has also been reported that zebrafish would preferentially use a specific side of the eye under certain circumstances (Sovrano, 2004). For understanding the relationship between specific brain regions and their corresponding physiological functions. A previous study ablated the medial and lateral pallium of goldfish to observe the experimental subjects' change of behavior. (Portavella et al., 2005). Since only bilateral lesions were performed in Portavella’s study, the left and right telencephalon's functional specificity could not be elucidated. In the present study, a unilateral ablation of the telencephalon has proceeded to study the functional asymmetric of the telencephalon.

6. Research aim and significance

This project was aimed to study the functional asymmetric of telencephalon on the spatial and fear memory using the transgenic zebrafish model with cold-shock induced structural inversion. A modified aspiration lesion paradigm was performed to ablate the telencephalon unilaterally. Animals were then subjected to behavioral experiments to evaluate the telencephalon ablation effect.

Results of the present study are not verifying our previous founding. But also provide further insight on the relevance of cerebral lateralization and brain function.

Material and Methods

1. Subjects

Both the wild-type AB strain zebrafishes and foxd3 transgenic zebrafishes were used in this study. The AB strain and foxd3 transgenic zebrafishes were purchased from the TZCAS & Dr. Yung-Shu Kuan (NTU) respectively. The foxd3 transgenic zebrafish express a green fluorescent protein in the parapineal, displaying the parapineal location for identifying the inversion of the telencephalon. Wild-type AB zebrafish was used as a control group to understand the cognitive function of general zebrafish. All fishes were housed in the Department of Life Science's experimental animal facility, National Taiwan Normal University. The breeding procedures were adapted from the guide book written by Westerfield (2000). The room temperature of the breeding room was maintained at a constant temperature of 28℃. The light/dark period was set to a 14/10 hours cycle with light period begun at 10 a.m. every day, and light was turned off at 12 p.m. Juveniles of the same date of hatching and from the same parent were housed in the same tank. Larvae were fed daily with paramecium, and adult fishes were fed twice a day, once with brine shrimp and once with artificial feed. All the experimental procedures were reviewed and approved by the National Taiwan Normal University Laboratory Animal Management Committee. It complied with the regulations for the use of laboratory animals set by the Council of Agriculture.

2. Thermal induced developmental inversion of the brain of foxd3

Given that the proportion of zebrafish with the right parapineal gland is quite rare under natural development, to accumulate sufficient individuals, it is necessary to increase the probability of zebrafish brain reversal by cold shock treatment. In general, zebrafish embryos are hatched in the water tank, with the temperature maintained at 28℃. The previous study reported cold-shock treatment could increase the probability of having brain inverse zebrafish. The hatching protocol was adapted from Dreosti (2014). Briefly, the embryos were transferred from a 28℃ water tank to a 22℃ cold water tank at the tail-bud stage of embryo development, soak in cold water for 3~4 hours, then returned to 28℃ water tank to continue the hatching.

3. Confirmation of zebrafish parapineal position

The transgenic Tg (*foxd3: GFP*) zebrafish used in this study has a green fluorescent protein expression in the brain's pineal complex. Therefore, under the fluorescence microscope (Olympus, BX60F, Japan), the green fluorescence in the parapineal was excited by the blue light. If the parapineal was located on the left side of the fish brain, this individual was defined as foxd3 strain zebrafish with parapineal on the left (foxd3_L), that was, the zebrafish without brain inversion; On the contrary, foxd3 strain zebrafish with parapineal on the right (foxd3_R) individual was cataloged to the brain inversion group. Each individual's parapineal position was confirmed using a fluorescence microscope should be carried out as soon as possible on the 4th to 6th day after fertilization. The location of the parapineal was confirmed using a fluorescence microscope. The typical pictures showing the right-parapineal (Rpp) and left-parapineal (Lpp) were summarized and shown in figure II. The whole pineal complex structure (pineal + parapineal) was illustration in the Figure II(a). The large round-shape nucleus in the center is the pineal gland, and the small nucleus highlighted with a red square is the parapineal. After the aforementioned cold-shock treatment, we obtained approximately around 6~10% brain inverted individuals.

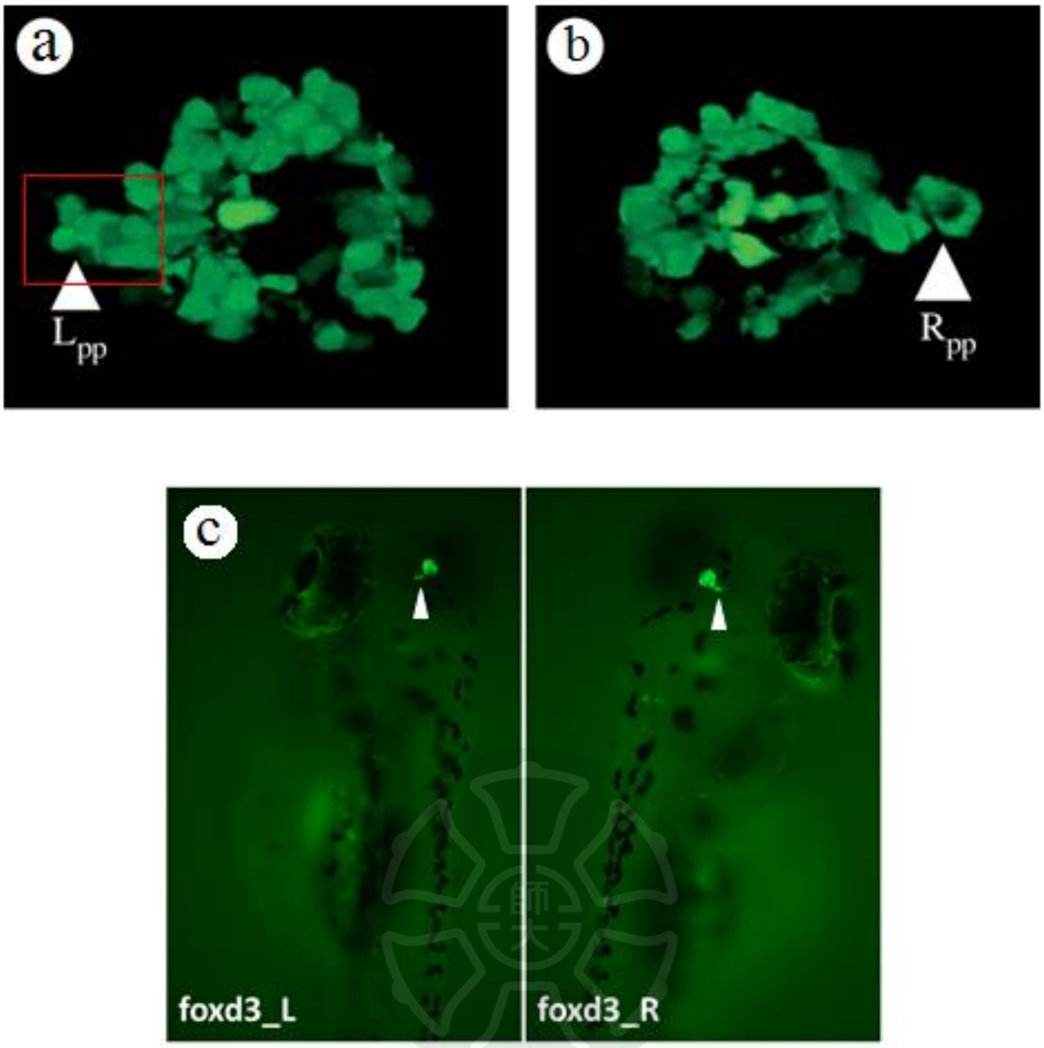


Figure II. The illustration of the pineal complex, and the typical figures of showing the parapineal position of the foxd3 TG zebrafish. As shown at (a) (b) the parapineal gland is a slender and narrow structure located obliquely behind the pineal gland (Facchin et al., 2009). (c) the typical photos of the left-side parapineal (foxd3_L) and right-side parapineal (foxd3_R) location in foxd3 TG zebrafish.

4. Locomotor activity test

It aimed to study whether a non-specific effect on zebrafish's locomotor activity existed in the transgenic zebrafish or after the telencephalon lesion treatment. Animals that showed abnormal locomotor activity were excluded from the subsequent behavioral experiment. The behavioral chamber was a circle shape, transparent acrylic made water tank. Its internal diameter was 24 cm, with the water level maintained at 8 cm high. There was a white-light lamp set above the water tank to provide the illumination. The digital camera was set directly beneath the lamp and on the center of the tank. The animal behaviors were analyzed the tracking detection system-software SMART 3.0 (PanLab Harvard Apparatus, MA, USA). It recorded the animal's position change per second and analyzed the zebrafish's horizontal movement distance the trajectory (adopted from Wu, 2008).

Only a single zebrafish was placed into the water tank for a 15 minutes recording at a time. When the zebrafish was placed into the water tank, its movement triggered the recording automatically. Once the recording was completed, the zebrafish was returned to its housing tank.

5. Novel tank diving test

It was designed for mimicking the fish's behavior under the natural environment and aimed to evaluate the anxiety level in zebrafish. Zebrafish prefer to stay in the lower half area rather than the upper half area for avoidance the attack from predators. The time spent in the lower half area positively correlated to the anxiety level of the zebrafish. The behavioral chamber is a transparent trapezoidal acrylic water tank (15.2 cm tall × 27.9 cm top × 22.5 cm bottom × 7.1 cm wide) and filled with 1.2-liter water (modified from Facchin et al., 2015). The video equipment was placed directly in front of the water tank. Each fish's recording time was 15 minutes which was divided into first, middle, and last intervals with 5 minutes each. The time spent in the upper half, and the lower half of the water tank was recorded and served as an index of anxiety level.

