博士論文

The neural substrate for internal-directional information exploitation revealed by dissection of the higher-order cognitive function in zebrafish

（ゼブラフィッシュの高次脳機能の解析によって解き明された身体方向情報の活用のための神経基盤）

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Abstract

The quantitative observation of behavior as an observable outcome of brain function at the organism level has been one of the most powerful tools of modern neuroscience to understand cognitive functions. Zebrafish has been drawing attention in behavioral neuroscience. Anatomical and lesion studies have revealed the evolutionarily and functional homologous brain regions to the mammalian counterparts. Several behavior paradigms of adaptive learning, e.g. active avoidance, revealed that zebrafish has the conserved cognitive function in learning and memory. However, the higher-order cognitive functions of it are poorly known. In this research, I aimed at addressing the question of whether the different aspects of higher-order cognitive function are conserved in zebrafish and tried to provide some new insights into the mechanism by the newly designed behavioral paradigms with high-throughput behavioral analysis.

Many studies have shown that zebrafish can be trained by aversive reinforcement learning by using noxious stimuli as a reinforcer. However, aversive stimuli may transfer the state of the animal into fear or anxiety, and concomitantly induce defensive behavior such as freezing or agitation. Consequently, they could interfere with behavioral measurement or even cause the failure of subsequent behavioral training. Therefore, a reinforcement behavior paradigm with positive reward would be more favorable for studying the higher-order cognitive function. To achieve this goal, I devised an automated system to train zebrafish through appetitive reinforcement training by precise control of reward dispensing on a millisecond scale in aquatic environment. The system was applied to the operant conditioning paradigm by associating the positive valence (reward) with visual information from the environment. The system automatically trained the fish without artificial bias at high training frequency by dispensing the rewards only when fish choose a correct assigned color

cue. The results showed that zebrafish is capable of associating the reward with the assigned visual cue. The learner fish reduced error of color selection within several training sessions with declined latency of choice response and achieved around 80%-plateau correct response within a week by repeated training. Inspired by the result, I then modified the system for addressing the question in higher-order brain function.

Working memory is one fundamental function among higher-order brain functions, defined as the function to hold the information for a second-scale period and keep its amenability to further processing. To test the working memory, I modified the hardware with the extended designs for establishing delayed nonmatching-to-sample (DNMTS) task. The DNMTS task is designed here specifically for visual-spatial working memory examination with a reference stimulus and a set of answer stimuli. By comparing the answer stimuli color with the reference color, the fish needs to reach the arm that displays the different colors from the reference to earn the food reward. As the fish needs to hold the information for comparison, the span of working memory can be detected by the different delay periods. The delay was set to 0 s in introductive sessions for teaching the rule, and gradually increased to measure the maintenance ability of visual-spatial working memory. The results demonstrated that zebrafish was almost unable to complete the task even for the introductive session. The mean success rate was around 0.5 to 0.6 which was not significantly different from a chance level with the binomial test. Consequently, fish showed no statistical significance in learning among the trials with further delay durations in the test period. Although I failed to train fish in DNMTS, the results revealed that this type of task, which demands fish to use information by reasoning with the implicit rule, may have surpassed the upper limit of the cognitive capacity of zebrafish.

Attention control is essential as a prior step to decision making and is highly related to behavioral flexibility. The brain is a resource-limited processor. Therefore, attention control is

important to save the calculation power for the right purpose by selectively extracting useful information alone. To test if zebrafish hold the function in attention control, the rule-shift task (RST) was designed to examine the capacity of zebrafish to attend to specific information among two intermingled rules. I selected the navigation behavior, to conceptually separate the information of the internal source (idiothetic) or external source (allothetic) in information exploitation by behavioral paradigm design. It is crucial for animals to adapt to the variable environment by using both these two classes of information and keep the flexibility to switch between them. Therefore, in Rule 1, fish was requested to follow internal-directional (idiothetic) information, e.g. right turning. In contrast, in Rule 2, the fish needed to ignore the internal information and to attend to the visual (allothetic) environmental cues, e.g. red color at the goal. The correct response was rewarded to reinforce the behavior. Once fish achieved the learner criteria in one rule, another rule was imposed on the task without notification. The fish needed to recognize the change of the hidden rule by the error feedback from the result of choice. Our data showed that fish could reach the learner criteria in both rules, the latency of choice decreased as the number of training trials increased. Furthermore, fish adapted more quickly to the altered rule when it experienced the rule changes repeatedly, indicating the capacity of retrieving the rule memory to prioritize information selection.

The Habenular-interpeduncular nucleus (Hb-IPN) circuit integrates information from multiple sensory inputs, basal ganglion, and forebrain. With the characteristic left-right asymmetric axonal projection pattern and asymmetrical representation in sensation, the Hb-IPN circuit is a great candidate as the neural substrate in directional information exploitation and attention control between internal-external information. I, thereby, specifically perturbed the neural activities in the subregion of the dorsal habenula (lateral and medial subregions) which are specifically connected with the dorsal/intermediate and intermediate/ventral interpeduncular nucleus pathway (dHbL-d/iIPN, dHbm-i/vIPN)

respectively to test the possible involvement of the circuit for attention regulation. Surprisingly, I found that the zebrafish with silenced dHbL-d/iIPN showed specific impairment in learning by internal-directional information exploitation but kept intact capacity of learning by external-cued associated information. Control analyses showed that the dHbL-d/iIPN silenced fish were indistinguishable from the control group in locomotion activity, behavioral flexibility, and body coordination, showing that the dHbL-d/iIPN pathway is indispensable in self-directional sensation. The two parallel pathways from the dHb to the IPN are also well known for regulating the winner/loser conflict outcome. Therefore, the dHbL and dHbM could play together as a switchboard for the synergistic control of both social behaviors (dominant or submissive) and attentional orientation (internally or externally directed).

Table of Content

Chapter 1. Introduction

Define Problems

We are living in the information age, the era that is empowered by computers with an overwhelming amount of information. The size of transistors manufactured nowadays smaller than a single neuron (10 to 100 microns), making the microprocessors with even more calculation units (for example, intel Core i7, 10^7 transistors) than a tiny brain, such as the brain of drosophila (Zheng et al., 2018) or zebrafish larvae (Friedrich et al., 2013) (10^5 neurons). However, even for these powerful microprocessors, they hardly reach the computational power of the simplest brain that can achieve complex functions with low energy cost. This makes us wonder what is the principle designs in the brain that lead to efficient computation? And how the cognitive function emerged in the course of evolution?

A good way to answer these questions is to select the right model animals which acts as the representative over the branches of the phylogeny tree, and then select the proper behavioral responses elicited as the outcome of its neural framework. The conserved behavior phenotype made possible even by the primitive neural design provides us with a higher chance that we could gain insight into the essential basic neural designs conserved in the complex system (Figure 1.1).

Higher-order cognitive function refers to the control of behavior implementation in non-automatic fashion. Although it has not yet been defined rigorously in the neuroscientific community; it can be characterized as the ability to coordinate thought and act toward achieving goals, especially while animals face to complex environmental inputs and execute behavior with voluntary effort (Miller and Wallis, 2009). Because of the complexity it has, it is difficult to address the components and the underlying neural substrates to understand how it works. Although the related studies have been conducted in primates and rodents (Chudasama, 2011; Cristofori et al., 2019; Diamond, 2013; Diamond and Lee, 2011; Hofmann et al., 2012; Stoet and Snyder, 2009), the complexity of the brain brings the extra challenge to researchers for understanding the neural designs of the higher-order cognitive functions. In this study, I use zebrafish to address the question by analyzing behavior requiring the higher-order cognitive functions, to add the fundamental knowledge for the future mechanistic study by taking the advantage of the zebrafish's relatively primitive brain. Specifically, I have tried to answer the questions "what is the level of the higher-order cognitive function that zebrafish can achieve?" in order to infer the possible neural substrates engaged in each component of the higher-order cognitive function.

The behavioral analysis with the correct selected animal model could rectify the reductionist bias in neuroscience

Neuroscientists have developed various tools to analyze the brain functions at different levels ranging from subcellular levels such as the molecular changes, synapstic plasticity, single-cell recording to connectivity study to the system levels such as large-scale recordings with calcium imaging, positron emission tomography (PET) imaging, functional magnetic resonance image (fMRI) and electroencephalography (EEG). Furthermore, the cutting-edge techniques, e.g. optogenetic or chemogenetic tools, to perturb the activities of functional units have broadened the spectrum of study. Such field is promptly developing in the past two decades. A general belief in the field is that if we comprehensively obtain the information of every neuron and connection in the brain, we can reconstruct the brain function and know how it works, namely, to decipher how the brain controls behaviors. However, is it true?

A study published by Eric Jonas and Konrad Paul Kording applied the modern neuroscientific approaches for understanding a microprocessor: MOS6502 in running 3 video games for simulating the process of brain research with up-to-date tools. The results ironically illustrated that even if they are capable of gaining comprehensive knowledge at the level of connections (connectome) and all the type of transistors (neurons); breaking the single transistors (neurons) to test if the behavior (if the processor can still boot the game) is altered; detecting every activity of single transistor (single-neuron activity) or larger-scale activity (local field potentials) from a region of the processor, it is difficult to understand how the artificially organized system functions to generate behavior (Jonas and Kording, 2017). Furthermore, MOS6502 is a much simpler computational unit than the simplest brain of a living organism. From the study, it is easy to notice the insufficiency of the approaches to understand the function of a brain. It is critically hard to interpret the causality of behaviors of the whole packaged system from the lower-level properties (Badre et al., 2015; Carandini,

2012; Cooper and Peebles, 2015; Gomez-Marin et al., 2014). However, among all these modern analyses in Jonas and Kording's paper, the lack of detailed behavioral analysis from the hypothesis-driven perspective could be the missing piece of the puzzle to link the lines of evidence.

The statement that the reductionist bias in neuroscience can be corrected by the understanding of behavior is advocated with the argument which elucidated the emerged substitution bias derived from reductionism: what is true for a circuit is not true for the behavior. Great increasing the size of neural data cannot substitute the conceptual framework to explain the behavior per se (Krakauer et al., 2017). The relationship between the neuronal/circuitry level and behavior is similar to the idea held in Anderson, 1972, who described the hierarchy establishment of the extensive research (on contrary to the intensive research which studies for the fundamental laws). The major point argued in the article is that the reductionist hypothesis cannot be the reciprocal meaning of a constructionist, i.e. the behavior outcome follows the fundamental law at the neuronal level but, in turn, it is hard to reconstruct the complex system only by knowing the fundamental rules. A simple analogy could help us to imagine that if we break down a submarine (complex system) into pieces and study hard on it, we could know all the detail of the parts such as engine or propeller, every part to assemble the engine such as screws, the matters to produce the part such as alloy. Nevertheless, it is hard to reconstruct a submarine from the knowledge without its blueprint (Figure 1-2 A). In other words, in a complex system, the scale in the hierarchy restricts the way to understand the phenomena: without the knowledge to know what is a submarine, and what a submarine can do, it is impossible to infer how these materials work together for a system. Here I would like to quote David Marr's method in the level of understanding of complex systems which provides the clear explanation for solving the reductionist bias in neurophysiology (Marr, 1982/2010) - "they were only describing

(cellular activity), not explaining (cognitive function)". He defined the understanding of neurophysiology with 3 levels, computation, algorithm, and implementation (Figure 1-2 B-D). The first level tries to answer the questions such as "what is the goal for computation?" and "what are the characteristics of computation, such as the logic of strategy or the effects of the computation can carry out. The second level explains the algorithm for eliciting the computation, namely, what is the transformation from the input to the output? And the final level aims to understand how the algorithm can be implemented by the physical elements. In Marr's idea, it is hard to infer from the neural hardware what algorithm applied for the emergence of behavior as compared to the opposite direction from the understanding of the computation (Bickle, 2015; Krakauer et al., 2017; Marr, 1982/2010). We can get the point with a good analogy like knowing how to play Shoji (algorithm). Compare to study what materials made each piece of Shoji, it is easier to know the rule by watching people play with it and see how the pieces are moved. The idea is applied as the core of this study. As a result, the study is conducted by addressing the issue that the higher-order cognitive function of the brain from the hypothetical experimental design on animal behaviors.

The advantages of zebrafish in system neuroscience

Zebrafish is a powerful animal model in developmental biology by its short development period, transparency for convenient observation, and amenability for genetic manipulation. Recently, it has been drawing attention to the neuroscience community because of its special traits bringing advantages in brain study. First, a large collection of transgenic lines makes the specific neuron identification possible for the mechanistic study (Baier and Scott, 2009; Kawakami et al., 2010; Scott et al., 2007; Tabor et al., 2019). The zebrafish mutation project at Wellcome Sanger Institute has generated mutations covering 60% of zebrafish protein-encoding genes gives the chance for the loss-of-function approach to link the neural system

to the genetic level (https://zmp.buschlab.org/). Second, the cutting-edge neuroscience tools such as optogenetics, chemogenetics, calcium image are available in zebrafish (Amo et al., 2014; Godoy et al., 2015; Wyart and Del Bene, 2011). Third, the brain size of zebrafish is much smaller than rodents with < 0.5 mm thickness and only 1.5 mm length in larvae; < 2 mm thickness and 4.5 mm length in adults (Wullimann et al., 1996). The number of neurons is only on the order of 10^5 in larvae and 10^7 in adults (Friedrich et al., 2013; Hinsch and Zupanc, 2007). Also, the transparency in larvae and the pigment mutation lines for adult fish benefit the image observation both in the neuronal recording by calcium imagine and in circuitry identification (White et al., 2008). Last but not least, pioneer studies in behavior has revealed that zebrafish display complex behaviors including association learning, kin recognition, territory behavior, social hierarchy, and spatial navigation (Agetsuma et al., 2010; Amo et al., 2014; Chou et al., 2016a; Gerlach et al., 2008; Karnik and Gerlai, 2012; Nakajo et al., 2020, 2020; Prober et al., 2006; Saverino and Gerlai, 2008). Following the discussion in the previous paragraphs, understanding the function of the brain needs a selection of the proper animal model following the reductionist idea and with the reference to evolution, assuming that evolution is the driving force to shape the optimal solution to adapt to the environments. With a similar goal, the evolved function for achieving the goal converges with similarity, for example, fish swim in the aquatic environment, it needs fin to get fast movement. The functions here include the morphological traits and skillsets to adapt to the environment such as fin and swimming bladder for morphological adaptation in the aquatic environment and the control of fin flipping and inflate the swimming bladder as the skill sets. For the morphology, we could see the molecular evidence, morphogen, is often conserved across the species, for instance, fibroblast growth factor (Fgf) is well-known to regulate the development of limbs across different species of tetrapod (Cooper et al., 2012; Purushothaman et al., 2019; Xu et al., 1999), but it has also been found in wing development

of avian (Montero et al., 2001) as well as the fin development in teleost (Shibata et al., 2016). On the other hand, the examples of neural functional regulations can also be found in many studies, e.g. the vocal signal is controlled through hindbrain rhombomere 8-spinal compartment that is generally conserved among bird, amphibian, primate and, interestingly, fish, although the ways to generate the voice are different, birds and mammals control the vocal through the control of respiratory activity, whereas fish control its pectoral fin to generate sounds (Bass and Chagnaud, 2012). The emergent of neural function shared the original element to achieve the same goal in the course of evolution. In both morphology and behavior, we can see the evolutionary evidence across the species which provides the strong inference that reductionist thinking can be applied. As a result, zebrafish can serve as a powerful tool in neuroscience not only by taking the advantage of methodology but most importantly as the simplest model to infer the principle design for the emergence of complex behavior.