6. T-maze test

This experiment was used to examine the spatial memory of the normal and brain inversed zebrafish. It was adapted from the rodent T-maze test with mild modulation (Wu, 2008). Unlike the rodent studies, in which a positive reinforcer such as food was applied. Since zebrafish preferred to stay in a deep water zone, the T-maze's two goal ends were equipped with a deep or a shallow water reservoir. Animals were subjected to the training several times. A learning curve could be drawn and showing a decrease of time spent in entering the right target ends. Our previous study showed the zebrafish failed to obtain good learning results on the left target area (Wu, 2008). Therefore, only right target area setting was applied in the present study. The detailed schema of the experimental equipment is shown below:

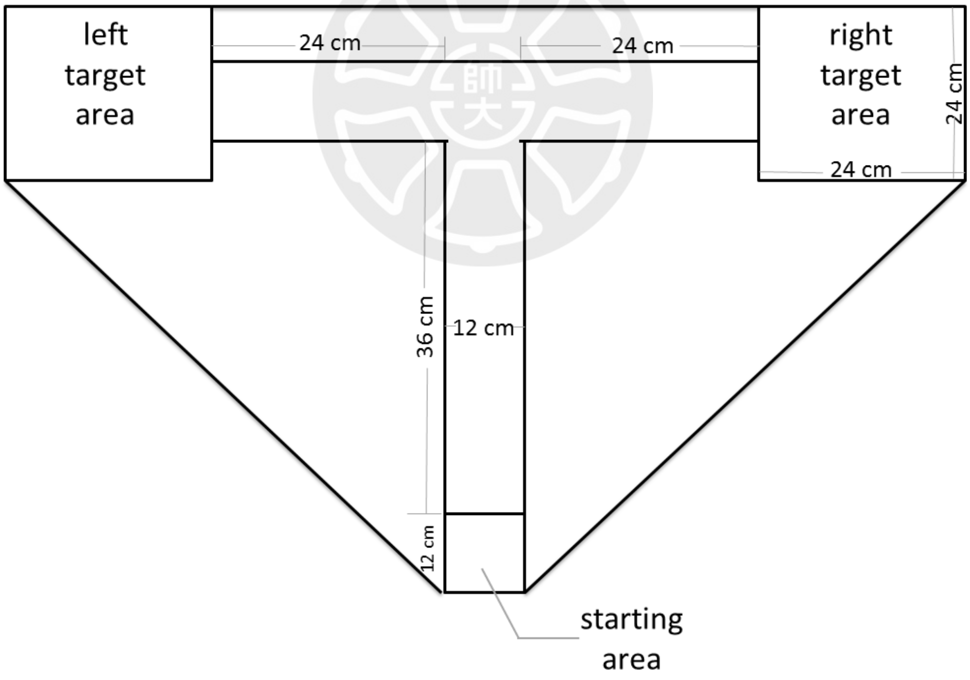
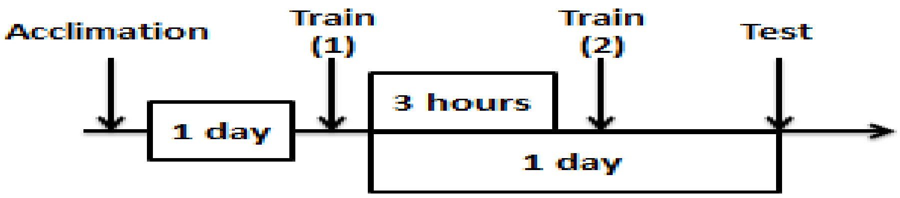


Figure III. The scheme diagram of the T-maze.

It contains two square shape target areas (left & right, with a side of 24 cm), two alleys connects to the target areas (with a length of 24 cm), one main alley (12 cm wide and 36 cm long), and the starting area (with a side of 12 cm).

The experimental procedure was summarized as below:



On the first day, all fishes were sequentially placed in the maze for acclimation, and the process was repeated in the next two consecutive days as the train (1) and train (2). A test was given to the zebrafishes on the third day. The time spent on entering the correct goal end (with deeper water) was recorded and analyzed as escape latency. The shorter the escape latency represented the better spatial memory.

The experimental procedure of the T-maze test is described as below::

Phase 1: Acclimation

1) Adjusted the water temperature to 28 ± 1℃

2) Placed the animal into the starting area and allowed it to stay for 30 seconds.

3) Pulled off the partition so that the animal could swim freely to the T-shaped area.

4) The animals were allowed to swim freely in the T-shaped area for 5 minutes, and returned to the housing tank immediately after acclimation.

Phase 2: Training

1) The training-1 was proceed 24 hours after the acclimation, with the water temperature maintained at 28 ± 1℃

2) Placed the animals in the starting area and allowed them to stay for 30 seconds.

3) Remove the partition of the starting area so that the animal could swim out to the T-shaped area freely.

4) The latency of the animal reaching the goal area was recording and the animals were allowed to swim freely in the T-maze for 5 minutes.

5) Return the animals to the housing tank.

6) The training-2 was proceeding at three hours after the training-1 with exactly the same procedures as training-1.

Remark: If the animal did not enter the target area within 5 minutes, It would be guided to the target area with a net and let them stay in the target area for 2 minutes.

Phase 3: Testing

1) The testing was performed 24 hours after the training-1 (21 hours after the training-2).

2) Placed the animal in the starting area and allowed it to stay for 30 seconds.

3) Pulled off the partition so that the animal could swim out to the T-shaped area for searching the goal area.

4) Recorded the first time that the animal entered the target area and stayed more than 30 seconds after the partition was opened.

6) The experiment completed once the animal reaching the target area and stay for 30 seconds; the experiment was also be halted if the fish unable to enter the goal area within a 5 minutes trial.

7. Inhibitory avoidance test (IA)

It is adapted from the well-established rodent learning and memory model and aimed to explore the establishment of fear memory in zebrafish (Ng, 2011). Zebrafish preferred to stay in the deeper water area. An electrical shock was applied as a negative reinforcer to the zebrafish. The tested fish had to refrain from swimming from a shallow compartment into a deep compartment in order to avoid receiving an electric shock. The illustration of the behavioral chamber is shown in the figure below. It contains a deep compartment and shallow compartment which are separated with a sliding door.

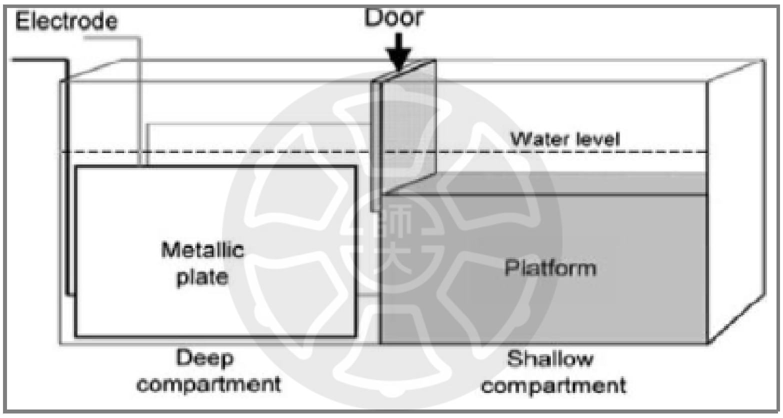
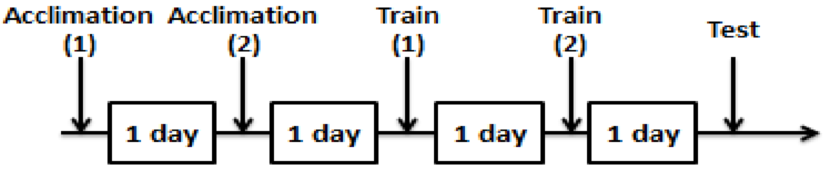


Figure IV. The scheme diagram of the inhibitory avoidance (adapted from Ng, 2011)

The water level was maintained at 2 cm and 8 cm depth in shallow and deep compartment respectively. Two metal plates were installed in the deep water area to connect with the electrode. The shock was set to 1.5 V / cm; with the 10 ms interval; the total shock time was 5 seconds. The experiment contains three phases, including acclimation, training and testing. The experimental process is summarized as follow:



Phase 1: Acclimation

1) The fish was placed in the shallow compartment with the sliding door being closed, and allowed to swim freely for 1 minute.

2) The sliding was opened and animals were allowed to access the deep compartment freely.

3) Fish could move freely inside the behavioral chamber for 5 minutes.

4) Removed the fish from the chamber and returned it to the housing tank.

5) The acclimation-2 was performed 24 hours after the first acclimation with the same procedure as acclimation-1.

Phase 2: Training

1) The training-1 was performed 24 hours after the acclimation-2.

2) The fish was placed in the shallow compartment and allowed to stay for 1 minute.

3) Open the sliding door and animal is allowed to access the deep compartment freely.

4) Once the fish entered the deep compartment, the door was closed immediately, and a 5-second electric shock was given.

5) Removed the fish and returned it to the housing tank.

6) The training-2 was performed 24 hours later with the same procedure.

Phase 3: Testing

1) The zebrafishes were subjected to the testing 24 hours after the training-2.

2) The animals were placed in the shallow compartment and stay for 1 minute.

3) The sliding was opened and fish could move freely to the shallow compartment. The latency of reaching the deep compartment was recorded and analyzed.

4) The experiment completed once the animal reaching the deep compartment; the experiment was also be halted if the fish unable to enter the goal area within a 5 minutes trial.

8. Telencephalon lesion surgery

The zebrafishes were anesthetized with the 200 ppm MS-222 (Sigma, E10521, Germany, CAS NO: 886862). Once anesthetized, the fish was fixed on the operating table with the dorsal side facing up. The body was covered with water-soaked cotton pads to ensure that the body surface moisture during the operation. Oxygenated water with 100 ppm MS-222 was continuously perfused from the fish's mouth to avoid hypoxia and maintain anesthetic status during the procedure.

The operation was performed under a dissecting microscope. First, used a scalpel to scrape off the head's skin and carefully cut the cranial bones. Used a 10 mL plastic syringe and butterfly needle tube as the suction device, and used a glass capillary as a needle device at the end of the butterfly needle tube. Aspirate a specific half of the telencephalon according to experimental needs. After the telencephalon was damaged, the cranial bones and head skin were carefully reset. A drop of Vetbond™ Tissue Adhesive (084-1469SB, 3M, U.S.A.) was added to the surgical site to facilitate tissue healing. The zebrafish were placed in the rearing tank immediately after the operation for a total of 5 days. Animals were then subjected to subsequent behavioral experiments after the 5-day recovery period. The animals were divided into a sham group, a right-side telencephalon lesion group (R-lesion group), or a left-side telencephalon lesion group (L-lesion group) based on the experimental design. The detailed treatment of each group is summarized below:

Group	**Anesthetized**	**Cranium opened**	**Telencephalon ablated**
Sham	Yes	Yes	No
R-lesion	Yes	Yes	Yes (right-side only)
L-lesion	Yes	Yes	Yes (left-side only)

9. Statistic

This research's experimental results were analyzed by the statistical software GraphPad Prism 8, and the data were all presented by Mean ± SEM. With the P-value ≤ 0.05, was regarded as a statistically significant difference. In the locomotor activity test and anxiety-related experiments, one-way ANOVA compared each group's swimming distance. In cognition-related experiments, including the T-maze test and inhibitory avoidance test, two-way ANOVA is used for statistics. Differences between groups and comparisons before and after learning within groups were evaluated based on multiple comparisons.

Results

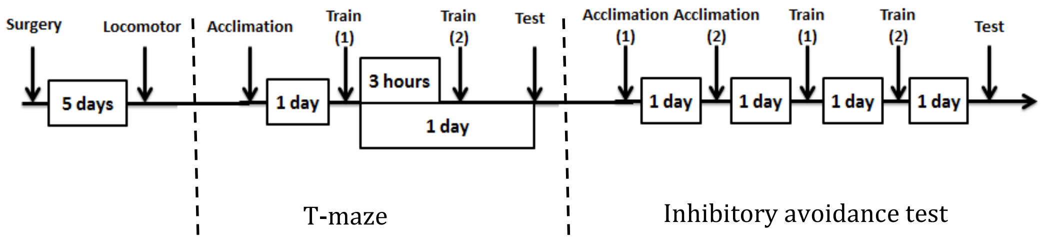
Experiment-1: Explore the effect of unilateral telencephalon damage on zebrafish without brain reversal.

Rationale:

Our previous results showed that the left side telencephalon dominates spatial learning, and the right side telencephalon dominates spatial memory. Therefore, this study adopts the unilateral telencephalon lesion method and examines how zebrafish's spatial memory and fear memory are affected.

Procedure:

Wild-type AB strain zebrafish were randomly distributed to one of the following groups: the sham, left-side telencephalon ablation group (L-lesion), and right-side telencephalon ablation group (R-Lesion). All the surgical treated zebrafishes go through a 5-day recovery period. Afterward, they were assigned to the locomotor activity test to examine the possible non-specific surgical treatment effect on zebrafish' motor function. It is an essential step for avoiding the confounding effect of surgical treatment on the T-maze test and the inhibitory avoidance test results. The experimental process is listed as follows:



Animals were euthanazited with MS-222 and hypothermia immediately after completing the last behavioral experiment, They were then soaked in formalin, and the brains were removed after the tissue fixation was completed. Each brain was photographed and recorded for evaluating the lesion size and location.

Results:

In figure 2a, no significant difference in the distance moved was found among the three groups (Kruskal-Wallis statistic: 0.000 and $P > 0.9999$). We concluded that the operation did not influence the spontaneous movement ability of the zebrafishes. In the T-maze test, AB-sham and AB L-lesion groups significantly decrease their latency after training (two-way ANOVA, $P_{(\text{AB sham})} = 0.0004$, $P_{(\text{AB L-lesion})} = 0.0020$), only R-lesion group didn't significantly decrease (two-way ANOVA, $P_{(\text{AB R-lesion})} = 0.6861$). This result suggests the right side telencephalon ablation impaired the acquisition of spatial memory (figure 2b). According to linear regression of telencephalon lesion size and special learning effectiveness in AB-sham (figure 2c), the equation of the line is: $Y = 3.225X + 23.80$ ($P < 0.0001$, $R^2 = 0.7467$). The higher the percentage of telencephalon lesion, the longer the T-maze's escape latency, which meant that the spatial learning ability was reduced apparent with the increase of lesion size. In the inhibitory avoidance test (figure 2d), AB-sham an AB L-lesion groups significantly increase their escape latency after training (two-way ANOVA, $P_{(\text{AB sham})} < 0.0001$, $P_{(\text{AB L-lesion})} < 0.0001$), but R-lesion group didn't significantly increase (two-way ANOVA, $P(_{\text{AB R-lesion}}) = 0.9628$). This result showed that the right side telencephalon ablation also impaired the acquisition of fear memory. A trend is found in the linear regression of telencephalon lesion size and fear learning effectiveness in AB (figure 2e), the equation of the line is: $Y = -3.216X + 233.7$ ($P = 0.0001$, $R^2 = 0.6125$). The higher the percentage of telencephalon lesion, the shorter the escape latency time spent in the IA task.

Experiment-2: Evaluation of the anxiety-like behavior between foxd3_L & foxd3_R in zebrafishes using novel tank test.

Rationale:

Previous studies used a novel tank diving test to examine the brain reversal effects on the anxiety-like behavior in zebrafish. Results showed the "entries in the top half" and "duration spent in top half" were elevated in the brain inversion zebrafish (Facchin et al, 2015). We decided to confirm whether a similar behavioral phenotype has also occurred in the Tg (*foxd3: GFP*) zebrafish, and it is the fundamental step of the study.

Procedure:

Each fish were placed in the tank and recorded for 15 minutes. When analyzing, we will artificially split the 15 minutes into three intervals with five minutes each to understand the changes in the anxiety level of zebrafish in the tank.

Results:

The results were shown in figure 3. In a novel tank diving test, the top half duration in the foxd3_R group was significantly lower than other groups in the third five minutes (Kruskal-Wallis test: 6.337, P value = 0.0421). This result suggested that zebrafish with structural inversion have a higher level of anxiety, which is consistent with the previous study. We concluded that the Tg (*foxd3:GFP*) zebrafish used in this experiment had a similar behavioral phenotype with the same strain of zebrafish used by other research institutions.

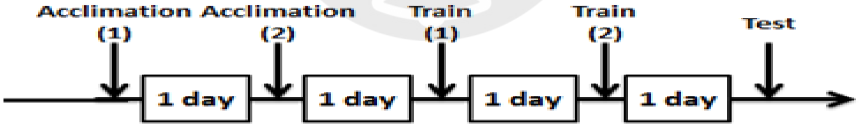
Experiment-3: Evaluate the possible effect of brain inversion on the inhibitory avoidance learning in zebrafishes.

Rationale:

The purpose of this experiment is to test whether wild-type AB strain zebrafish and Tg (*foxd3: GFP*) zebrafish have innate differences in inhibitory avoidance learning. On the premise that no intrinsic difference in inhibitory learning was found, we could ensure that the between-group difference in learning effectiveness in subsequent cognitive experiments has resulted from the experimental manipulation.

Procedure:

Additional AB wild type, foxd3_L, and foxd3_R zebrafish were prepared and subjected to the behavioral experiments at the stage of fully adult. The general procedure included two-days of adaptation, two-days of training, and a single test session. The experimental procedure is showed as follows:



Results:

The results were shown in figure 4. All groups had significantly increased latency after training, The mean of latency in AB group elevated from 16.38 to 112.5 (N = 16, P value = 0.0129), foxd3_R group elevated from 16.58 to 187.4 (N = 12, P value = 0.0001) and foxd3_L group elevated from 18.81 to 163.9 (N = 16, P value = 0.0001). No significant difference in inhibitory avoidance learning was found among groups (two-way ANOVA, P-value = 0.2956). From the results of this experiment, it could be seen that without any surgical treatment, whether the strain or brain development was reversed or not, it would not affect the zebrafish's fear learning in inhibitory avoidance test.

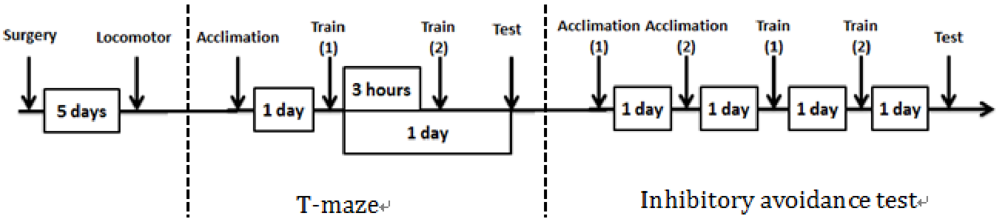
Experiment-4: Explore if the same side telencephalon dominates left-side parapineal transgenic foxd3-GFP strain zebrafish's cognitive function.

Rationale:

According to the results of experiment-1, the right side telencephalon has a dominant effect on learning in the wild-type zebrafish. We examined whether the transgenic foxd3-GFP zebrafish was consistent with the wild-type zebrafish trend that one side of the telencephalon dominates the cognitive function.

Procedure:

Additional transgenic foxd3-GFP strain zebrafish were prepared and distributed into the sham group, R-lesion group, and L-lesion group. Individuals who had apparent motor dysfunction after surgery were excluded from the subsequent behavioral experiments. The experimental procedure is summarized as below:



All experimental animals were sacrificed and soaked in formalin after the behavioral experiment, and then their cerebrum was collected and subjected to the lesion side verification.