Zebrafish could be a breakthrough in higher-order cognitive brain function study with a comprehensive view across brain nuclei

From the 1900s, the higher-order cognitive function has been defined and tested from many different aspects from clinical examination to animal models. It took a long time till people realized that the higher-cognitive function is executed by the frontal lobe. As a result, amounts of studies from primates and humans highly focused on the subregion of the area to scrutinize the subcomponents of it. However, with the help of advanced research tools, the increasing number of works have recently revealed that the higher-order brain function is actually orchestrated by many other brain areas rather than a frontal lobe alone.

One of the recent studies used head-fixed mice in virtual reality (VR) for the two-choice rule-shift task, which required the mice to select the discriminated size or pattern of visual

stimuli by rolling a ball (Biró et al., 2019). They found that the mice learned the task under VR condition and mice tended to reduce the reaction when the selection contains conflict information. By using optogenetics, they inhibited the medial prefrontal cortex (mPFC) at the moment when the mouse was rewarded by choosing the correct answer and showed that the learning rate was reduced only when the rule was switched. By analyzing the type of error, it was indicated that the mice created more errors in updating the value rather than inhibiting the old rule, showing the involvement of mPFC in behavioral flexibility (Biró et al., 2019). Additionally, recording from the mPFC in plus-maze with rule-shift task (spatial and cued rule) showed that the mPFC encodes the spatial position in the maze and replayed the trajectory activity in the awake immobility period (Kaefer et al., 2020). Interestingly, they found that the replay rate is positively correlated with the rule-shift performance, indicating the role of the mPFC spatial information in flexible behavior. From the other study, rats were trained for go/nogo task with reversal-learning task under head-fixed condition. The neural activity recording at somatosensory (S1) and orbitofrontal cortex (OFC) demonstrated that the S1 responded to the onset of trial whereas the neurons in lateral OFC were activated with saliency and rule-switch (Banerjee et al., 2020). Chemogenetic silencing the lateral OFC significantly impaired the reversal learning. Also, the same group identified the projection from the OFC to the S1 conveyed the neural signal with expectation error, which regulates the rule update by remapping the subpopulation of neurons in sensory cortex (Banerjee et al., 2020).

In addition to the mPFC and the OFC, the thalamus also plays a pivotal role in higher-order cognitive function. Mice were assigned to get rewards in the dual-rule-switch task by selecting either the auditory (rule 1) or visual (rule 2) stimulus. Behaving animal with the EEG recording combined with optogenetic inhibition specifically in the mPFC-mediodorsal thalamus (MD) projection impaired rule-switch learning with high perseverative error (the

error emerged by applying historical rule) but maintained the sensory response intact (Marton et al., 2018). The neural activity in MD was also found in representing the sensory information. It was suggested by the perturbation results that it not only stabilized the relevant sensory information but also suppressed the irrelevant sensory information in PFC. By these processes, the MD neurons regulate behavior flexibility (Rikhye et al., 2018).

Furthermore, the dorsal striatum has been reported to be essential in strategy shifting by switching the egocentric or cued-dependent strategy. Specifically, by examining the mice with inactivated NMDA receptor, they found the mice did not show any deficit on initial learning or reversal learning, however, impaired strategy shift (Darvas and Palmiter, 2015). A comparison study by the cholinergic interneuron lesion in the dorsomedial (dmStr) or ventral striatum (vStr) further investigated the involvement of the striatum in behavioral flexibility in detail. They demonstrated that the cholinergic interneurons in the dmStr and the vStr were involved in a different type of sensory switch. The dmStr cholinergic interneuron is essential for the flexible behavior with previous irrelevant cue whereas the vStr cholinergic interneuron is specifically related to the shift to novel cue (Aoki et al., 2015b). Another study used pharmaceutical perturbation to silence the dmStr by activating the GABAergic interneurons. They reported that the general inhibition in the dmStr impaired the ability to shift to the new rule by the increasing regression errors, which is the error calculated for the failure of maintenance of the acquired new rule (Bissonette and Roesch, 2015). Despite the specific function of the dmStr in the control of behavioral flexibility is still controversial, it is the consensus that the dmStr is indispensable in reallocate the different information in new learning. Moreover, very recent research showed that rats with chemogenetic activation in the circuit of locus coeruleus to mPFC improved the performance in the rule-shift paradigm, which might be caused by the facilitation of the disengagement in the previous rule (Cope et al., 2019). In addition to that, the habenula-interpeduncular has recently been reported that

silenced the lateral part of dorsal habenula impaired zebrafish's ability to update the previously valued stimuli in reversal learning (Palumbo et al., 2020). This result suggested the midbrain nucleus is also involved in the flexible behavior.

These results have provided lines of evidence in the involvement of neural correlates in higher-order brain function, specifically related to updating the information and reallocating the attended information. These great works have brought the clue of the explanation of higher-order cognitive function, nonetheless, studying the function by zebrafish may give the advanced chance to integrate the evidence and shed the light on the mechanism explanation with a systematic view.

Significance and Aims

Analyzing behavior by designing a suitable task is the necessary step for understanding the higher-order brain function. Moreover, examining the brain function through zebrafish could give us the best chance to infer the neural design from a reductionist fashion. Nonetheless, rare studies had paid attention to the detailed behavior analysis with the system neuroscience view in zebrafish, especially for higher-order brain function. Therefore, in this study, two aims are employed to fulfill the purpose. Aim 1 employs the dissection of the defined higher-order brain function by the order of difficulties. Aim 2 employs the test of the involved elements for the specific higher-order brain functions.

Specific Aim 1. Construction of the automated system for sophisticate behavior task design

Purpose: A training system to monitor behavior without bias is the key to behavior examination at high precision. As a result, aim 1 essentially constructs the automated system which is high throughput for training without artifact by the experimenter and able to provide positive reinforcement training with flexible task design.

Specific Aim 2. Determination of the capability of higher-order brain function for zebrafish

Purpose: Aim 2 designs and implements the behavior task from low behavior level to higher behavioral level to test what is the limitation of higher-order brain function that the zebrafish can achieve.

Specific Aim 3. Determination of the neural substrate for specific higher-order brain function

Purpose: With the results from aim 2, aim 3 determines the neural substrates in the specific higher-order brain function under the framework of task design from aim 2 with intervention

approaches.

In this study, I examined the different levels of higher-order brain function through multiplex behavioral analysis. Accordingly, I revealed the capacity of cognition level in zebrafish and discussed the basic elements of the neural design and the origin of the brain that evolves to the brain with complex function. With the extended approaches, I identified the neural substrate for exploiting the internal information.

Definition of Terms

Behavior

Tinbergen defined behavior as "the total movements made by the intact animal" (Tinbergen, 1955). With the definition, inspired by Konard Lorenz, in his hierarchical model, the behavior is depicted as the outcome which is driven by the instinct impulse and follows the removing blocks of an internal stimulus with the mechanism as the checkered flag of the selection from the environmental stimulus (Tinbergen, 1955). For example, hunger plays the role of the energy source to provide the driving force as the instinct impulse. Then, a low level of glucose in the blood may act as the internal stimulus to trigger the release of blocks of action. Lastly, the internal releasing mechanism guides the behavior in action, such as the smell or the image of food. Although the idea was mentioned 65-years ago, it is reminiscent of the free energy principle that is advocated recently (Friston, 2009).

The definition of behavior, however, in this study, is defined with a more updated concept: "Behavior is the internally coordinated responses (actions or inactions) of whole living organisms (individuals or groups) to internal and/or external stimuli, excluding responses more easily understood as developmental changes" (Levitis et al., 2009). Notably, the definition generalized the subject for behavior from animal to an organism, including the group's response. Under this context, the total movement is rephrased to "internally coordinated responses (actions or inactions) to internal and/or external stimuli" from Tinbergen's definition. This includes the realms for those invisible movements of the intact organism, says that the behavior should include even the observed stationary state (no-go) for the organism as long as the neuron is activated (internally coordinated response) in response to the stimuli. Also, the stimuli here is generalized, conceptually includes both internal and external stimulus, because the stimulus can be derived from the internal

21

information of the brain. This idea influences the experimental interpretation of this study.

Higher-order brain functions

As mentioned above, the definition of higher-order brain function is not the consensus in the neuroscience community. I apply the operational definition to define it as the brain functions which implement the goal-oriented behavior, emerged by learning in a non-automatic fashion. In other words, it is a set of cognitive functions that are involved in non-instinct behaviors generated by learning and which keep the flexibility in responding to the stimuli.

Delimitations and assumptions of the study

In this research, all sampled fish were bred in the laboratory. Although the experimental zebrafish has been bred in the laboratory for many generations, here I assume that the zebrafish keep their wildtype traits with slow evolutionary speed, the traits not only include the morphological phenotype but also include the instinct behavior. Additionally, it is hard to comprehensively cover all categories of higher-order brain functions. Therefore, in this study, the examined higher-order brain function is limited to the specific categories where concerning the components of executive function that includes the elements of working memory, behavioral flexibility, rule engagement, and attention control.

Organization of the study

Chapter 1 has presented the introduction. Chapter 2 presents the Literature review of the current studies. Chapter 3 presents the methodologies applied in the thesis. Chapter 4 presents the research findings. Chapter 5 presents the conclusion, discussion, and hypothesis for future research.
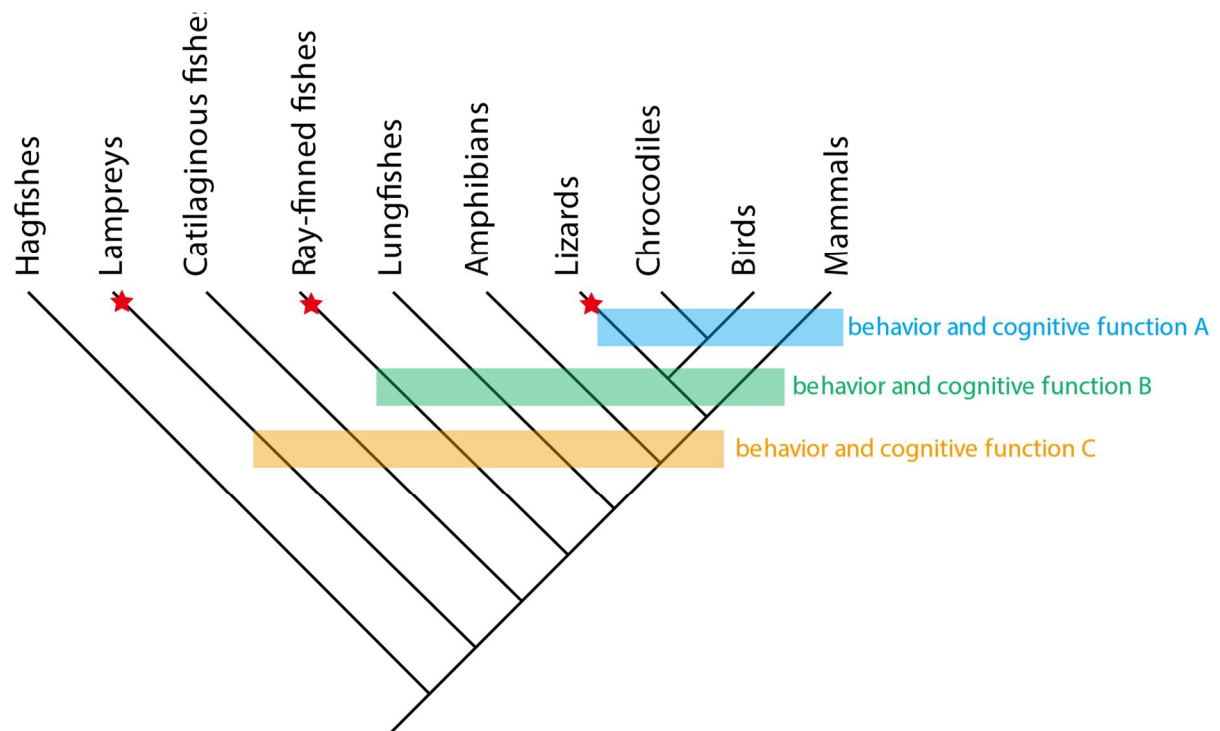
Figure 1-1. The emergence of certain behaviors or cognitive function in the phylogenic tree.