Results:

In figure 5a, no significant difference in the distance moved was found among the three groups (Kruskal-Wallis statistic: 1.153 and $P = 0.5617$). The results showed that the surgical treatment did not cause motor dysfunction. Neither sham nor lesion groups showed a difference in locomotor activity. The T-maze results demonstrated that foxd3_L sham and foxd3_L L-lesion groups significantly decrease their latency in the testing session (two-way ANOVA, $P_{(\text{foxd3\_L sham})} = 0.0019$, $P_{(\text{foxd3\_L L-lesion})} = 0.0033$). No change on the latency of the R-lesion group was found (two-way ANOVA, $P_{(\text{foxd3\_L R-lesion})} = 0.7878$). Results showed that the right side telencephalon ablation impaired the acquisition of spatial memory (figure 5b). Figure 5c showed the linear regression of telencephalon destruction ratio and special learning effectiveness in foxd3_L, the correlation coefficient is $Y = 2.504X + 57.11$ ($P = 0.0016$, $R^2 = 0.4743$). The result showed that the higher the percentage of brain damage, the longer the fish will spend in the T-maze, which means that the spatial learning ability has largely interfered. In inhibitory avoidance test (figure 5d), foxd3_L sham and foxd3_L L-lesion groups significantly increased their latency after training (two-way ANOVA, $P_{(\text{foxd3\_L sham})} < 0.0001$, $P_{(\text{foxd3\_L L-lesion})} < 0.0001$), but R-lesion group didn't significantly increase (two-way ANOVA, $P(_{\text{foxd3\_L R-lesion}}) = 0.9995$). Results suggested that the right side telencephalon ablation also impaired the acquisition of fear memory. Figure 5e was the linear regression of telencephalon lesion size and fear learning effectiveness in foxd3_L. The correlation coefficient is $Y = -3.360X + 229.9$ ($P < 0.0001$, $R^2 = 0.7192$). The larger the ablation area, the less escape latency in the inhibitory avoidance.

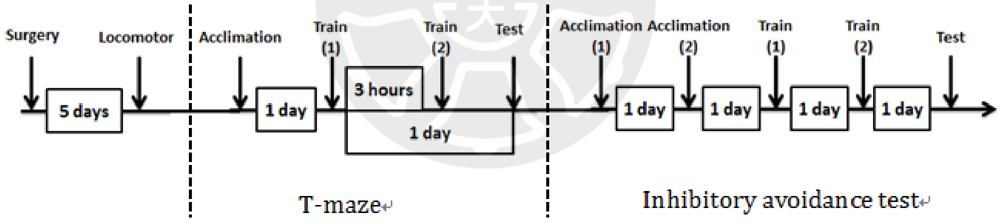
Experiment-5: Explore the functional cerebral lateralization using right-side parapineal transgenic foxd3-GFP strain zebrafish.

Rationale:

We hypothesized that functional lateralization exists in the telencephalon, which dominates the learning and memory in zebrafish. It is reasonable to assume that the telencephalon's functional lateralization will be reversed in the right-side parapineal transgenic foxd3-GFP strain zebrafish (foxd3_R). We examined this hypothesis using unilateral telencephalon ablation combined with behavioral experiments.

Procedure:

Only foxd3_R zebrafishes were used in this experiment. Animals were randomly assigned into sham control, R-lesion or L-lesion groups. The experimental procedure was similar to experiment-1 & -4.



Also, animals were sacrificed immediately after the last behavioral experiment, and its brain was dissected out and soaked in formalin for fixation. All the brains were subjected to lesion side verification.

Results:

No significant difference in the distance moved was found among the three groups (figure 6a, Kruskal-Wallis statistic: 1.751 and P = 0.4167). The possible confounding effect of the surgical treatment was excluded. In the T-maze test, the latency of the foxd3_R sham and foxd3_R R-lesion groups were significantly decreased after training (figure 6b, two-way

ANOVA, $P_{(\text{foxd3\_R sham})} = 0.0269$, $P_{(\text{foxd3\_R R-lesion})} = 0.0027$). The latency of the L-lesion group didn't significantly remain unchanged (two-way ANOVA, $P_{(\text{foxd3\_R L-lesion})} = 0.3286$). This result showed consistent with our previous observation on the wild-type zebrafish that a functional asymmetric exists on the spatial learning. It also supports our assumption that once the telencephalon is inversed, the dominant side is changed as well. Figure 6c showed the linear regression of telencephalon lesion size and special learning effectiveness in foxd3_R, the correlation coefficient is $Y = 1.581X + 20.96$ ($P = 0.0197$, $R^2 = 0.2803$). The larger the ablation area, the longer the T-maze's escape latency, which implies the spatial learning ability was impaired.

Results of the inhibitory avoidance test showed the latency of both the foxd3_R sham and foxd3_R R-lesion groups significantly increased after training (two-way ANOVA, $P_{(\text{foxd3\_R sham})} < 0.0001$, $P_{(\text{foxd3\_R R-lesion})} < 0.0001$), but not in the L-lesion group (two-way ANOVA, P($_{\text{foxd3\_R L-lesion}}$) = 0.9965) (figure 6d). These results provided further evidence that functional asymmetric did exist in the telencephalon. The dominant side of the telencephalon also changed in the foxd3_R zebrafishes and damage to the left side of the telencephalon interference acquisition of fear memory. Figure 6e showed the linear regression of telencephalon lesion size and fear learning effectiveness in foxd3_R. The correlation coefficient is $Y = -2.030X + 188.1$ ($P = 0.0070$, $R^2 = 0.3562$). The larger the ablation size inversely correlated with inhibitory avoidance learning.

We summarized the results of present study in the following table.

Strain	**Group**	**T-maze**	**IA**
Wild-type	Sham	✔	✔

AB **(Normal telencephalon)**	R-lesion	✘	✘
	L-lesion	✔	✔
foxd3_L **(Normal telencephalon)**	Sham	✔	✔
	R-lesion	✘	✘
	L-lesion	✔	✔
foxd3_R **(reversal telencephalon)**	Sham	✔	✔
	R-lesion	✔	✔
	L-lesion	✘	✘

✔: intact ; ✘: impaired

Discussion

This study aimed to explore the functional asymmetric of the telencephalon on the learning and memory behaviors. Both the wild-type AB strain zebrafish fish and a transgenic foxD3:GFP zebrafishes were applied. The structural inversion was induced thermally during the hatching, and animals were subjected to the unilateral telencephalon ablation by the aspiration to examine whether the spatial memory or emotional memory are affected.

Results revealed a significant increase in anxiety-like behavior in the structural inverted transgenic zebrafishes (foxd3 strain zebrafish with parapineal on the right, foxd3_R) (figure 3), which showed consistency with previous studies (Facchin et al, 2015). There was no significant difference in locomotion among groups. Results also demonstrate that the right-side telencephalon dominates the inhibitory avoidance learning in both the wild-type and non-inverted transgenic zebrafish (foxd3 strain zebrafish with parapineal on the left, foxd3_L). In contrast, the inhibitory avoidance learning dominated by the left-side telencephalon of the foxd3_R group, which supports our hypothesis that a functional asymmetric of telencephalon on avoidance learning did exist; besides, the functional asymmetric on the spatial learning was also found (figure 6b, 6d). It is also dominated in the wild-type and foxd3_L group's right-side telencephalon, but not in the foxd3_R animals. In summary, we evidenced a functional asymmetric of telencephalon on acquiring both the spatial and emotional memories in zebrafish.

The structural inversion of the telencephalon does not affect the target side preference of the T-maze learning.

Numerous studies reported that vertebrates exhibit behavioral asymmetry (Andrew et al., 2002; Ghirlanda & Vallortigara, 2004; Vallortigara, 2000; Vallortigara & Rogers, 2005). In the study of chicks, they found that the left-eye chick (right hemisphere) has advantages in the use of distant topographical cues (Rashid & Andrew, 1989) and the exploration of complex stimuli (Vallortigara & Andrew, 1991). The right-eyed chick is better at controlling the decision-making and performing responses and, for example, avoiding pecking at the distractor target (Mench & Andrew, 1986), avoiding pecking at the familiar companion (McKenzie et al., 1998). An eye preference is found in the zebrafish. The right eye (RE) is used by zebrafish in the visual control of response (Sovrano & Andrew, 2006). When seeing an unfamiliar object for the first time, zebrafish usually use the right eye to make up decisions and decide whether to approach or flee. When the zebrafish encounters stimulus targets, it recognizes them with the left eye (LE) and checking whether the target was encountered before. The process of assessing novelty involves comparing the current perceptual input with previous experience or familiar objects. Which suggested a retrieval of the learned memory is required. Therefore, a functional linkage exists between the left & right eye preference and the learning and memory.

The functional asymmetric also found in the memory stage of the spatial memory in zebrafishes (Wu, 2017). The unilateral ablation of the right-side telencephalon could impair the retrieval of the learned spatial memory in the wild-type zebrafish (Wu, 2017). In addition, they found a statistical difference on the right-side and left-side learning in zebrafish's T-maze task. Zebrafishes failed to show an idea learning effect when the target area was located on the left side of the behavioral chamber (Wu, 2008). In the present study, the target area is set on the right-side only.

Interestingly, there was no change in the T-maze learning's right-side preference in the foxd3_R group than the wild-type and foxd3_L group (figure 2b, 5b, 6b). Regardless of whether it is wild-type, foxd3_R or foxd3_L groups of zebrafish, after a sham operation (open the skull without hurting the brain), all three groups showed learning effects on the T-maze task (figure 2b, 5b, 6b). These results suggest the structural inversion itself did not alter the right-side preference of the T-maze.

The foxd3_R and foxd3_L groups' unilateral ablation results confirmed that the right-side telencephalon dominates spatial learning, and the left-side telencephalon is essential for retaining spatial memory (figure 5b, 6b). The reverse of the dominant telencephalon in both the learning and retention was found in the foxd3_R group. Since we examined the right-target area setting only, the possible effect of unilateral ablation on the left-side learning remains uncertain. A further experiment is required for clarification.

The correlation between ablation size of the telencephalon and the learning effectiveness.

We performed a linear regression analysis on the relationship between the ablation area and the learning effect. A significant correlation coefficient was found (figure 2e, 5e, 6e). Our results demonstrated even a tiny size of ablation has a tremendous impairment effect on both the spatial and emotional learning. Previous study the effects of ablation of the medial telencephalic pallia (MP) and lateral telencephalic pallia (LP) in goldfish (Portavella et al., 2004). They found Emotional and spatial learning in goldfish is dependent on different telencephalic pallial systems (Portavella et al., 2005).