The colored bar represented the 3 imagined examples of behavior/cognitive function A to C conserved among the phylogenetic taxons. Red stars indicate the primordium of the specific function in evolution.
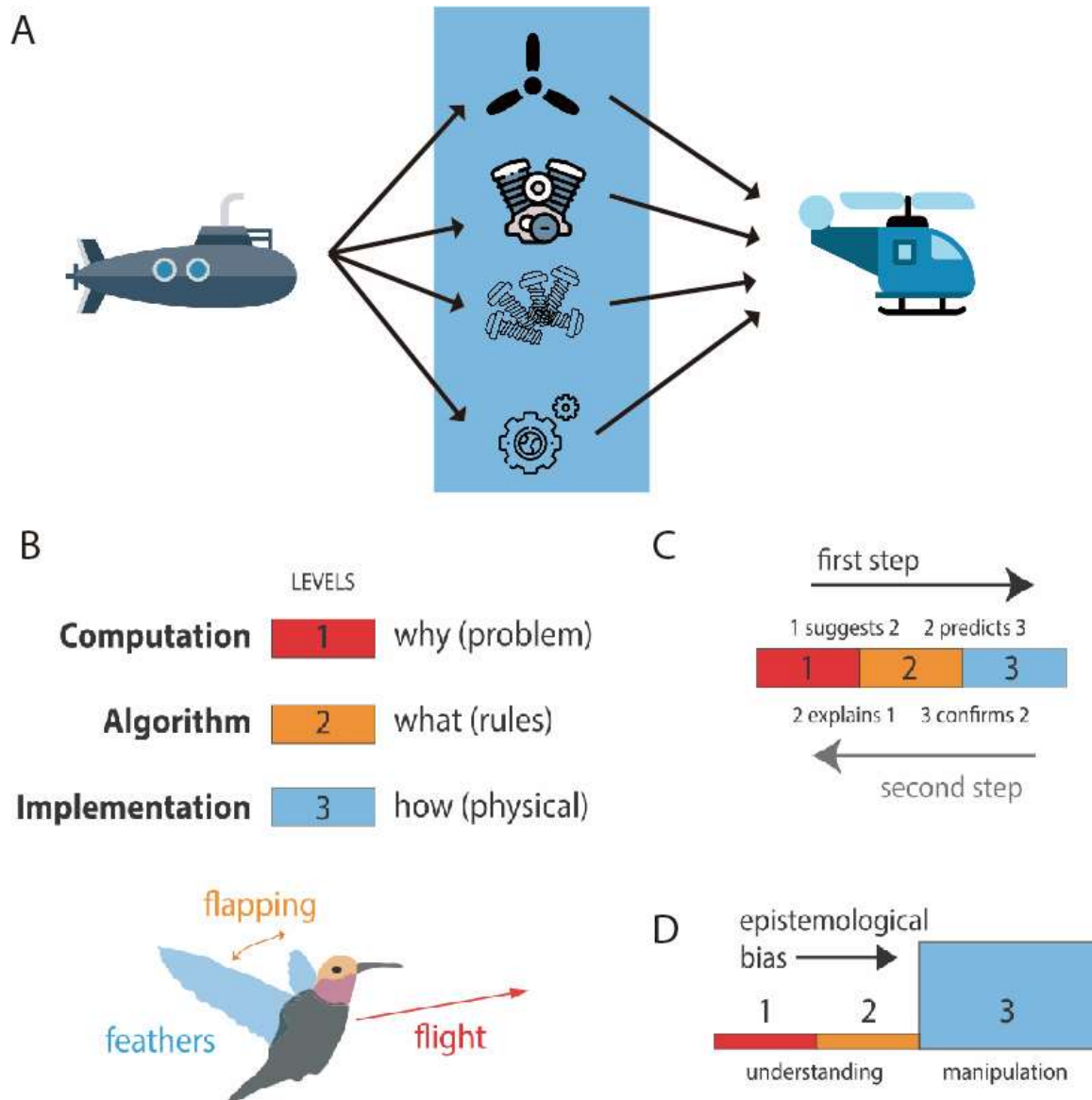
Figure 1-2. (A) The analogy of submarine illustrates the constructionist fallacy. Without knowing the fundamental properties of a system in a systematic view, it is difficult to reverse engineer the system in the correct way. (B-D) Marr's three-level analysis. (B) A bird flies (goal of computation problem) by flapping wings (algorithm) depending on the feature of feathers to work with aerodynamics (physical implementation). The feather contributes to the work of fly but understanding the feature of feathers does not bring the answer to why the bird flies and what makes it fly. (C) The relationship between the three levels is not arbitrary; The first step should come before the second step. First Step: the algorithmic level of

understanding is essential to interpret its mechanistic implementation. Second step: implementation level work feeds back to inform the algorithmic level. (D) The manipulation-based view for understanding induced by technology could cause epistemological bias. (Krakauer et al., 2017).

Chapter 2. Literature review

In this chapter, I will review the literature covering the following topics:

● Higher-order brain function – the executive function

● The neural correlates for executive function

● The animal models for executive function

● Navigation behavior as a tool for identifying the function of the information selection

● The candidate brain circuit serves the function of information selection

Higher-order brain function– the executive function

The executive function is one of the major higher-order brain functions which is depicted when animals perform under the supervisory and regulatory rule in measured and thoughtful processing; guiding behaviors when the organisms encounter the various information. In general, two concepts constitute the definition of executive function: Rule and control. For creating and modifying the rule, the function can be further divided into initiating, inhibiting, shifting, and relating: initiating rules to match the stimuli and behavioral goal, inhibiting rule for neglecting irrelevant stimuli, shifting rules for further adapting to the changes of stimuli-goal coupling, and relating the rules for a more complex strategy (Dale Purves et al. 2013, Chapter 13; Diamond, 2013). In the aspect of control functions, they are often related to the monitoring of the current state of the environment and control the engagement for certain behaviors. It recognizes rule changes to respond to the environmental alternation, and as such, the rule should be updated more frequently in a variable environment than the case in a more stable environment. Except for these categorized functions, working memory is considered as the center to keep the rule till the output of behavior outcome (Figure 2-1).

The executive function has been identified and classified from the human studies in the prefrontal cortex (PFC) damaged patient. PFC damage, however, with little overt impairment, often produces different levels of deficit included in the description of executive function, such as the inflexibility an environmental dependency syndrome or dysexecutive syndrome. Patients with the former syndrome could be triggered by the immediate environmental cue. For instance, when the patients see a pen and a book on a desk, they may start drawing on the book regardless of what the book is used for. On the other hand, patients who suffer from the dysexecutive syndrome fail to plan long-term goals and often quit tasks in the middle (Baddeley and Wilson, 1988). Imaging the executive function in the analogy of a traffic controller may easily get the point of how it presumably works. When the traffic is light, the controller does not need to function with much effort. Cars can follow a simple rule with less control. However, once it becomes heavier, to prevent the crash or the traffic jam, the controller needs to operate with sophisticated algorithms, such as prioritize a certain path and inhibit the others in the specific context. It is worth noting that this operation needs a model to accomplish it but does not exactly need the awareness of the operation. That is, consciousness may not need to be involved in the process. Although the executive function is described as a function for completing a certain behavior task, it is not a unitary function. Multiple subprocesses are integrated and interweaved together (Bellebaum and Daum, 2007). As follows, I review the psychological constructs under the framework of executive function.

Rule engagement

Since the executive function strongly links with rules, initiating the rule is the important factor of it. A rule could be described as the association between the specific information and goals, or values so to speak. By weighting the different strenghts of information-value association, a more explicit model of the world can be created. Again, I take the traffic controller as an

example. If the traffic controller needs to separate the bigger cars and the smaller cars to a different track (rule) for reducing the traffic congestion, the first thing to know is to measure the size or weight of the cars. Thus, the traffic controller selects the particular information (size or weight) for completing the task. However, the size of the car may not lead to the best solution for removing the traffic jam. Assuming the traffic controller is the machine that is capable of receiving the feedback outcome of the traffic situation. It estimates the different conditions to achieve the best outcome by simulated trial-and-error with a model. With many trials by testing the different information-value combination, it is possible to gradually descend the error toward the optimal solution. Therefore, as the scenario depicted, the establishment of a rule is a learning process by the judgment of probabilistic outcomes with discriminative stimuli. It must be noted that the established rules here, with the intact executive function, should maintain the flexibility to the states accordingly, which is the combination of contingency and outcome information as envisioned by Anthony Dickinson (Dickinson and Balleine, 1994). Therefore, the rule used here should be prone to goal-directed behavior rather than habitual behavior under this framework. This is a great concern of experimental design for the task of executive function.

From the observation of humans, patients with lateral prefrontal cortex damage show nearly intact behavior, at least in the superficial examination. However, they show little spontaneity in action with deficits in motivation and inert corresponding emotion (Dale Purves et al. 2013, Chapter 13). It is reported that the neurons, located in the different subregions of the prefrontal cortex, respond to the applied rules in two rules matching/nonmatching -switching task in monkey (Wallis et al., 2001). In contrast, through the fMRI recording in human, the ventral basal ganglion had been suggested to be involved in the rule establishment by mapping the stimulus to a specific response (Cools et al., 2004) (Figure 2-2).

Behavioral flexibility and Attention control

Behavior flexibility is the capability for changing the current attended rule or information to others. With the premise that the brain is engaged in the current state of an established rule, changing the attended new rule/information follows two steps: inhibiting the current rule and shifting to apply/establish the new rule. The former step can be classified with four forms of inhibition. 1) halting behaviors that are well trained or previously valid. 2) preventing irrelevant information from interfering with other processing. 3) restraining actions that are inappropriate in a given social context. And (4) removing irrelevant information from working memory (Dale Purves et al. 2013). Human studies apply the oddball task to test the inhibitory function. In the task, the participant needs to change their corresponding behavior to specific sequential information (e.g., showing the images of sequential squares varying in size and color, participant keeps pressing left bottom when the square is shown, whereas press right bottom when a circle shows up). Studies with the task demonstrated that the event-response potential (ERP) is raised with the well-characterized positive deflection at approximately 300 ms (P300) following the onset of the target stimulus (Polich, 2007). With the fMRI examination, it was shown that the dorsolateral prefrontal cortex and the parietal cortex is activated by the target-changing stimulation (Huettel and McCarthy, 2004). Besides, the dorsolateral portion of the striatum has been identified to be involved in flexibility control. By perturbation of direct pathway medium spiny neurons (dMSN) and indirect pathway medium spiny neurons (iMSN) respectively, the enhanced activity of dMSN facilitates the acquisition of reversal dual-action sequence, whereas activating iMSN reduced the acquisition in the same task (Laurent et al., 2017). Wisconsin Card Sorting Test (WCST) is widely used for testing behavior flexibility that requests subjects to sort cards by one of specific information on the card among distinct information (e.g., shape, color, or number).

Unbeknown to the subject, the rule frequently gets changed under the determined criteria, and the subject can only deduce the rule by trial and error. Subjects normally recognize the rule change and react with the updated information to correct the strategy for card sorting. Nevertheless, patients with the damaged prefrontal cortex and the temporal lobe continue to use the previously valid rule while the error reported in the choice (perseverative error) (Dale Purves et al. 2013, Chapter 13).

On the other hand, before the sensory information is used to form a rule, it may be processed for reducing the input noise to decrease the burden of computation. This process is the so-called attention selection. The current researchers postulated the model of attention control by separating the information selection into the early selection and the late selection based on the sequence of information flow (Figure 2-3A). The hierarchical model is now concluded that the sensory information can be analyzed in early selection (i.e. the sensory properties such as color in visual input) and the late selection serves for the complex analysis (e.g. the meaning of the stimuli). Researchers also postulated that the top-down control from the higher-level associated cortex area shapes the lower-level sensory region to facilitate the process (Figure 2-3 A-B). The neural substrate of attention control was firstly observed in human patients with hemisphere neglect syndrome. These patients with the brain lesion at the right parietal cortex tended to neglect the object on the left side (Grabowecky et al., 1993). Besides, the more detailed behavior observation for these patients revealed that they have no sensory deficit; in particular, when the moving fingers showed on their left or right side, they could report the moving finger without a problem. However, the patients neglected the left moving finger if the fingers showed at both sides simultaneously. From this, it is easy to note that, at least for visual-spatial attention in human, is controlled by the separated hemisphere and seemly asymmetrically regulate the left-right attention (neglect syndrome is prominently observed with a large population in the right parietal cortex damaged patients).

Also, the relative strength of stimuli is a key factor to elicit attention. In addition to the right parietal cortex as the neural correlate for attention control, the dorsal posterior parietal and the lateral occipital cortex have been reported to play a role in attention by examining the patient with Balint's syndrome. On the contrary to the neglect syndrome, patients showed the symptom that failed to extract the complete features of objects rather than spatial correlation. For example, when the patients saw the array of two-color objects, they could only report seeing one of the colors (Robertson and Treisman, 2006).

The regulators of attention mentioned in the above paragraph are mostly found from human studies in the behavioral task with instruction. These kinds of tasks are categorized as the endogenous control of attention, which has focused on voluntary attention control. However, the attention control can be triggered by the exogenous stimuli in the environment per se and it is the major category applied in the research by animal model. It has been reported that the right temporoparietal junction (TPJ) was strongly activated when the cues were popped up in the visual attention task (Corbetta et al., 2000). With the results, the researchers further interpreted that the TPJ areas are involved in responding to the shift of attention toward the new stimuli in the environment. As postulated by Jeremy Wolfe, the feature activation map for visual attention is constructed by the stimulus factors and top-down influence from the higher-level factors and behavioral goals (Wolfe and Utochkin, 2019; Wolfe et al., 2003). The stimulus factors here were suggested to be a set of feature-tuned maps such as color, shape, size, or motion. These factors were compared with the local differences to find the contrast on the local visual field among items to generate the saliency. On the other hand, the top-down activation carries the prior knowledge (value) to guide the establishment of a general activation map that reflects the probability of the target across the searching space. Therefore, with the guidance of the activation map, the features can be searched in order to allocate and relocate the attention (Wolfe and Utochkin, 2019).