In teleost fish, the telencephalon not only assemble neocortex but also the limbic system including the amygdala & hippocampus in mammals (Demski, 2013). It is well-known that brain trauma in the limbic system has a negative impact on cognitive function in humans (Sydnor et al., 2020). In mammals, the amygdala and hippocampus are both the essential structure of the limbic system and play an essential role in acquiring and retaining spatial and emotional memory. The amygdala is involved in both emotional behavior and memory (LeDoux, 1995). Damage of the amygdala leads to the loss of the learned conditioning response (Ambrogi Lorenzini et al., 1991). The hippocampus participates in the spatial learning (O'Keefe & Nadel, 1978) and associative memory (Eichenbaum et al., 1992). Previous studies have suggested that in teleost fishes, the lateral and medial telencephalic pallium should be homologous to the hippocampus and the amygdala, respectively (Portavella et al., 2004). In fact, in the study of the goldfish model, it has been proved that whether it is the destruction of the lateral or medial telencephalic pallium, it will cause the loss of the learned conditioned escape response. (Portavella et al., 2004). In other words, partial brain damage is sufficient to produce significant damage to specific cognitive functions. In this study, we damaged the entire unilateral telencephalon which include both the lateral and medial telencephalic pallium. Which could explain the robust results obtained in both the spatial and emotional memory. From our research results, it could be confirmed that unilateral telencephalon lesion does have a negative impact on spatial and emotional memory.

Continuing the aforementioned point, these results evidence the importance of the telencephalon of the learning and retention of spatial and emotional memories. We want to mention the possible measurement error of this study. The lesion area was measured almost two weeks after the ablation. Previous study reported that the telencephalon of zebrafish regenerated after ablation (Schmidt et al., 2014). We cannot exclude the possible confounding effect of regeneration on our behavioral data. Also, a modified measurement paradigm using the intact side of the telencephalon as the lesion area's control and baseline for calculating the lesion area. It could only provide an indirect measurement of the lesion area size. An over-estimation or under-estimation of the lesion area may occurred. In the subsequent study, we suggest the lesion area should be measured immediately after the lesion and using another landmark area such as the mid-brain to serve as the baseline for evaluating the lesion area's size.

Summarizing the results of this research, we found that if the right side of the zebrafish with normal brain development was damaged, it would have a negative impact on the ability of spatial learning and fearful learning. In contrast, if it was a zebrafish with reversed brain development, it was the damage to the left side of the brain that affected the individual's learning ability. This phenomenon shows that a certain side of the brain dominates the individual's learning, and this laterality is obviously determined by innate development and cannot be changed by acquired experience.

Functional domination of telencephalon may exist in the learning and memory processing of zebrafish.

The possible functional domination of telencephalon in vertebrates was widely studied using newborn chicks. The chicks could catalog into left-eye or right-eye chicks based on their preferential use of the eye. Scientists found that the left-eye chick (right hemisphere) has advantages in the use of distant topographical cues (Rashid & Andrew, 1989;

Morandi-Raikova & Mayer, 2020) and the exploration of complex stimuli (Vallortigara & Andrew, 1991; Andrew et al., 2009) or space (Andrew, 1991; Morandi-Raikova & Mayer, 2020). The right-eyed chick is better at controlling the reaction, and at the same time, decides what kind of reaction to execute based on the selected clues (for example, avoiding pecking at the distractor target(Mench & Andrew, 1986; Chiandetti et al., 2017), avoiding pecking at the familiar companion (McKenzie et al., 1998; Salva et al., 2012). Besides, study also showed that higher levels of aggressive behavior occur in hens when the left, rather than the right, eye is in use. Hens that respond to a playback of an aerial predator alarm call by rolling their head to one side to look up are more likely to use their left eye for this purpose (McKenzie et al., 1998).

Unfortunately, the long hatching period and thick eggshell obstacle the studying of the molecular and functional mechanism. Recently, the advantages of shorter hatching time, transparent eggs, undecoded genome sequence, and easier transgenic manipulation make the zebrafish an ideal model for studying the functional domination of telencephalon. Most importantly, tthe eye preference in zebrafish is not only exhibited in the larvae stage but also sustains to the fully adult stage (Sovrano & Andrew, 2006). Our previous study using *in vivo* electrophysiology recording demonstrated the difference in the high-frequency stimulation-induced telencephalic long-term potentiation between left and right telencephalon (Wu et al., 2017). Unilateral ablation of the telencephalon possesses impairment in the zebrafish's spatial learning and memory in a different manner (Wu, 2008). The present study's results strengthen this point of view and evidence functional domination of telencephalon does exist in zebrafish. We suggest a telencephalic default pathway for processing spatial and emotional learning may exist. One important question that remained is whether the innate pathway requires the postnatal experience. Unfortunately, due to the present experimental design limitation, only adult wild-type and fox_D3 animal were examined. We are not able to draw out a conclusion.

In the future study, the telencephalon inversion effects on the behavioral asymmetric can be examined in the larvae of both the wild-type and fox_D3 zebrafish. If the innate pathway requires acquired experience, we anticipate that there will be no difference in the preference of T-maze learning among wild-type and fox_D3 groups. Otherwise, we shall not exclude the possibility of an innate pathway underlie. Furthermore, a more precise lesion paradigm such as laser lesion could be applied with the fox_D3 animals. The fluorescence level of GFP could be served for determining the location and the size of the ablation. Which could obtain a reliable correlation analysis for the lesion size and functional impairment.

In conclusion, we suggest the functional lateralization of the telencephalon in the learning and memory does exist in the zebrafish. We also found that even a tiny ablation was performed may have a tremendous impairment effect on both the spatial and emotional learning.

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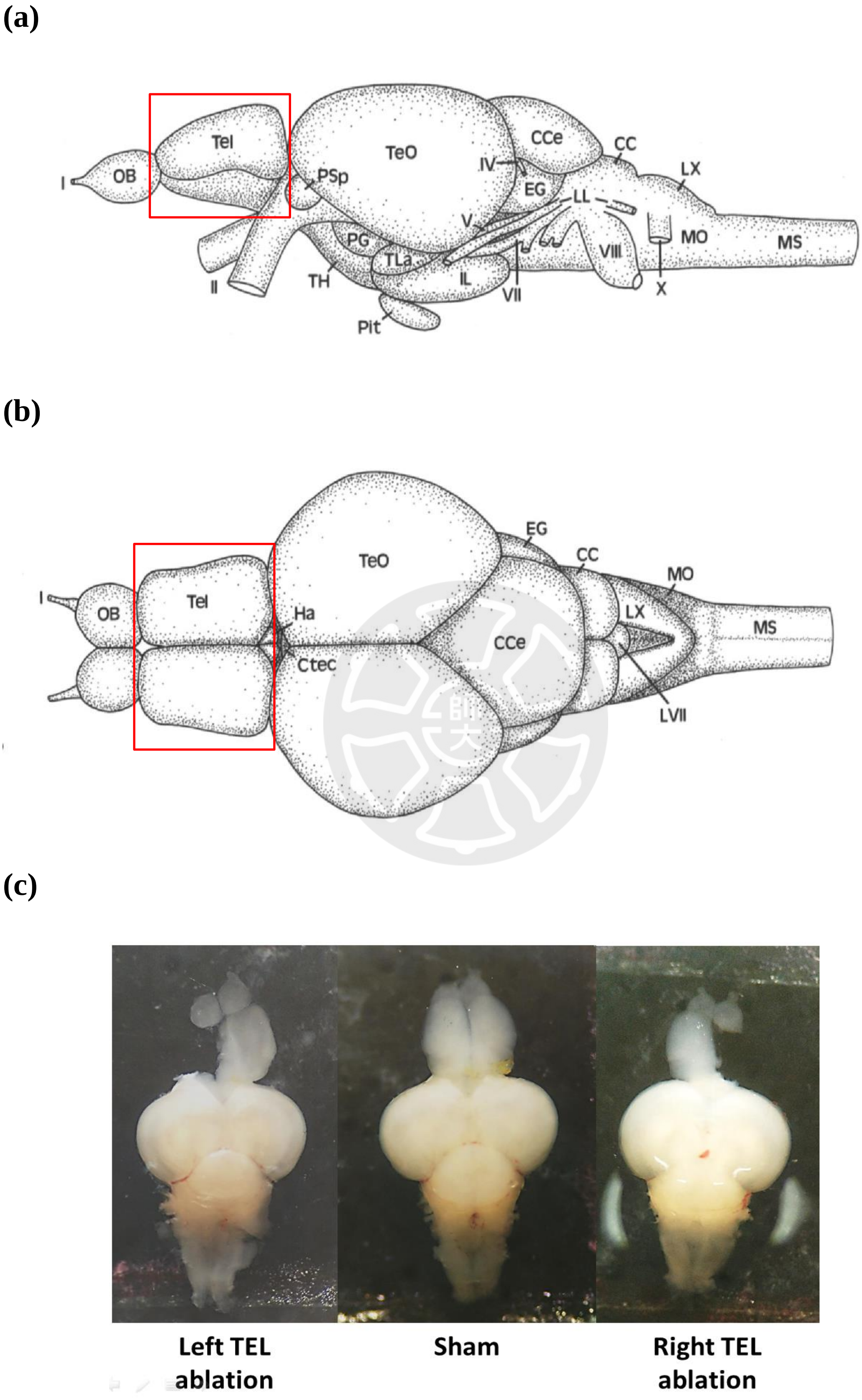
Figures



Left TEL ablation

Sham

Right TEL ablation

Figure 1. Schematic diagram of the results of zebrafish brain surgery.

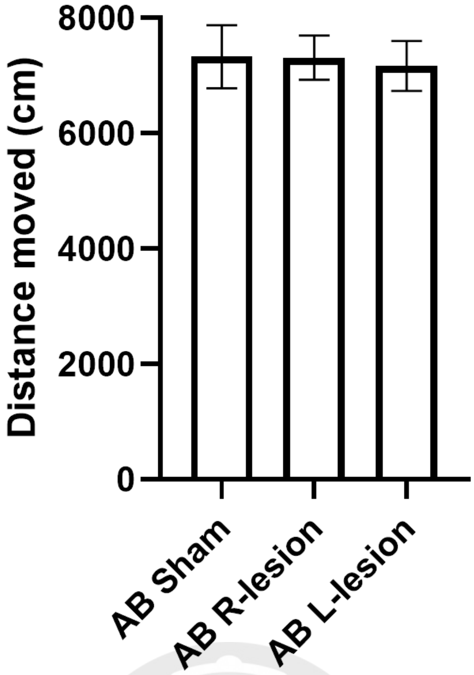
Figure 1 shows the typical brain structure of a zebrafish and the situation after surgical destruction of the telencephalon.