Working memory

As described previously, the working memory is the necessary element to complete the executive function by maintaining the rule and relaying the information to behavior. Working memory, defined as the temporarily maintained memory that can be operated for the computation in the brain, keeps the information online for implementing the corresponding behavior. It is distinct from short-term memory that only holds memory without manipulation. The working memory and short-term memory can be distinguished by Simon's task and Hearts and Flowers task in the human study. In the former task, the subject is requested to apply two rules: stimulus 1 press on the right; stimulus 2 press on the left. On the contrary, the latter task requests the subject to respond to stimulus 1 by pressing the same side as the stimulus and press the opposite side for responding to stimulus 2. Simon's task only requires the subject to keep the information in mind whereas the Hearts and Flowers task requires to manipulate the stored information with directional judgment. Evidence showed that the costs associated with the manipulation were greater in young children while the costs associated with the required memory capacity were larger in adults (cost is normally counted by the response time, the higher cost reflects the slower response time) (Davidson et al., 2006; Diamond, 2013). The model of working memory was postulated by Alan Baddeley and Graham Hitch in the 1970s that consists of three capacity-limited memory buffers and one central executive system. The three memory buffers handle the distinct type of information with iterated loops, of which a phonological loop holds the sounds representation, a visuospatial sketchpad hold visual and spatial representation and an episodic buffer hold the integrated information with time. On top of it the central executive system serves the resource allocation of the memory buffer and perform the manipulation (Baddeley, 2003) (Figure 2-4). Each buffer system is able to rehearse the

information by the iterated loop, and also processes the information into long-term storage. On the contrary, Nelson Cowan hypothesized another model that the working memory was postulated as the activated state from the storage pool of long-term memory (Cowan, 1998) (Figure 2-5). Compared with Baddeley's model, Cowan believes that the pool of the memory is unlimited, however, a prompt decay from the activated state causes the temporary representation of the specific information unless the activated state is rehearsed. Also, in his model, the executive control is suggested to be able to hold the different types of information simultaneously. It is easy to infer that the neural substrate for working memory is separated from long-term memory in Baddeley's model whereas they are intermingled in Cowan's model. Consequently, Baddeley's model also divides the function of store and rehearsal in different regions. Instead, Cowan's model proposes that they can happen in the same region. Tasks with a retention interval inserted between the representation of objects are used to test the working memory and search for the neural correlates. Researchers recorded the neurons in the dorsolateral prefrontal cortex (DLPFC) of monkey, and showed that the neurons were activated during the retention interval but not for the rest of time (Funahashi et al., 1989). The fMRI study in human showed that the activity of the DLPFC is higher when the subject manipulated the working memory rather than just maintained it (Postle et al., 2000). Recently, the DLPFC has also been reported to be involved in long-term memory (Blumenfeld and Ranganath, 2006). Moreover, the thalamic mediolateral nucleus has been found to be participating in working memory (Watanabe and Funahashi, 2012). The lines of evidence now appear to favor Cowan's model that the working memory could be the activated state from the long-term memory pool.

The neural correlates of executive function

In this section, I shortly summarize the current finding of neural correlates for executive functions. As briefly mentioned previously, observation from prefrontal cortex (PFC) damaged patients and the lesion studies, revealed that the PFC is essential for intact executive function (Barbey et al., 2012). Patients with the damaged PFC is diagnosed with the dysexecutive syndrome (Baddeley and Wilson, 1988). In particular, the damage of the orbitofrontal cortex (OFC) can impair the learning, correction of stimulus-reinforcement association, and motivated behavior (al-Adawi et al., 1998; Rolls, 2013), the dorsolateral prefrontal cortex (DLPFC) lesion may cause impairment in goal-directed behavior (al-Adawi et al., 1998), cognitive control (Larson et al., 2006), inhibition (Picton et al., 2007), and working memory (Cristofori et al., 2019; Reid-Arndt et al., 2007). Neural recording in primates also confirmed the involvement of the PFC within different levels of executive function (Blumenfeld and Ranganath, 2006; Cools, 2004; Funahashi et al., 1989; Huettel and McCarthy, 2004; Postle et al., 2000; Wallis et al., 2001) (Figure 2-6). The PFC may act as the role in the "top-down" process that maps between sensory input, thoughts, and action. Compared with the "bottom-up" process, the top-down process flexibly arranges the association for the non-automatic behavior. As a result, several models have been postulated to explain the involvement of the PFC in executive function. Dehaene and Changeux hypothesized the two levels network model in 1989, they proposed the linkage of decomposed features (input) and output are established at the first level by a 'winner-take-all' network. In addition, the model included the executive layer for rule coding (level 2) that controls the flow of information between input and output (Figure 2-7 A). On the other hand, Arthur Shimamura lately proposed the dynamic filter model that posed the PFC as the function for filtering out the specific information by the reciprocal interaction with the posterior associate cortex

(Shimamura, 2000) (Figure 2-7 B). Moreover, a modified model was suggested by Miller and Cohen that the PFC was proposed to maintain and complete the whole ensemble of network activation of the associate cortex by forming the rule map (Figure 2-7 C) (Miller and Cohen, 2001) (Miller and Wallis, 2009).

In addition to the PFC, the executive function has been suggested to link to other brain areas (Andrés, 2003; Collette et al., 2006), for instance, the attention control is well-known to be associated with the right parietal cortex and the temporoparietal junction (Corbetta et al., 2000; Grabowecky et al., 1993; Robertson and Treisman, 2006). The PFC strongly connected regions, such as the hippocampus and amygdala were revealed to be engaged in the executive function for motivated behavior and working memory (Barbas and Zikopoulos, 2007). Furthermore, the impairment in the PFC-striatal loop showed the deficits in information inhibition, multitasking, and high perseverative error by examining the behavior of the patient with Parkinson's disease (Heyder et al., 2004). Besides the networks of the pallium and the basal ganglia, some distal regions to the cortex have also been suggested to be engaged in executive function. For instance, the results from the damaged cerebellum and neuroimaging demonstrated that the cerebellum was involved in related to planning, rule shifting, abstract reasoning, working memory, and verbal fluency (Bellebaum and Daum, 2007). The deficits were strongly observed in the acute lesion but attenuated with time after the lesion. This phenomenon was mentioned in the context of the cortico-cerebellar interaction in information processing that the cortico-cerebellar loop is closely intertwined to many brain nuclei and caused different cognitive deficits. Although the evidence from the cerebellum lesion studies was controversial in terms of planning, inhibition, and abstract reasoning, the cerebellar involvement in working memory was constant among studies (Bellebaum and Daum, 2007). It was concluded that the cerebellum might contribute to both rehearsal and storage of working memory (Bellebaum and Daum, 2007).

Although lines of evidence have shown the distinct aspects of the neural substrate in the subcomponents of executive function, the information is still fragmental and hard to be integrated to conclude a comprehensive understanding. The complexity of the function puzzles the investigation of the mechanism. Therefore, instead of human study under the low-resolution approaches, additional approaches with different angles of thinking is needed to shed the light on it.

The animal models for executive function

The executive function is defined and mostly identified by the human study. However, with the advantage of reproductivity and experimental controllability, animal models have been widely used to dissect the components of executive function and contributed to the understanding of the mechanism. In this section, I review the studies in animals with the framework of executive function defined by the human study.

In the late 1860s, Eduard Hitzig and Gustav Fritsch conducted the first study of the frontal lobes in dogs, revealing that the dog with the damaged posterior frontal lobe led to a lack of voluntary control, nevertheless, the reflexive response was held. Consistently, the stimulation on the posterior frontal lobe led to the limb movements on the opposite side of the stimulated lobe. In contrast, the lesion of the anterior frontal lobe did not show any deficit in movement nor loss of the apparent sensation. Moreover, stimulating the same area did not generate any obvious muscle movement. This result gave rise to the very original hypothesis that the anterior frontal lobe is associated with the higher-order brain function rather than the control of body movement. With the lesion of the subregion on the prefrontal cortex, David Ferrier and Leonardo Bianchi experimented with monkey, showing the damage of the areas caused the failure to recognize the learned object, to guide behavior from

experience, to show affective responses, and to perform coherent behavior (Dale Purves et al. 2013, Chapter 13). Lately, the subcomponents of the executive function have been separately examined in animal models.

The attention task has been adopted in monkey and revealed that the PFC lesion caused the similar deficit to the neglect syndrome in human. Nevertheless, the controversial evidence showed that the unilateral lesion of the dorsolateral or the ventrolateral PFC does not disrupt their attention orientation, instead, it sabotaged the flexibility in the relocation of attention (Rossi et al., 2009). In rodent's five-choice reaction time task (5-choice task), in which the animal was assigned to react with the corresponding location of visual stimulus by nose-poking, rats with a lesion of the mPFC showed declined accuracy with high perseverative error in this task. In particular, the distinct subregion of mPFC: the prelimbic cortex (PrL), the anterior cingulate (area Cg1), and the ventral infralimbic (IL) cortex are associated with the deficits in increased perseverative responses, response accuracy, and the inclined number of premature responses respectively (Chudasama et al., 2003). Furthermore, the pharmaceutical approaches by local infusion of dopamine D1 receptor agonist in mPFC revealed the biphasic effects on attention control (low dosage improves the performance but impairs it with high dosage). Moreover, the manipulation of the serotonin (5-HT) and acetylcholine(ACh) showed the influence on the 5-choice task with the increased attention selectivity by activating serotonin receptor 1A and 1B and treating with low dosage ACh (McGaughy et al., 2002; Meltzer et al., 2003).

Unlike humans typically use the Wisconsin card sorting test (WCST) for detecting the rule engagement and flexibility of the rule and attention shift, studies in monkey used the set-shifting task to detect these functions. The patterns of visual stimuli such as the superimposed stimulus with line and shape are used in this task. The rule is shifted in the task and can be separated into two types: the intra-dimensional shift and the extra-dimensional

shift that requires the animal to shift attention between the different stimuli within the same stimulus dimension (e.g. the vertical line or horizontal line) or to shift between the stimulus dimensions (e.g. the shape and line). The lesion in the lateral PFC in marmosets showed the selective deficit in extra-dimensional shifts whereas the lesion in orbital PFC demonstrated the additional perseverative error in intra-dimensional shift (Roberts et al., 1988). Moreover, results from the dopamine and the norepinephrine global lesion in the medial, the dorsolateral and the orbital prefrontal region also caused the deficit in the extra-dimensional shift. On the contrary, 5-HT depletion in the PFC specifically produced the perseverative deficit in the reversal task. The same task has been brought into rat study by changing the stimulus dimension from the different property of vision into the stimulus dimension in textures and odors (Ng et al., 2007). The experimental results generally supported the same observation in the monkey that the mPFC lesion impaired the ability to shift attentional set whereas the orbital PFC lesion caused the increased perseverative error in reversal trial.

Finally, the working memory task has been adopted in rats, monkeys, and chimpanzees. Damaging the PFC in monkeys and chimpanzees showed the deficits in spatial delayed response that typically requests the animal to find the reward where the location had been presented for a short period before the requested search. The advanced study with more specific lesion demonstrated that the dorsolateral PFC ablation by toxic excitation or cooling, impaired the intact function in delayed response task (Bauer and Fuster, 1976; Goldman-Rakic, 2011; Jacobsen, 1935). Although these studies did not strictly match the specific definition of working memory in the human study, it has generally concluded that the PFC, in non-human primates, serves the function of storing internal representation and bringing it online to guide future behavior. On the other hand, a lesion in the medial prefrontal cortex (mPFC) showed the impairment of working memory in alternative T-maze and foraging tasks in rodents. In the former task that the rodent was rewarded by entering the different arm

comparing the searched location in the previous trial in sequence whereas in the latter task, many arms were baited and the animal needed to keep track of the arms to visit the unbaited arm for gaining the reward. Specifically, the lesion of the prelimbic cortex (PrL) but not the anterior cingulate (area Cg1) (the mPFC in rodent comprises the PrL, area Cg1, and the genu of the corpus callosum), impaired the working memory in delayed response task (Chudasama, 2011). Furthermore, regarding the sensitive response of catecholamine in humans, pharmaceutical manipulation of the DLPFC in monkeys, as well as the mPFC in rodents, had been applied for further proof for the engagement of the brain regions in working memory. Results demonstrated that the dopamine depletion affected the working memory performance in delayed response task (Chudasama, 2011). Interestingly, the dopamine manipulation in the PFC only selectively affected the spatial working memory rather than the working memory for self-ordered searching, that serves for the memory of operational sequence established with consistent order (Floresco et al., 2006). This result implies that the various type of working memory may be regulated by a more specific circuit.

Based on the evidence from the animal model, the more specific function among the different sub-regions in the PFC has been revealed. The neuropharmacological experiments combining with the specific behavioral task brought a clearer insight into the players in terms of neurotransmitters in executive function. However, these studies mostly addressed the question in primates and rodents. Although the complex function can be achieved in these animals to mimic the human study, the complexity of the brain and the size of it provide the extra difficulties to disentangle the brain computation with clear explanation.

The executive function in zebrafish

Zebrafish has been gradually applied in neuroscience study not only due to its advantage as a great tool in amenability of genetic manipulation, transparency, and small size for wide-

field observation but more importantly because the more and more results suggested the homology of zebrafish to mammal both in brain anatomy and functions (Agetsuma et al., 2010; Aizawa et al., 2011; Amo et al., 2010, 2014; Aoki et al., 2015a, 2013; Bloch et al., 2020; Ganz et al., 2015; Geng and Peterson, 2019; Kozol et al., 2016; Mueller, 2012; Okamoto, 2014).

Results from the recent behavior task implied the possibility that zebrafish may hold the ability to implement, at least part of, the executive function. Reinforcement learning by the association of food reward and colored visual cue showed that zebrafish was capable of discriminating the color and could keep the behavioral flexibility to apply the intra-dimensional shifts among different colors (Agetsuma et al., 2010; Aoki et al., 2013; Avdesh et al., 2012; Colwill et al., 2005; Levin and Chen, 2004; Parker et al., 2013). Although these studies lacked the injection of the well-designed distractor as control, they implied that the zebrafish conduct the attention selection with a specific feature. Furthermore, a three-choice version of 5-choice task was conducted with zebrafish and demonstrated that the zebrafish successfully achieved the goal by attending the salient cue with presumably sustained attention (Parker et al., 2012). Interestingly, low dose amphetamine reduced the anticipatory response in the task as similar to the results shown in other animal models and humans as the reduction of impulsive control (Parker et al., 2012, 2013). The go/no-go task by active avoidance training also indicated that the zebrafish can inhibit certain behavior with associated information after reinforcement training (Agetsuma et al., 2010; Aoki et al., 2013). These studies provided the behavioral observation that zebrafish may be capable of using the higher-order brain function to guide behavior. However, none of them exactly conduct the specific examination of the subcomponents in executive function. Therefore, the specific design of the task in zebrafish for investigating the executive function becomes extremely critical for funding the basic knowledge in the future study.

Navigation behavior as a tool for identifying the function of the information selection

It is crucial for animals to attend to appropriate information for survival (Dall et al., 2005). However, identifying the underlying cognitive processes of information selection and utilization is difficult, especially in behaving animals under variable circumstances. Navigation behavior, such as foraging, is excellent to study how animals are engaged in attending to specific information to achieve favorable outcomes. The choice to use different navigation strategies, *i.e.* whether to depend on landmarks or dead-reckoning, allows to conceptually divide information in terms of differences of the source (McNaughton et al., 1991). In landmark navigation, animals perceive the external cues through primary sensory modalities, such as olfaction, hearing, and vision. In contrast, dead-reckoning relies on an integrated internal signal such as vestibular and motor command signaling (Taube and Burton, 1995). The distinct disposition to utilize information in spatial learning has been revealed in behavioral studies, whereby rats tend to use environmental cues (allocentric) more preferentially than integrated self-direction (egocentric) and mice use co-existing navigation strategies (Packard and McGaugh, 1996; Rondi-Reig et al., 2006; Tolman, 1949; Tolman et al., 1947). Nonetheless, the neural substrates for adopting received information into a decision-making strategy remain unknown. Information selection is one of the critical elements in rule engagement and attention control in executive function. By specifically dissecting the navigation strategy, I will focus on investigating the neural substrate of the specific information selection in this study.

The candidate brain circuit serves the function of information selection

The habenula-IPN circuits as a candidate neural substrate for internal/external information selection

The habenula is the nucleus located in the diencephalon, bridging neural signals between the limbic forebrain and the hindbrain monoaminergic nuclei (Sutherland, 1982). The habenula of zebrafish can be separated into the dorsal and the ventral habenula (dHb and vHb)(Amo et al., 2010). The dorsal habenula can be further subdivided into the medial (dHbM) and lateral (dHbL) subregions (Aizawa et al., 2005a; Doll et al., 2011)(Figure 2-8A-C). The efferents of the dorsal habenula almost exclusively project to the interpeduncular nucleus (IPN) through the fasciculus retroflexus (Aizawa et al., 2005a; Bianco et al., 2008; Gamse et al., 2005a). We have previously shown that in zebrafish the dHbL and the dHbM respectively innervate the dorsal/intermediate and the intermediate/ventral IPN (d/iIPN and i/vIPN respectively). The dHbL-d/iIPN neurons project to the dorsal tegmental area (DTA), whereas the i/vIPN neurons project to the median raphe (MR) (Figure 2-8B and C) (Agetsuma et al., 2010; Aizawa et al., 2005a; Bianco et al., 2008; Chou et al., 2016a; Tomizawa et al., 2001). The dHb responds to multiple sensory stimuli such as olfactory and visual stimuli (Dreosti et al., 2014a; Jetti et al., 2014a; Krishnan et al., 2014; Miyasaka et al., 2009a; Zhang, 1996). The IPN is implicated in the regulation of fear behavior and social conflict (Agetsuma et al., 2010; Chou et al., 2016a; Duboué et al., 2017; Mathuru and Jesuthasan, 2013; Nakajo et al., 2020). The neural activity in the IPN has been linked to head direction in rats (Clark and Taube, 2009). Accordingly, the Hb-IPN circuit is a strong candidate for the selection of external information (environmental sensation) and internal information (self-directed information, e.g. egocentric direction information). In this study, I hypothesized that the habenula-interpeduncular nucleus is involved in the attended information selection, playing the

fundamental rule for higher-order brain function.

In this study, I hypothesized that the higher-order brain function is conserved in zebrafish. Specifically, I hypothesized that the zebrafish is capable of updating the attended information with flexibility, holding working memory online for guiding the correct behavior and using rules to facilitate the selected information. With the assumption, I further hypothesized that except the telencephalon, the habenula-interpeduncular nucleus is involved in the attended information selection, playing the fundamental rule for higher-order brain function.
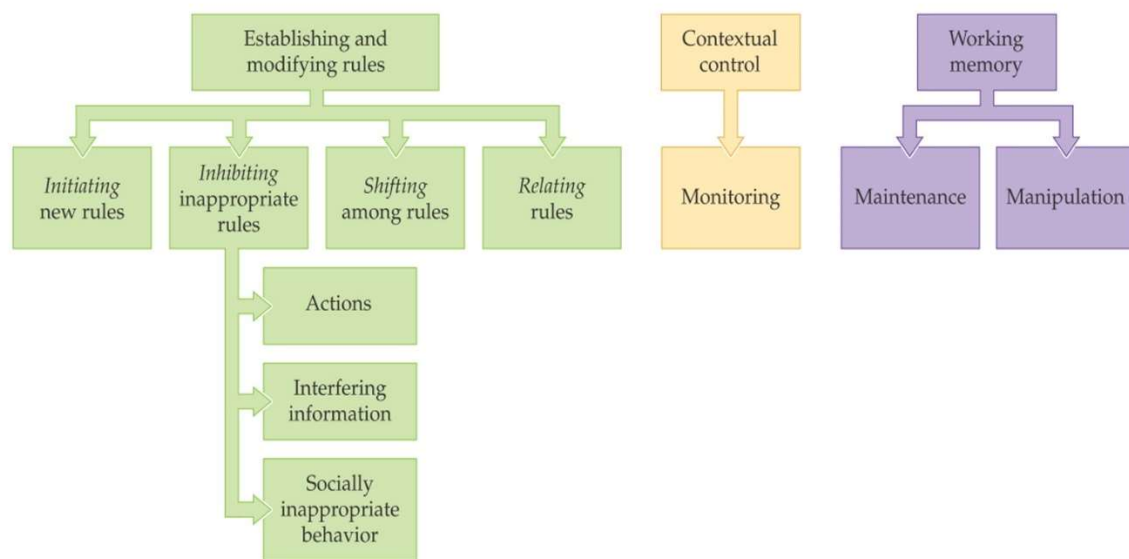
Figure 2-1. A taxonomy of executive function. (From Dale Purves et al. 2013)

A
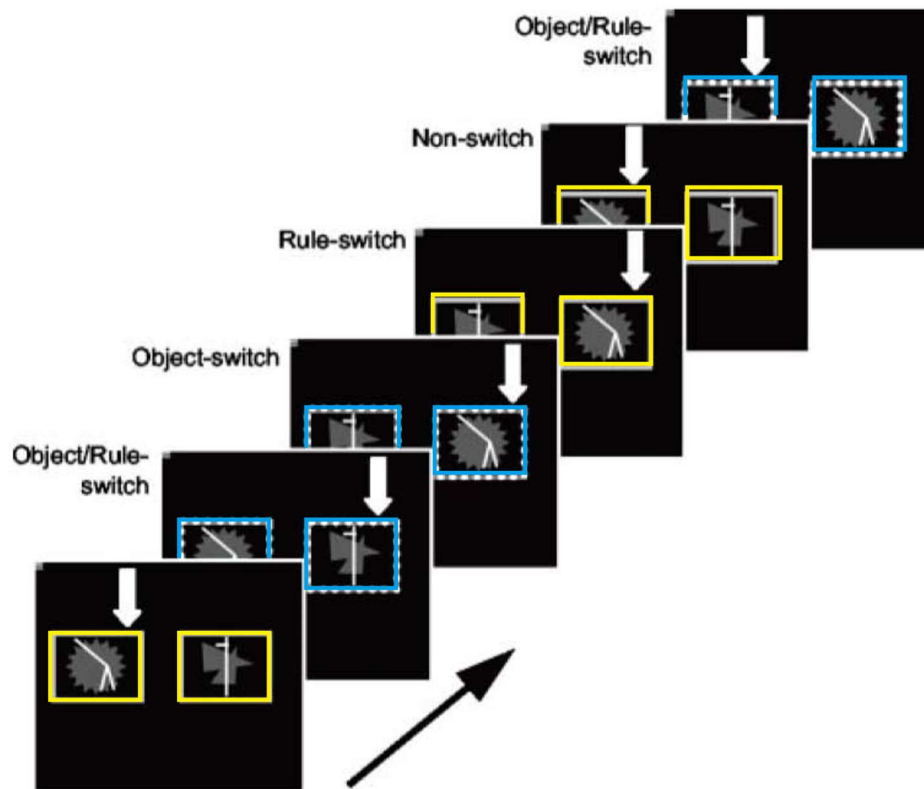
B
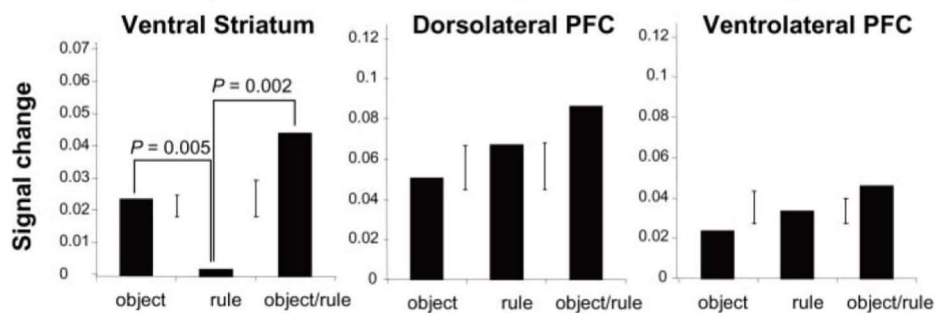
Figure 2-2. (A) Example of the task. The task allows the separated investigation in object shift and rule shift. The white arrow indicates the correct selection. The yellow outlined box cues the subject to choose the same object as the previous trial. The blue outlined box cues the subject to switch to the other object (rule shift). (B) The ventral striatum showed the specific response in object switch rather than rule switch. On the contrary, the dorsolateral PFC responded to all kinds of switches. (Modified from Cools et al., 2004)

A

Top-down control

Later selection

Earlier selection

Executive functions

High-level analysis

Sensory and perceptual analysis

Sensory inputs

Response

B

Attentional control network

Bottom-up stimulus processing

Generic sensory input
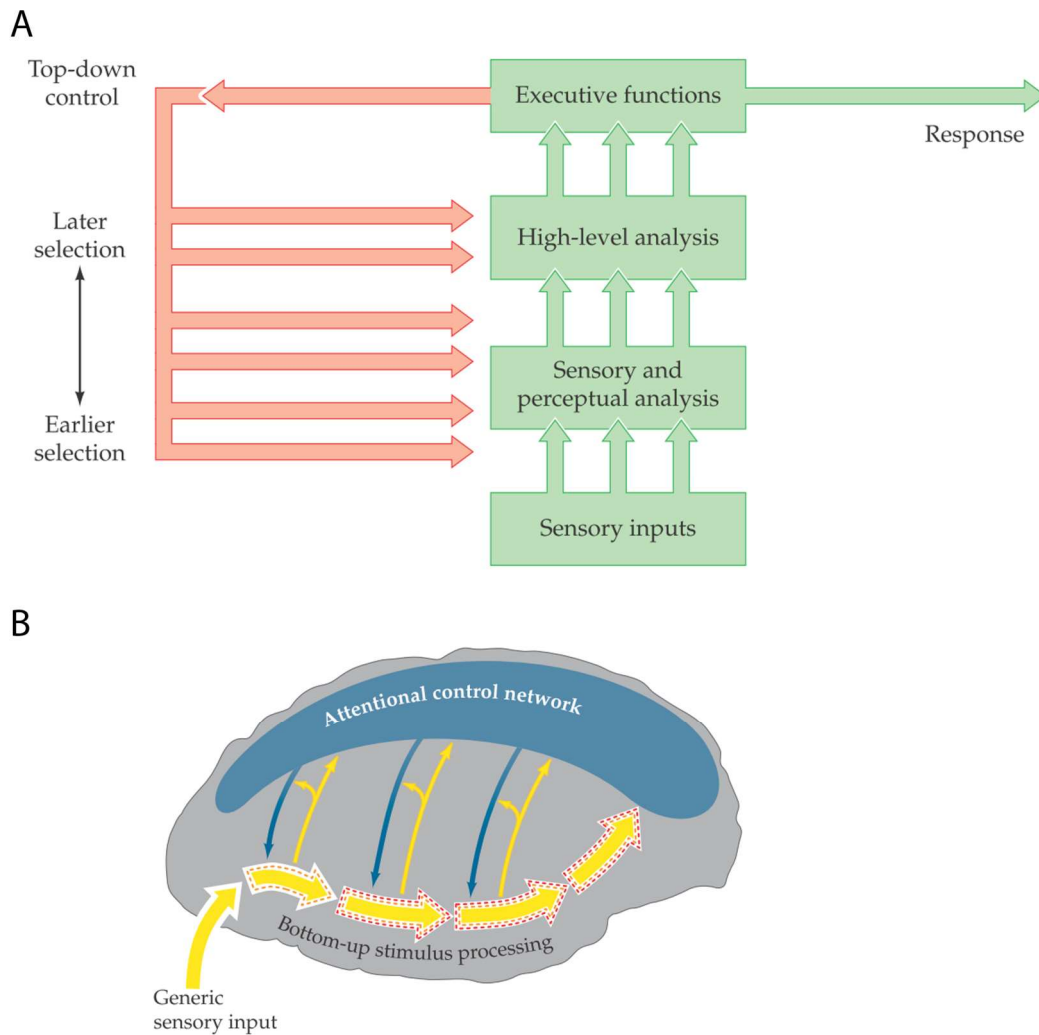
Figure 2-3. Model of information processing in selective attention. (A) Extended model from the attention filter model (Broadbent, 1985, Treisman, 1960). The current model incorporates the top-down control guided by the executive functions. The model proposes the bottom-up sensory input undergoes the earlier selection to later selection. Following the increased level, the attended information is selected to fulfill the demand in the environmental context. (B) The scheme represents the systematic idea that the brain regions are involved in the stream of attention process. The thick yellow arrow indicates the generic sensory input goes along with the bottom-up stimulus processing with the different levels of selection (dotted outline). The blue region indicates the top-down attention control network which interacts with the