(a) Side view of zebrafish brain (Wullimann et al., 1996)

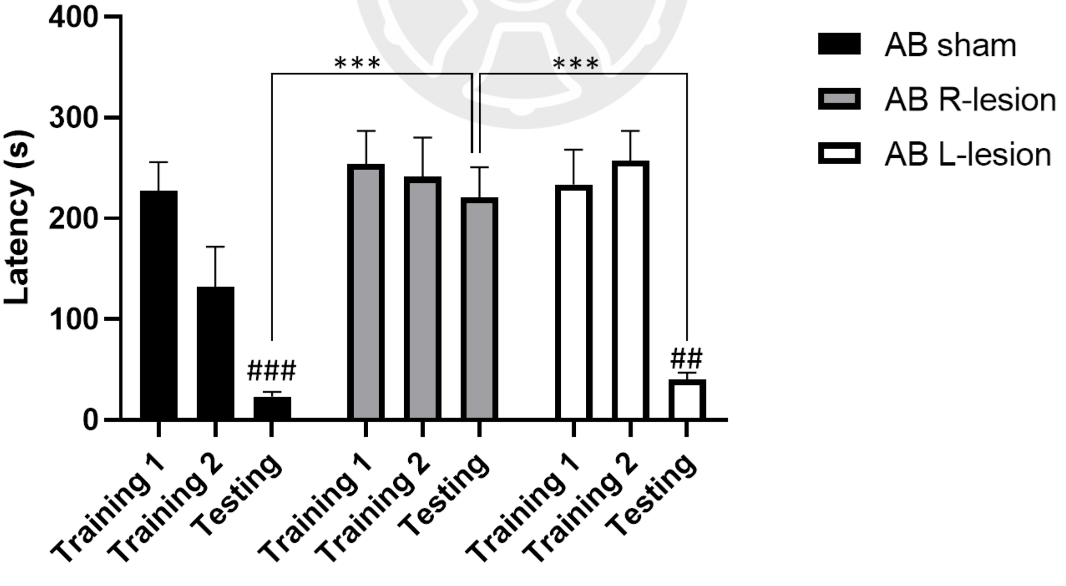
(b) Top view of zebrafish brain (Wullimann et al., 1996)

(c) The sham group was undamaged, and the brain was intact. In the other groups with unilateral destruction, a specific side of the telencephalon was missing.

(a)


8000
6000
4000
2000
0
Distance moved (cm)
AB Sham
AB R-lesion
AB L-lesion

(b)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Latency (s)
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Training 1
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Testing
Training 1
Training 2
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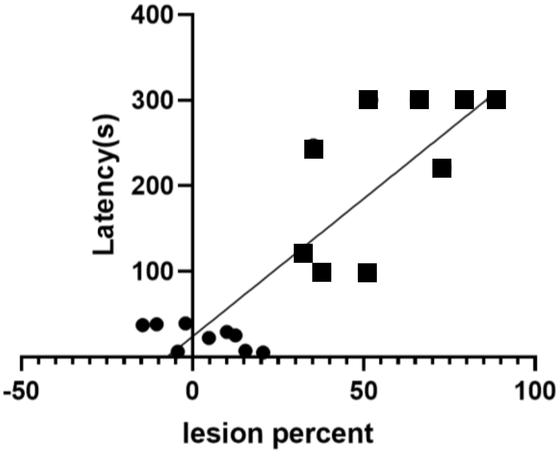
Training 1
Training 2
Testing
AB sham
AB R-lesion
AB L-lesion

(c)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Latency(s)
-50
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50
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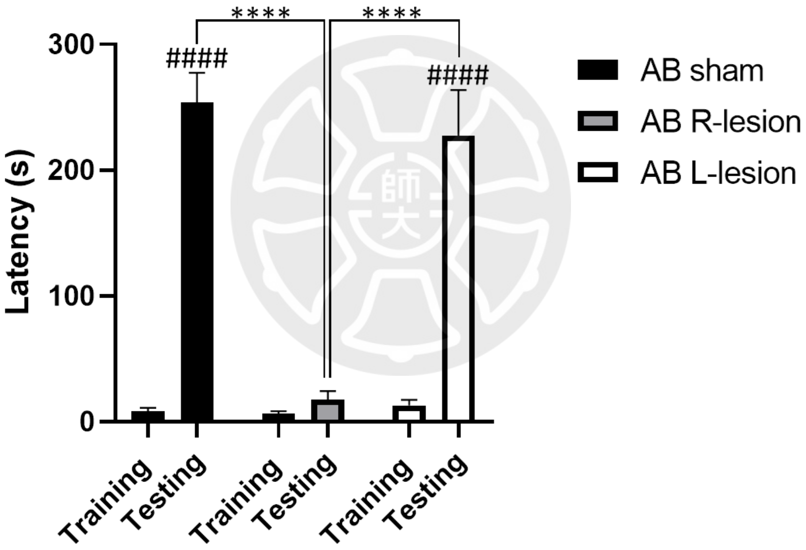
lesion percent

(d)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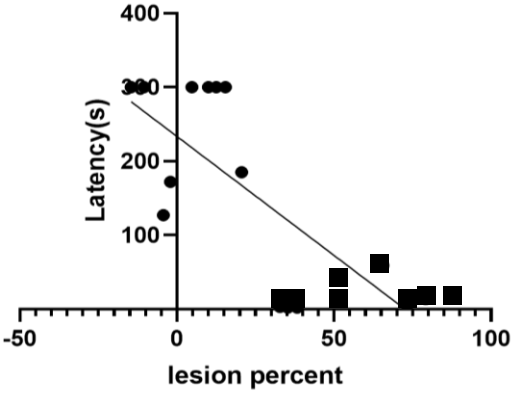
Latency (s)
AB sham
AB R-lesion
AB L-lesion
Training
Testing
Training
Testing
Training
Testing

(e)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Latency(s)
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lesion percent

Figure 2. Lesion of the right side of the telencephalon will lead to obstacles in the learning ability of AB wild-type zebrafish.

(a) Spontaneous motor ability of wild-type AB strain of zebrafish after operation. No significant difference on the distance moved was found among three groups. Each vertical bar expressed as mean ± SEM (Each group n = 9)

(b) Evaluate the effect of unilateral telencephalon lesion on the learning of spatial memory in AB zebrafish. The latency time of the sham group and the L-lesion group was significantly reduced during the test phase (two-way ANOVA, $P(_{\text{AB sham}}) = 0.0004$, $P(_{\text{AB L-lesion}}) = 0.0020$), but the right destruction group did not. Each vertical bar expressed as mean ± SEM (*: compared with different groups, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$；#: compared with training 1, # = $p < 0.05$, ## = $p < 0.01$, ### = $p < 0.001$) (●: sham group；■: R-lesion group)

(c) Linear regression of telencephalon destruction ratio and spatial learning effectiveness in AB. The equation of the line is: $Y = 3.225X + 23.80$ ($P < 0.0001$, $R^2 = 0.7467$)

(d) Evaluate the effect of unilateral telencephalon lesion on the learning of fear memory in AB zebrafish. The latency time of the sham group and the L-lesion group was significantly increased during the test phase (two-way ANOVA, $P(_{\text{AB sham}}) < 0.0001$, $P(_{\text{AB L-lesion}}) < 0.0001$), but not in R-lesion group (two-way ANOVA, $P(_{\text{AB R-lesion}}) = 0.9628$). (*: compared with different groups, **** = $p < 0.0001$；#: compared with training, #### = $p < 0.0001$)

(e) Linear regression of telencephalon destruction ratio and fear learning effectiveness in AB. The equation of the line is: $Y = -3.216X + 233.7$ ($P = 0.0001$, $R^2 = 0.6125$) (●: sham group；■: R-lesion group)

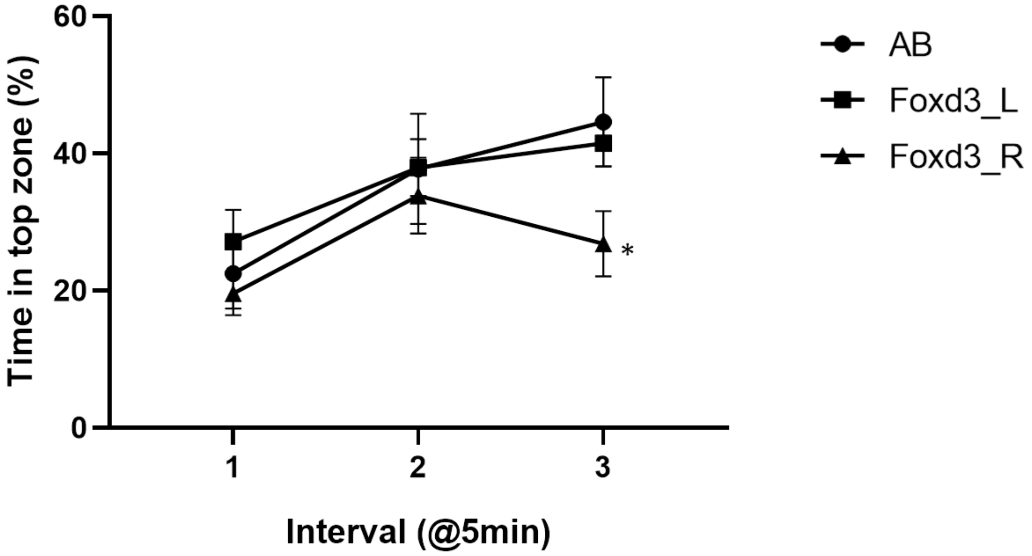


Figure 3. Zebrafish with brain inversion will have higher anxiety levels than normal zebrafish.

Compare each group on novel tank diving test. The time spend in the top zone was significantly decrease in the foxd3_R group at the third 5min interval (Kruskal-Wallis test: 6.337, P value = 0.0421). Each data points of the polyline expressed as mean ± SEM. (AB n= 10, foxd3_L n= 14, foxd3_R n= 14；* = $p < 0.05$)

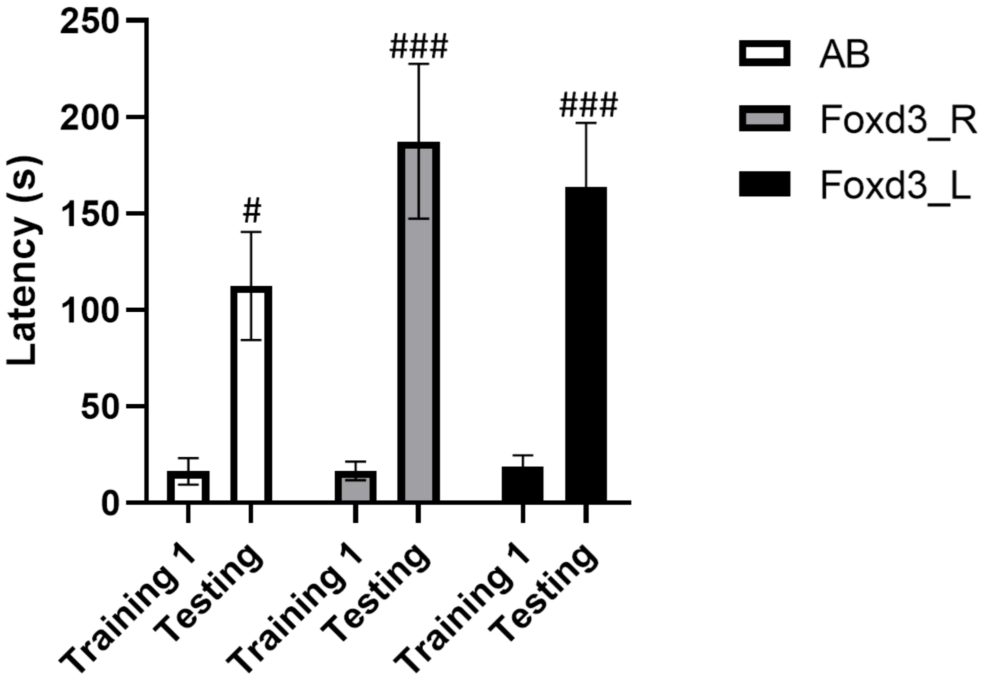
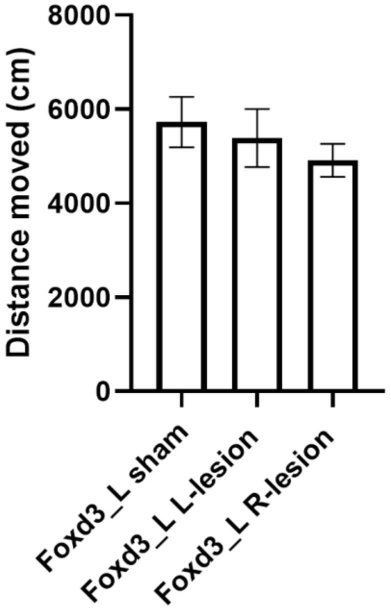


Figure 4. Under the premise of the brain is not damaged, the learning ability of fear memory among different zebrafish is consistent.

The results of the inhibitory avoidance test compared AB wild-type, normal foxd3 and brain-reversed foxd3 zebrafish (AB n = 16, foxd3_R n = 12, foxd3_L n = 16). The latency time of each group had increased after training ($P_{(AB)} = 0.0129$, $P_{(foxd3\_R)} = 0.0001$, $P_{(foxd3\_L)} = 0.0001$), but there was no difference among the groups (two-way ANOVA, P value = 0.2956).

(a)



8000
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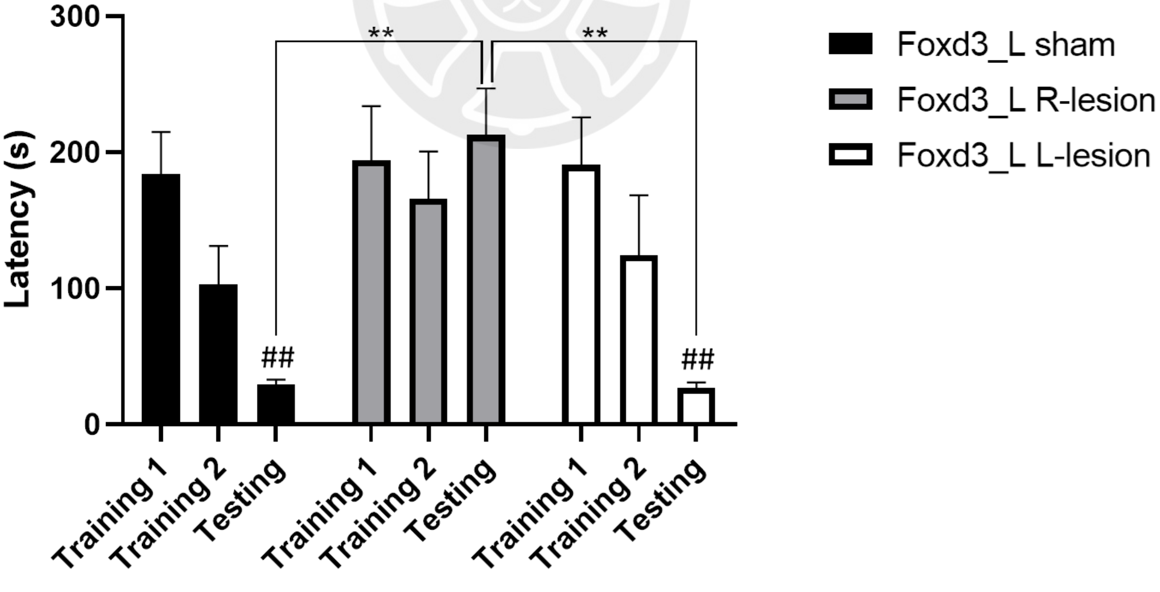
0
Distance moved (cm)
Foxd3_L sham
Foxd3_L L-lesion
Foxd3_L R-lesion

(b)



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Latency (s)
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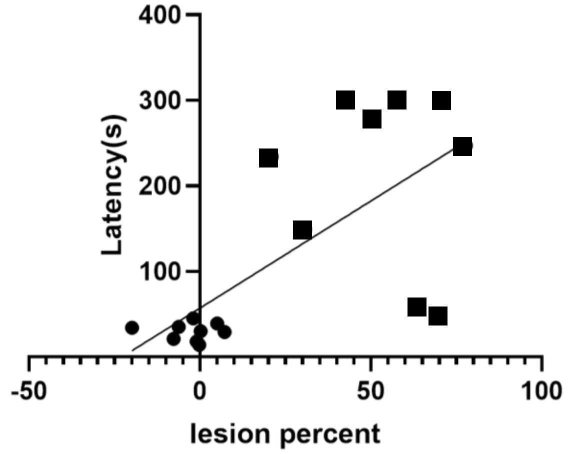
Training 1
Training 2
Testing
Training 1
Training 2
Testing
Training 1
Training 2
Testing
Foxd3_L sham
Foxd3_L R-lesion
Foxd3_L L-lesion

(c)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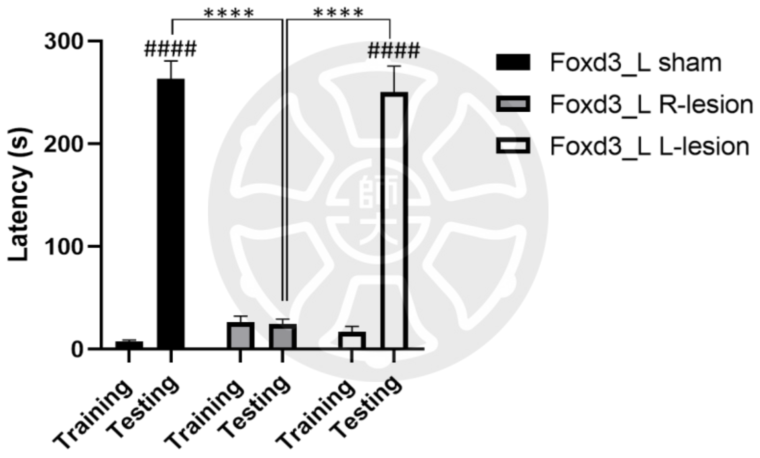
Latency(s)
-50
0
50
100
lesion percent

(d)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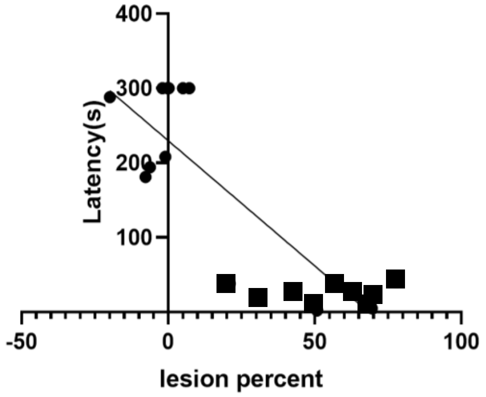
0
Latency (s)
Training
Testing
Training
Testing
Training
Testing
Foxd3_L sham
Foxd3_L R-lesion
Foxd3_L L-lesion

(e)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Latency(s)
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lesion percent

Figure 5. The cognitive function of different strains zebrafish is dominated by the same side telencephalon.

(a) Spontaneous motor ability of foxd3_L strain of zebrafish after operation. No significant difference on the distance moved was found among three groups. Each vertical bar expressed as mean ± SEM (Each group n = 9).