bottom-up process at different levels. (From Dale Purves et al. 2013)
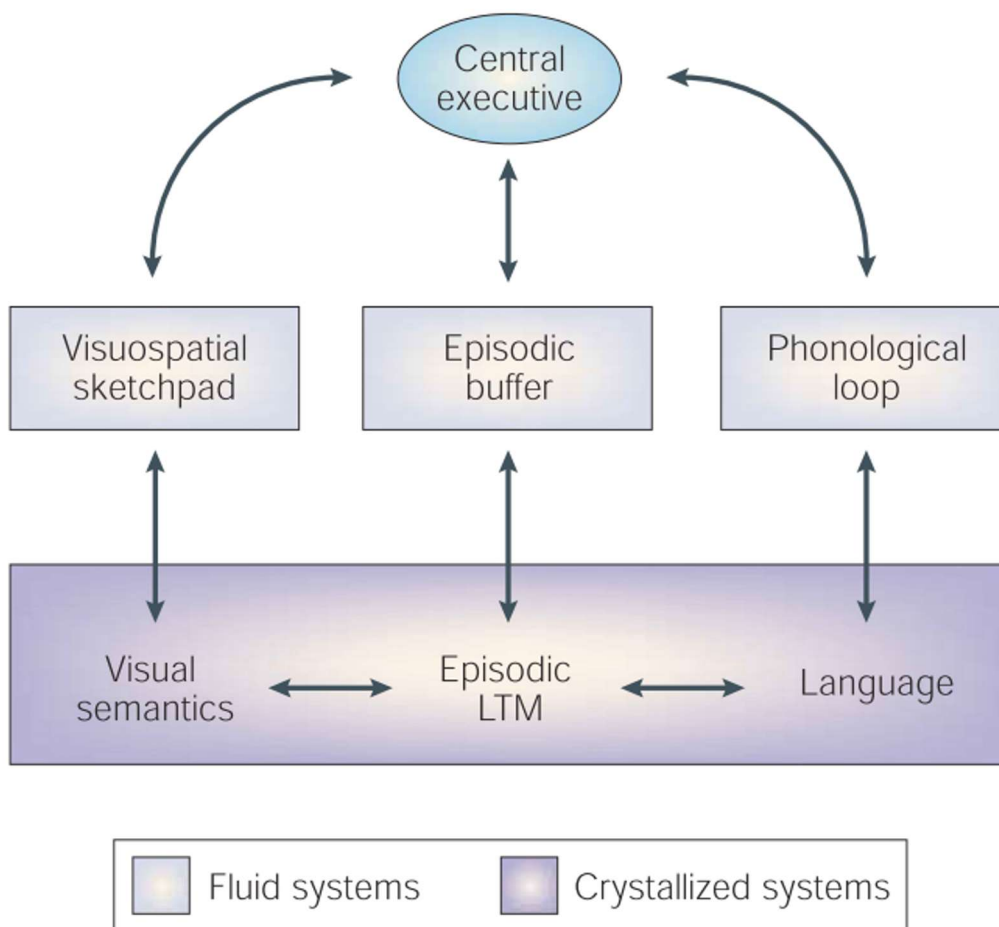
Figure 2-4. Baddeley and Hitch's working memory model. The dark purple region on the bottom of the scheme represents long-term or crystallized knowledge. The episodic buffer provides an interface between the sub-systems of working memory and long-term memory. (From Baddeley, 2003)
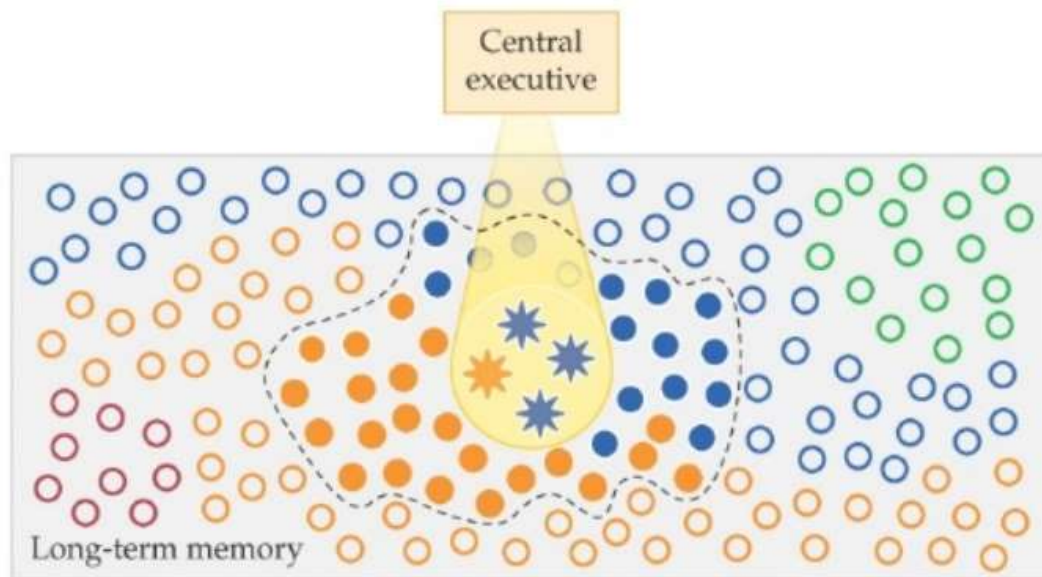
Figure 2-5. Cowan's working memory model. The working memory is proposed as the activated state from long-term memory storage (filled circle). The central executive pulls the subset of working memory into attention (stars). The different color in the long-term memory storage represents the different type of memory. (From Dale Purves et al. 2013)
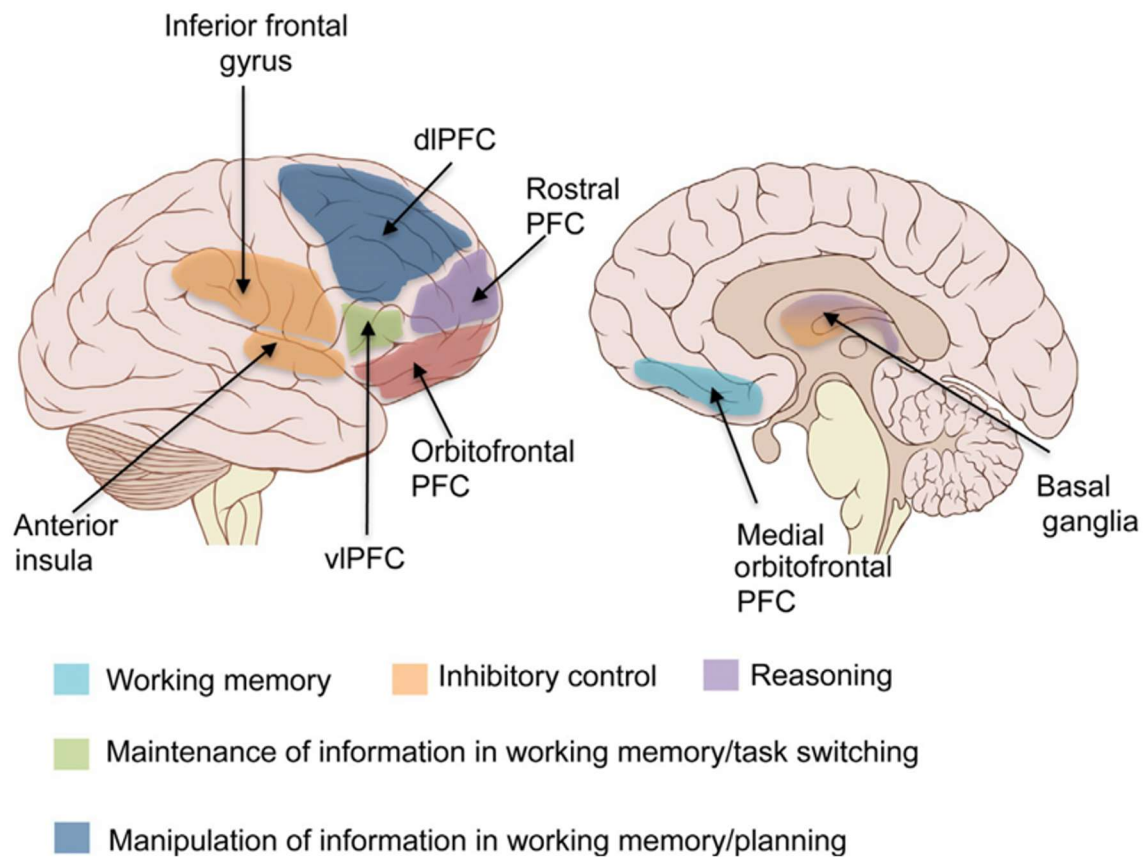
Figure 2-6. Summary of neural correlates of the subcomponents of executive function based on lesion and neuroimage studies. (From Cristofori et al., 2019)

A

Reinforcement

Level 2

Rule-coding units   Memory units

Objects

Input layer

Output layer

Actions

Level 1

B

Dynamic filtering theory

Prefrontal cortex

Prefrontal cortex

Posterior assn cortex

Posterior assn cortex

Time 1 ⟶ Time 2

C

PFC

C2

C3

C1

R1

R2
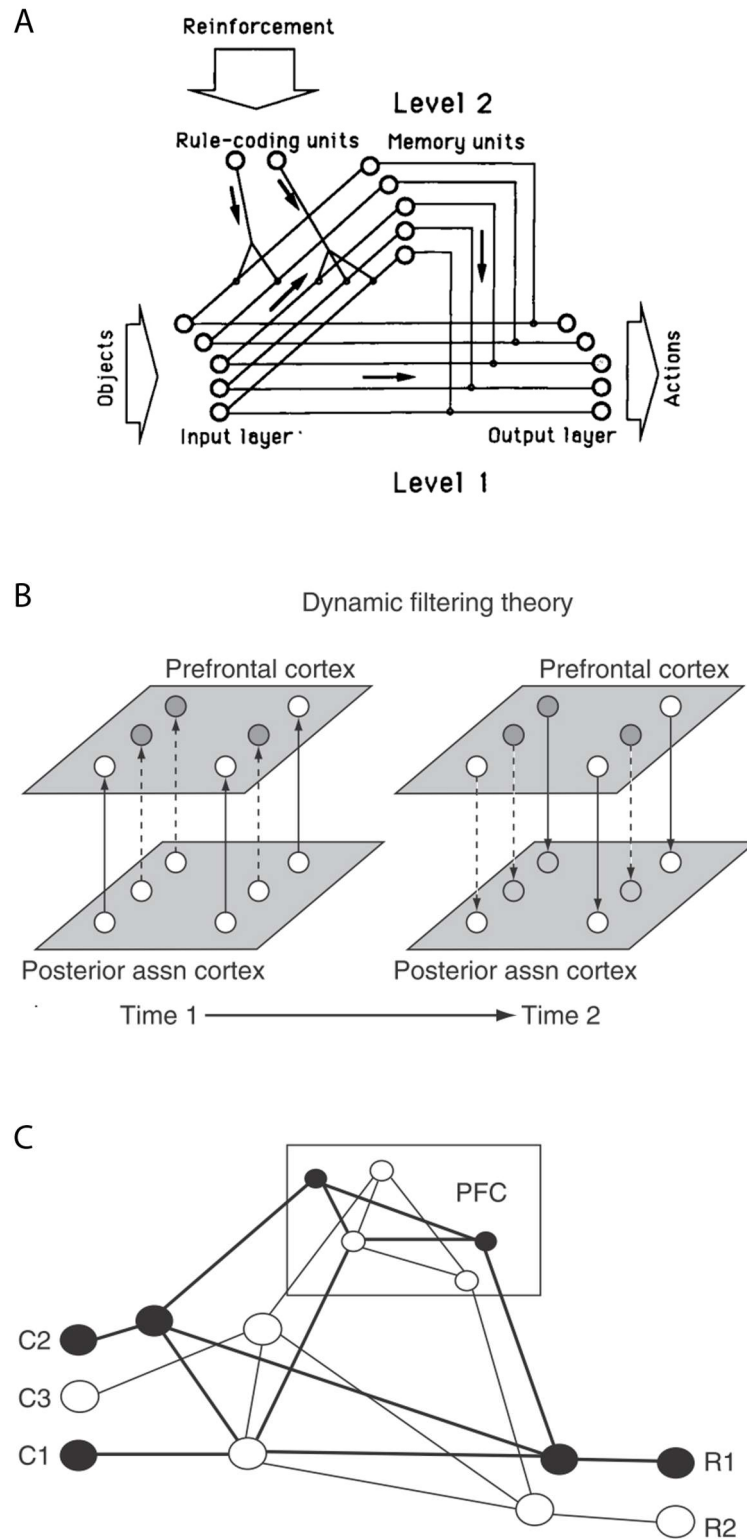
Figure 2-7. Models of the PFC function. (A) The model of the PFC function was proposed by