(b) Evaluate the effect of unilateral telencephalon lesion on the learning of spatial memory in foxd3_L zebrafish. The latency time of the sham group and the L-lesion group was significantly reduced during the test phase (two-way ANOVA, $P_{(\text{foxd3\_L sham})} = 0.0019$, $P_{(\text{foxd3\_L L-lesion})} = 0.0033$), but the R-lesion group did not. Each vertical bar expressed as mean ± SEM (*: compared with different groups, ** = $p < 0.01$；#: compared with training 1, ## = $p < 0.01$) (●: sham group；■: R-lesion group)

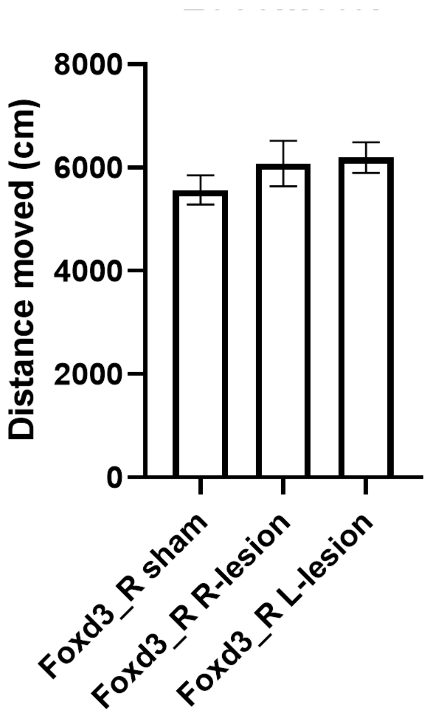
(c) Linear regression of telencephalon destruction ratio and spatial learning effectiveness in foxd3_L zebrafish. The equation of the line is: $Y = 2.504X + 57.11$ ($P = 0.0016$, $R^2 = 0.4743$).

(d) Evaluate the effect of unilateral telencephalon lesion on the learning of fear memory in foxd3_L zebrafish. The latency time of the sham group and the L-lesion group was significantly increased during the test phase (two-way ANOVA, $P_{(\text{foxd3\_L sham})} < 0.0001$, $P_{(\text{foxd3\_L L-lesion})} < 0.0001$), but not in R-lesion group (two-way ANOVA, $P_{(\text{foxd3\_L R-lesion})} = 0.9995$). (*: compared with different groups, **** = $p < 0.0001$；#: compared with training, #### = $p < 0.0001$)

(e) Linear regression of telencephalon destruction ratio and fear learning effectiveness in foxd3_L zebrafish. The equation of the line is: Y =

$-3.360X + 229.9$ ($P < 0.0001$, $R^2 = 0.7192$). (●: sham group；■: R-lesion group)

(a)


8000
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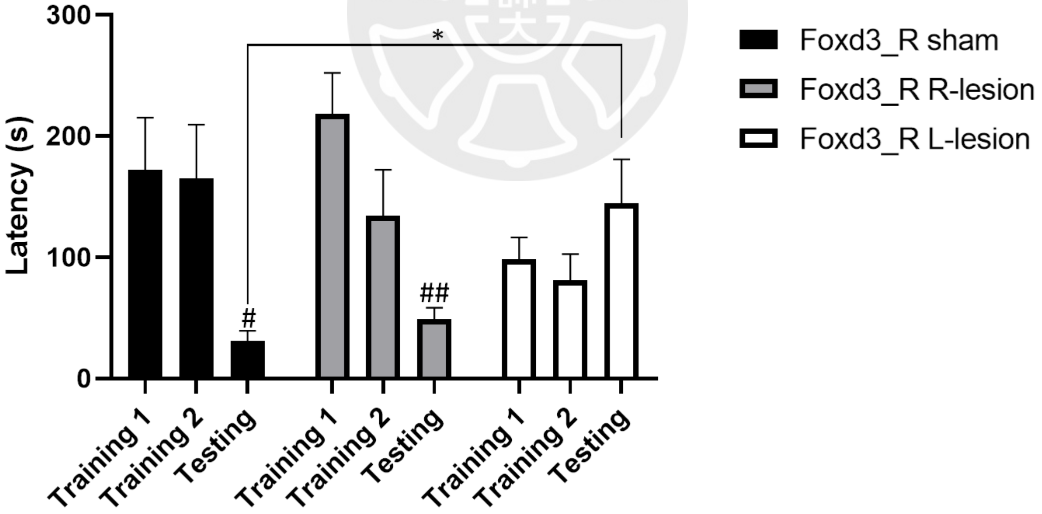
Distance moved (cm)
Foxd3_R sham
Foxd3_R R-lesion
Foxd3_R L-lesion

(b)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Latency (s)
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Training 1
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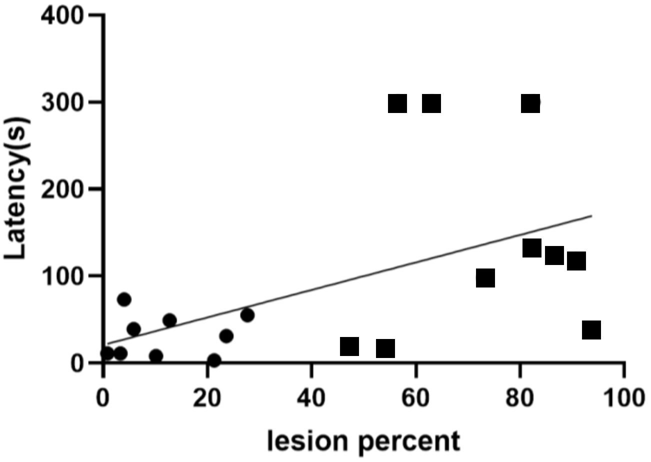
Training 1
Training 2
Testing
Foxd3_R sham
Foxd3_R R-lesion
Foxd3_R L-lesion

(c)


400
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Latency(s)
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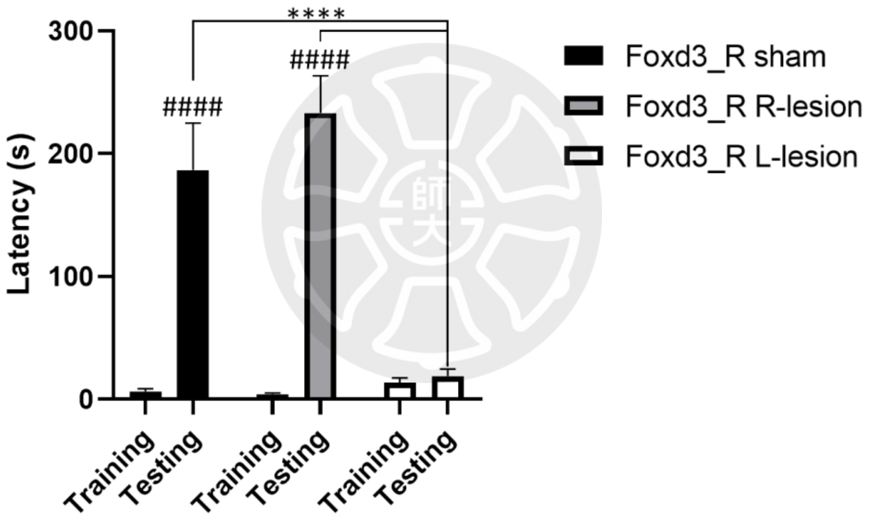
20
40
60
80
100
lesion percent

(d)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Latency (s)
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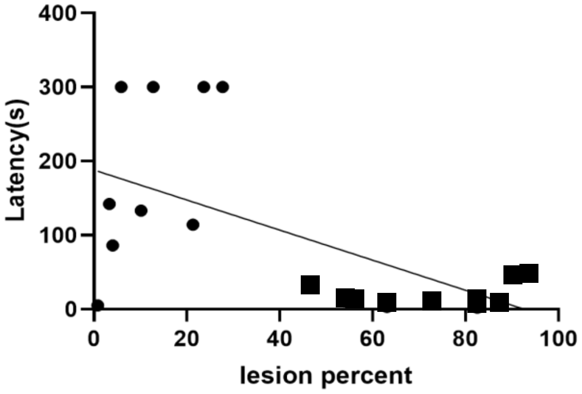
Foxd3_R sham
Foxd3_R R-lesion
Foxd3_R L-lesion
Training
Testing
Training
Testing
Training
Testing

(e)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Latency(s)
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60
80
100
lesion percent

Figure 6. Functional cerebral lateralization in brain inverted zebrafish also reverses in explicit behavior.

(a) Spontaneous motor ability of foxd3_R strain of zebrafish after operation. No significant difference on the distance moved was found among three groups. Each vertical bar expressed as mean ± SEM (sham n = 9, R-lesion n = 9, L-lesion n = 10).

(b) Evaluate the effect of unilateral telencephalon lesion on the learning of spatial memory in foxd3_R zebrafish. The latency time of the sham group and the R-lesion group was significantly reduced during the test phase (two-way ANOVA, $P_{(\text{foxd3\_R sham})} = 0.0269$, $P_{(\text{foxd3\_R R-lesion})} = 0.0027$), but the L-lesion group did not. Each vertical bar expressed as mean ± SEM (*: compared with different groups, * = p < 0.05；#: compared with training 1, # = p < 0.05, ## = p < 0.01) (●: sham group；■: L-lesion group)

(c) Linear regression of telencephalon destruction ratio and spatial learning effectiveness in foxd3_R zebrafish. The equation of the line is: $Y = 1.581X + 20.96$ ($P = 0.0197$, $R^2 = 0.2803$).

(d) Evaluate the effect of unilateral telencephalon lesion on the learning of fear memory in foxd3_R zebrafish. The latency time of the sham group and the R-lesion group was significantly increased during the test phase (two-way ANOVA, $P_{(\text{foxd3\_R sham})} < 0.0001$, $P_{(\text{foxd3\_R R-lesion})} < 0.0001$), but not in L-lesion group (two-way ANOVA, $P_{(\text{foxd3\_R L-lesion})} = 0.9965$). (*: compared with different groups, **** = p < 0.0001；#: compared with training, #### = p < 0.0001)

(e) Linear regression of telencephalon destruction ratio and fear learning effectiveness in foxd3_R zebrafish. The equation of the line is: Y =

$-2.030X + 188.1$ ($P = 0.0070$, $R^2 = 0.3562$). (●: sham group；■: L-lesion group)