Dehaene and Changeux (level 2). The isolated objects (input) are generally associated with

the output in level 1. However, the PFC was proposed to modulate the selection of specific input according to a specific rule. (From Dehaene and Changeux, 1989) (B) Shimamura's dynamic filter model. At time 1, the posterior associated cortex (posterior assn cortex) activates the specific subset of PFC according to the task demand (bottom-up). At time 2, the feedback projections from the PFC facilitate the necessary information flow by selecting the relevant (light circle) and inhibiting the irrelevant neural activities from the posterior associated cortex (dark circle) (top-down). (C) Miller and Cohen's model. In this model, the PFC was proposed to form a rule map after learning to guide the cue-state-response association. The PFC forms the task model by reinforcement learning and can be activated by a subset of the cue. Therefore, with the specific activity that activates the subset of task-model relevant activity, the cue-state-response is strengthened. Thick line and filled circle indicate the activated projection. The light line and light circle indicate the inhibited projection. (From Miller and Wallis, 2009).
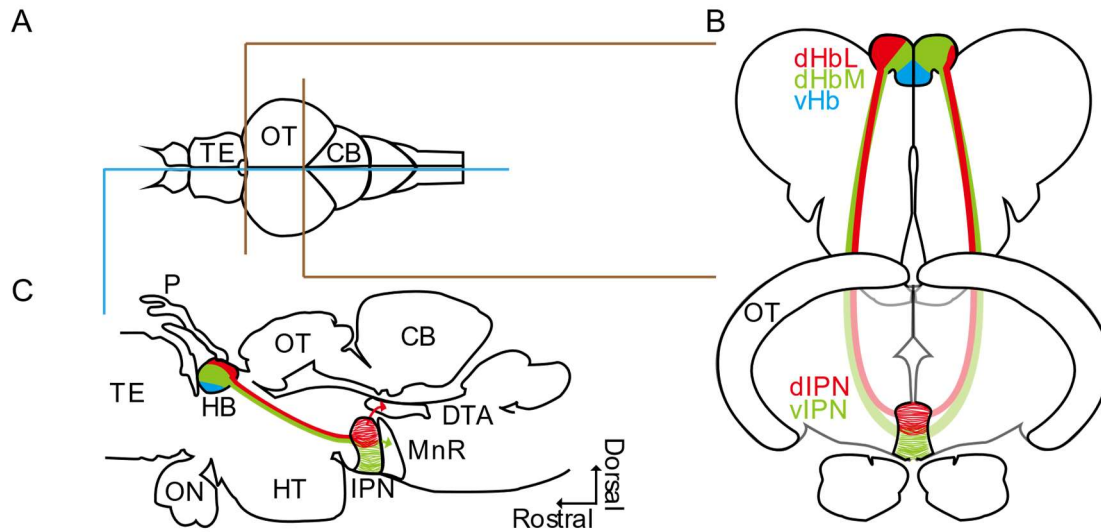
Figure 2-8. The habenula-interpeduncular circuit. (A-C) Schematic illustration of the zebrafish habenula subnuclei-interpeduncular nucleus circuits. (A) Dorsal view of the adult zebrafish brain. Brown lines, cross sections. Blue line, sagittal section. (B) The dHbL and dHbM project respectively to the d/iIPN (red) and i/vIPN (green). The vHb is colored in blue. OT, optical tectum. (C) Sagittal view of the adult zebrafish brain. The dorsal tegmental area (DTA) receives the efferent from d/iIPN (red arrow), while the dHbM-i/vIPN pathway is connected to the median raphe (green arrow). TE, telencephalon. OT, optical tectum. CB: cerebellum. ON, optic nerve. HT, hypothalamus. HB, habenula. IPN, interpeduncular nucleus.

Chapter 3. Methodology

Experimental model and details

The study used adult wild-type (WT) zebrafish (RIKEN-Wako, Saitama, Japan) and transgenic (Tg) zebrafish lines. Transgenic strains, *Tg(narp:GAL4VP16); Tg(UAS:TeTxLC)* and *Tg(narp:GAL4VP16); Tg(UAS:nfsB-mCherry)* (dHbL-silenced) were applied in this study (Agetsuma et al., 2010). Fish were maintained in 7-L tanks with continuous water exchange at 28.5°C under the 14-hour light/10-hour dark cycling. All protocols were reviewed and approved by the Animal Care and Use Committees of the RIKEN Center for brain science.

We separated the *Tg(narp:GAL4VP16); Tg(UAS:TeTxLC)* zebrafish and their wild-type siblings in adulthood by genotyping, due to the lack of a fluorescence reporter. Conversely, *Tg(narp:GAL4VP16); Tg(UAS:nfsB-mCherry)* zebrafish were selected by their fluorescence patterns from larva stage.

The plus-maze system

The plus-maze was installed in a 300x300x100-mm square transparent acrylic tank by separating the inner space into four arms (260-mm in length with 45-mm space between walls) (Figure 3-1A, the white part inside the tank). The food-dispensing units were set at the ends of the four arms (Figure 3-1A, dark gray). They were made of polypropylene tubes (PP) 30 mm in diameter and 50 mm in height, attached with a white plastic mesh (N-3, aperture size, 1.8x2.8 mm, Daipla corporation) on one flanking side (Figure 3-1B). We inserted a vacuum cleaning tube and tube with an LED light horizontally and the reward tube (tilted at 30 degrees) into the lid of the PP tube (Figure 3-1B). A display was placed within a 3-mm

space at the bottom of the tank to show the distinct color patterns on different locations and was controlled by custom-made software. The port of the tube on each food-dispensing unit was connected to one end of an electrical magnetic valve (AB31-01-3-AC100V, CKD corporation), which was controlled by a Digital I/O Device (USB-6501, National instrument) by TTL signal from the computer. The other end of the valve was connected to the 100 mL food reservoir (reward delivery valve) or controllable electrical pump (vacuum cleaning valve) (Figure 3-1B). With the proper adjustment of the parameters, we could precisely control the liquid flow in the tank. The 5-mW red LED light (wavelength, 616 nm) was directly inserted into the LED tube sealed by silicone gel for waterproofing and was connected to a Digital I/O Device under computer control (Figure 3-1B). A pair of speakers was used to generate a sound cue, which was directly linked to the computer sound port, and was controlled by our software. To avoid interference from the environment, the whole system was set up in a wooden box with a web camera (c920, Logitech) on top of the maze, controlled by the computer. Our custom-made software was written using Matlab and LabVIEW. The software integrated functions including displayed pattern generation, sound pattern generation, object detection, hardware control (magnetic valve and pump), and real-time behavior quantification according to the desired behavioral conditions.

Reward quantity control

A stable quantity of reward is crucial for successful reward-based reinforcement training. Therefore, we adjusted the amount of reward before new training started. Condensed frozen baby brine shrimp was diluted 90 times by fish water and was kept in a food reservoir (Figure 3-1A) and suspended by air bobbling. Before the start of training on each day, the supplier tubes needed to be completely rinsed by the food-reward to remove the bubbles in the tube.

A custom-made calibration program was then initialized before calibrating for the equal amount of food-reward delivery across each food-dispensing unit. The basic valve opening duration was initially set to 180 ms. We checked the number of baby brine shrimps to approximately 40-50 per ejection and adjusted the duration of opening until each food-dispensing unit delivered an equal amount of food. A vacuum cleaning system was not used in the rule-shift task because the small amount of food could be fully consumed during each trial by the fish.

Operant behavior training

To fully fit for purpose of precise measurement and high throughput training, the plus-maze system was applied for operant conditioning. As mentioned above, the fish behavior in real-time by the recording from the web camera to get the real-time information of fish e.g. position, and fed it into the customized software for the hardware control. In operant behavior training, the system displayed the visual cued pattern (blue and red) at the vertical arm of the current position of the fish. The task required the fish to swim into the correct colored arm within 15 seconds to gain the food reward. Before the start of the training, the amount of reward was adjusted as mentioned in the above section. Fish were transferred from the home tank to the test tank for acclimation at least for 10-minute with a gray background (Figure 3-2A). Following it, we started a 30-trial baseline training to detect the nature preference and performance odds. In the baseline session, we conducted fish only with visual stimuli but without reward release. Within approximately 20-second inter-trial-interval, the background color was displayed in gray (Figure 3-2A). Instead, I gave the terminal region of vertical arms with either red (RGB: 255, 43, 43) or blue (RGB: 43, 43, 255) color in pseudo-random order. Following the baseline session, the 6-consecutive training sessions

with reward were performed. The training session is designed with a maximum of 6 sessions per day (180 trials) (Figure 3-2B). The inter-session-interval was placed with a duration of a minimum of 10 minutes between sessions (Figure 3-2B). The same training was repeated until the fish reached the learner criteria (Assuming a 0.5 probability of binominal distribution in the target selection with a 95% confidence interval (CI), the learner criterion was set as achieving either the condition of 9 consecutive correct choices in 10 trials (0.9 moving average of success rate: averaged by consecutive frame) or 21 discrete success trials in a single session (30 trials, 0.7 accumulated success rate).

Rule shift task

Zebrafish were allowed to adopt two different rules that were distinguished by attended information. In rule 1, the reward was associated with an egocentric direction, *e.g.*, turning left at the cross point of the plus-maze, regardless of the goal colors. In contrast, rule 2 required fish to select an allocentric cue to gain the reward, *e.g.*, turning toward the arm with the blue target disregarding which side, the left or right, the blue target was located (Figure 3-3A). Hence, the fish is trained to either use internal information (left or right) based on the awareness of self-direction or external information acquired from the environment (red or blue color). We began the trial with a 1-second sound cue (440 Hz, 74 dB) and simultaneously showed the triangular color images (color blocks) at the bottom of the ends of the arms right angle to the arm where the fish was initially located. Following the assigned rule, the correct selection resulted in rewards release. In contrast, an incorrect selection immediately turned off the color images and shifted the training step into an inter-trial interval period. Regardless of the rule, the colored target images (color blocks) were always shown at the onset of each trial (Figure 3-3C). The trainee fish, therefore, needed to ignore irrelevant external

information during the directional-rule task (Rule 1) but followed external information during the cued-rule task (Rule 2). After the baseline training sessions (30 trials per session) with no reward (two sessions for each rule), the reward-reinforced training started on day 2. In a standard training day, a maximum of 6 sessions, which consisted of 180 trials, were performed. The time of training for a trial is around 1 minute including the 20-second inter-trial interval, the variable period of arm selection, and the 15-second reward period. With the 10-minute inter-session interval, we gave around 3-hour training each day. Once the fish had achieved the learner criterion, we shifted to a different rule in the following session (Figure 3-3D-E). Assuming a 0.5 probability of binominal distribution in the target selection with a 95% confidence interval (CI), the learner criterion was set as achieving either the condition of 9 consecutive correct choices in 10 trials (0.9 moving average of success rate: averaged by consecutive frame) or 21 discrete success trials in a single session (30 trials, 0.7 accumulated success rate). We separated training into 2 phases: the introductory period and the rule-shift test period (Figure 3-3E). The introductory session was given to train fish for understanding the rules. In the rule-shift test sessions, we examined the ability of fish to adapt to rule-shifts. In the introductory session, we used a stricter standard, in which the fish were required to achieve the learner criterion twice in a training day so that they could be considered a stable learner. We then shifted the rule for the stable learner in the next session. In contrast, in the rule-shift test sessions, once the fish had achieved the learner criterion, we shifted the rule in the next session (Figure 3-3E). By examining the learning process in the rule-shift task, we could clearly monitor the ability of fish to shift the utility of internal (directional) or external (cued) information. In addition, the behavioral flexibility between rules can be evaluated by repeated rule shifts.

Parameters and hardware calibration in the rule-shift task

To achieve the rule-shift task, we optimized the parameters of the plus-maze system to stabilize the training conditions. The trial was initialized with a sound cue and pattern display when the fish swam into zone 1 in the initial position fixed condition (Figure 3-3A) or any randomly chosen terminal end of the arms in a condition with a randomized initial position (Figure 3-3B). The sound cue was set to 440 Hz, 74 dB for 1 second. Two triangular colorized blocks were used to define the selection region to determine the choice of fish. We defined red in RGB value (255, 43, 43) and blue with (43, 43, 255) (Figure 3-1C). Because heat is continuously generated by the display, we installed the electric fan to blow the air through the 3 mm gap between the display surface and the bottom of the tank to maintain the water temperature at 28±2°C for the long-term training. The positions of the red and blue blocks were always set in the right-angle arms to the arm where fish started. The determination of red or blue in these flanking right-angle arms was in random order. By image subtraction, we extracted the fish shape from the background and kept monitoring the position of it by calculating the center of gravity to compute the behavioral performance. The real-time performance is quantified by the percentage of successful choice of 10 trials as the moving average. The performance was re-set to zero when a new training day started but was maintained across sessions on the same day.

Delayed-non-matching-to-sample task

To test the visual-spatial working memory, the delayed-non-matching-to-sample (DNMTS) task was designed by modifying the experimental condition in the plus-maze system. The

whole set of systems is as same as mentioned above. However, in the DNMTS task, instead of a single visual stimulus, two stimuli (sample stimulus and test stimulus) were given with the interval time (Figure 3-4A). The DNMTS requested the fish to select the different colored stimuli between the first and second stimuli. In practical, the task was divided into two phases of training. The first training phase was designed to introduce the non-matching rule to the fish, which I set the delayed time to 0 seconds and lately to 0.2 seconds by the judgment of the learner criteria in the averaged success rate 0.7 in consecutive 20 trials (Figure 3-4A training phase 1, 3-4C). The sample stimulus was designed to fill all of the four arms in the plus-maze to make sure the fish could receive the stimulus at any position. In the first training phase, 3 arms, including the terminal end of the arm where the fish was located and the flanking arm of it, were displayed with color block as the test stimulus. The Fish's position was displayed the colored block as same as the sample stimulus color to help fish engage the rule in an easier way (Figure 3-4A training phase 1). The second training phase was designed after the successful training of the first training phase. Comparing to the first training phase, the second training phase showed the color blocks (test stimulus) only at the terminal end of the flanking arms of the arm where the fish was located when the trial start (Figure 3-4A training phase 2). Moreover, the delayed period (retention interval, RT) was elongated with 6 levels: 0.2, 1, 2, 3, 4, and 5 seconds, which was randomly selected during the test. As such, the second training phase was designed for testing the working memory span of zebrafish. For the training trial, a 90-second inter-trial interval was inserted between trials. A maximum of 6 training sessions was performed for one day's training to maintain the motivation for the whole training process (Figure 3-4A-C).

Eye-body movement coordination test

Wild-type fish and *Tg(narp:GAL4VP16); Tg(UAS:TeTxLC)* zebrafish were used in the eye-body movement coordination test. Before the experiment, the fish was moved into a 1000 mL beaker with fish water and placed on a display, showing the gray (RGB [40, 40, 40]) background. To record the video, a web camera was held on top of the beaker and controlled by the custom-made program. Fish were allowed to swim freely in the environment. After a 15-minute acclimation, we started the recording sessions for each fish. The recording was conducted in 10 frames-per-second with 3 minutes for each session with at least 3-minute inter-trial intervals. Videos were annotated by convolution neural network auto-annotation algorithms (Nath et al., 2019) with 9 points, including front left eye, back left eye, front right eye, back right eye, mouth, head, center, pelvic fin, and tail. These 9 points were used to calculate the 6 directional vectors (eye [R] by the front right eye and the back right eye, eye [L]) using front left eye and back left eye, head by the mouth and head, center by the head and center, and tail by the center and tail). Directional vectors were used to calculate the angle changes between 2 frames of the recording (200 milliseconds). The radian of the angle change was calculated to determine the correlation of eye-body locomotion.

Nitroreductase-Mtz-based inducible dHbL neuron-specific cell ablation

We applied the same approach as in Agetsuma et al., 2010. The *Tg(narp:GAL4VP16; UAS:nfsB-mCherry)* fish specifically expressed nitroreductase/mCherry fusion protein in adult dHbL neurons. Fish were treated with 10 mM metronidazole (Mtz) for 24 hr and kept in fish water

for 7 days for cell-autonomous apoptosis induced by nitroreductase. After Mtz-induced neuron ablation, fish were imposed on the standard rule-shift task.

Immunohistochemistry

I conducted the immunohistochemistry (IHC) staining for detecting the expression pattern and level of tetanus toxin light chain (TeTxLC) in this study. The fish was sacrificed by cold shock and immediately used teleost Ringer's solution and fresh 4℃ paraformaldehyde (PFA) solution for heart perfusion. After the gill became colorless, the fish was decapitalized and fixed in 4℃ paraformaldehyde (PFA) overnight. The fixed fish head was washed with phosphate-buffered saline (PBS) and dissect the brain tissue with fine forceps. The brain was transferred to the 15% sucrose buffer and gradually replace to 30% sucrose buffer. The brain was then placed to OCT for cryosection. Samples were sliced into 45 um thickness slices which were washed in PBS-T for IHC staining. To detect TeTxLC, the goat anti-tetanus toxin (Novus Biologicals, NB100-63239) was used in 1:500 diluted in blocking buffer. The secondary antibody was selected for with anti-goat Igg conjugated with Alexa 488 for fluorescence signal detection under microscopy.

Quantification and statistical analysis

Data in this study were handled by a customized program in R. We conducted all statistical analyses, including the Wilcoxon rank sum test, Fisher's exact test, and Z-test using this program.
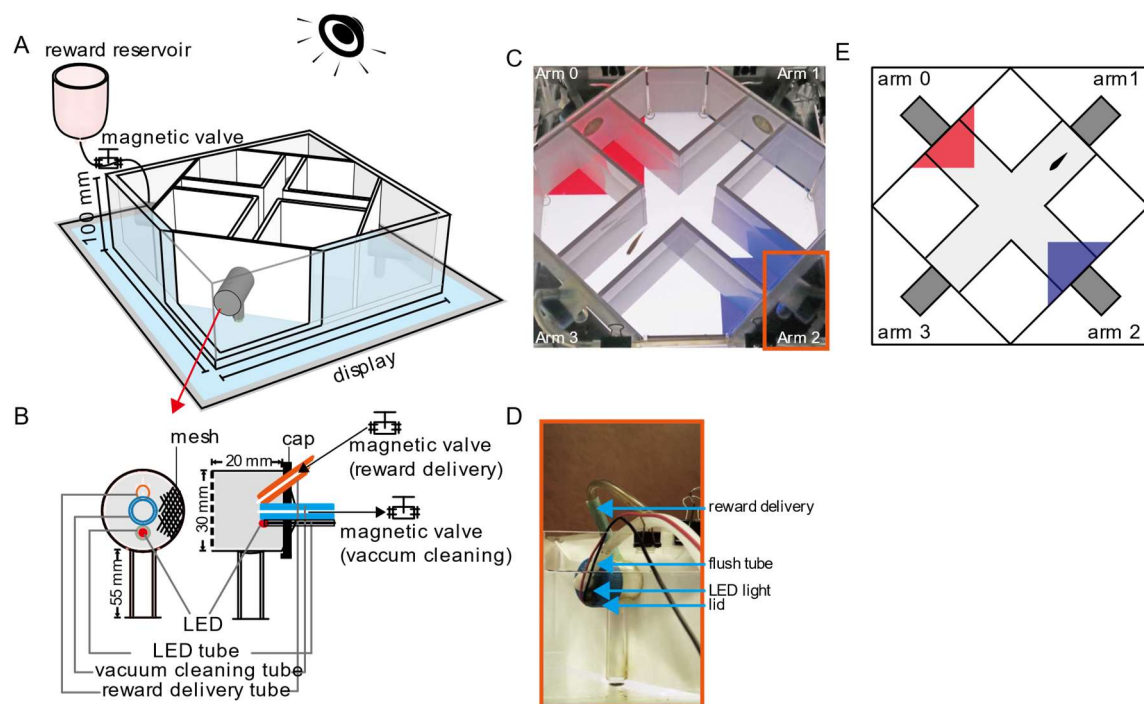
Figure 3-1. The design of plus-maze. (A) Schematic view of the plus-maze system. (B) The design of the food dispensing unit. Left, frontal view. Right, lateral view. (C) Top oblique view of the training space with color blocks displayed at the ends of the arms 0 and 2. Orange inset, location of the food-dispensing unit. (D) The side-view of the food-dispensing unit. (E) Top view of the plus-maze with the displayed color blocks. The arms were numbered in clockwise order from 0 to 3 with the left-top arm assigned 0.
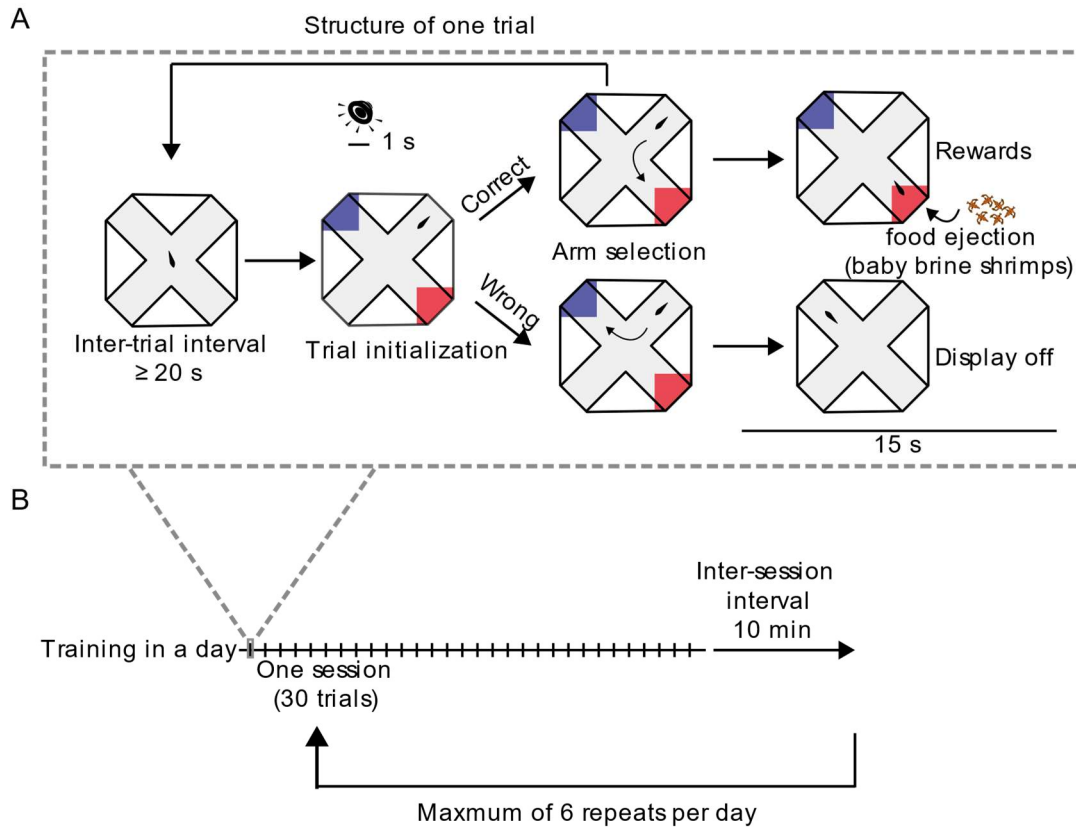
Figure 3-2. Task design of operant behavior training. (A) The structure of one trial. (B) Scheme of the task design by session.
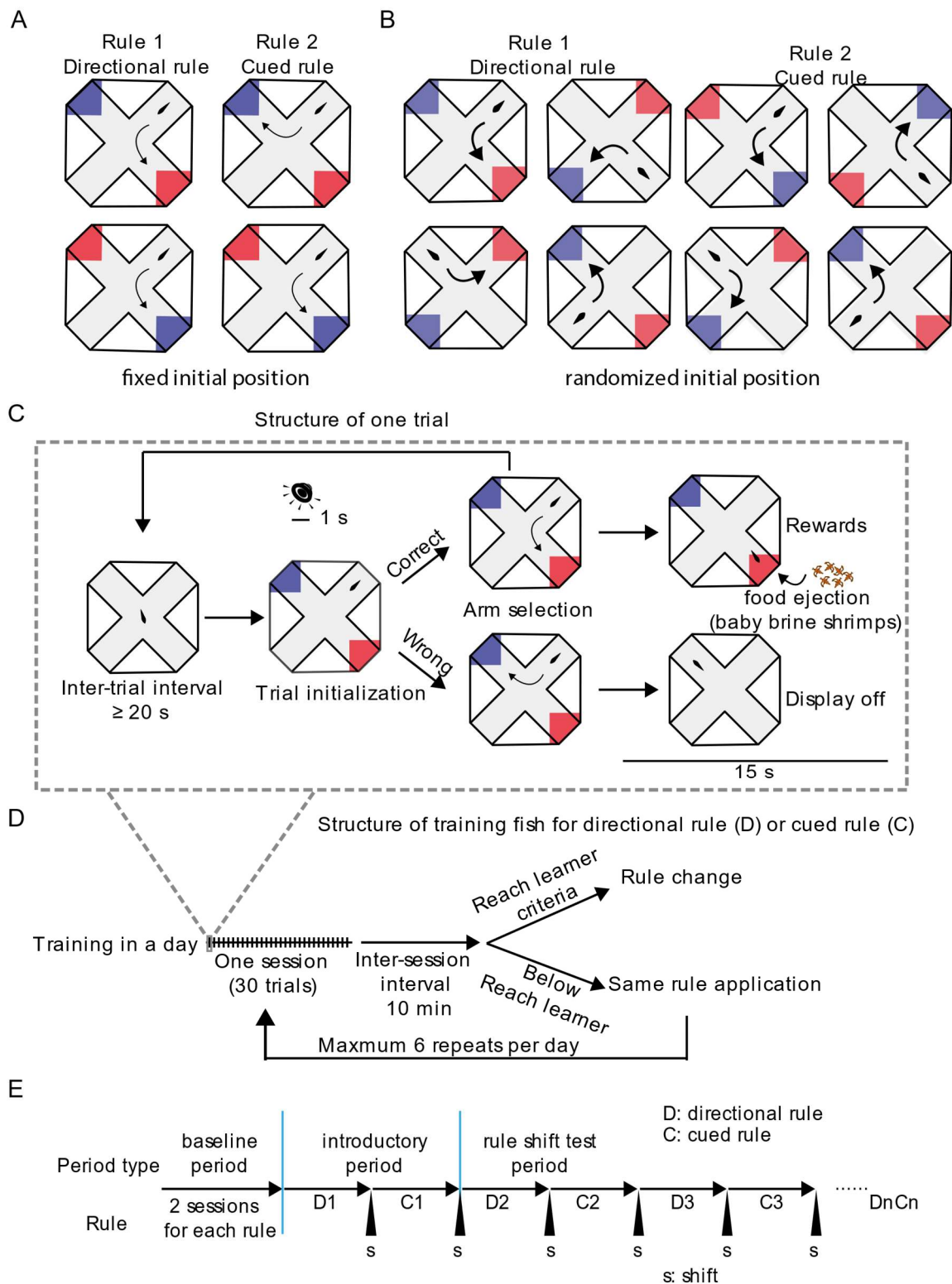
Figure 3-3. Structure of rule shift task. (A) The illustration of rules. Left, two examples of the

directional rule, where a fish has to turn left regardless of goal colors. Right, two examples of

the cued rule, where a fish has to turn to the blue goal regardless of turning direction. (B) A

schematic illustration of rule-shift tasks with randomized initial positions. Trials were initiated while the trainee fish reached the end of any arm within a given period after the inter-trial interval. The color blocks are shown in the arms at a right angle to the arm where the fish was initially positioned. The colors were assigned randomly between blue and red. The examples show correct left-turning decisions using the directional rule (Rule 1) and correct turning decisions to the blue block in the cued rule (Rule 2). (C–E) The rule-shift task training framework. (C) The training structure in one trial. A trial of the rule-shift task consists of the inter-trial interval with the background bottom color (light gray), the selection period that starts with a beep sound (440 Hz) for 1 second, and a 15-second reward period. Depending on whether the choice was correct or incorrect, the plus-maze system either ejects food in a designed amount or turns off the display of the color blocks. (D) A maximum of 180 trials over 6 sessions were performed during one training day. After inter-session intervals of 10-minute, the rules were changed in the next session if the performance reached the learner criterion, whereas the same rule was maintained if the performance was kept below the learner criterion. (E) The architecture of the rule-shift task. Three types of periods were designed for different purposes. In the baseline period, 2 sessions for each rule (4 sessions in total) were conducted for adaptation without reward. The introductory period was performed to allow fish to understand the rules. Next, the rule shifts were tested during the rule-shift test period. Rule types, *i.e.* directional and cued, were represented by D or C with the rule switch number. The learner criterion was set to 0.9 success rate within 10-trial moving average (averaged success rate with 10-trial continuous moving window) or to 0.7 success rate in the session average (averaged success rate by 30 trials). In the introductory period, it was necessary for the fish to achieve the learner criterion twice during a training day to confirm rule learning was properly established. In contrast, in the rule shift period, if fish achieved the learner criterion, the rule was changed in the subsequent session. If the fish

achieved the learner criterion in the final session of the day, the same rule would be tested

on the next training day to ensure the learning capability.
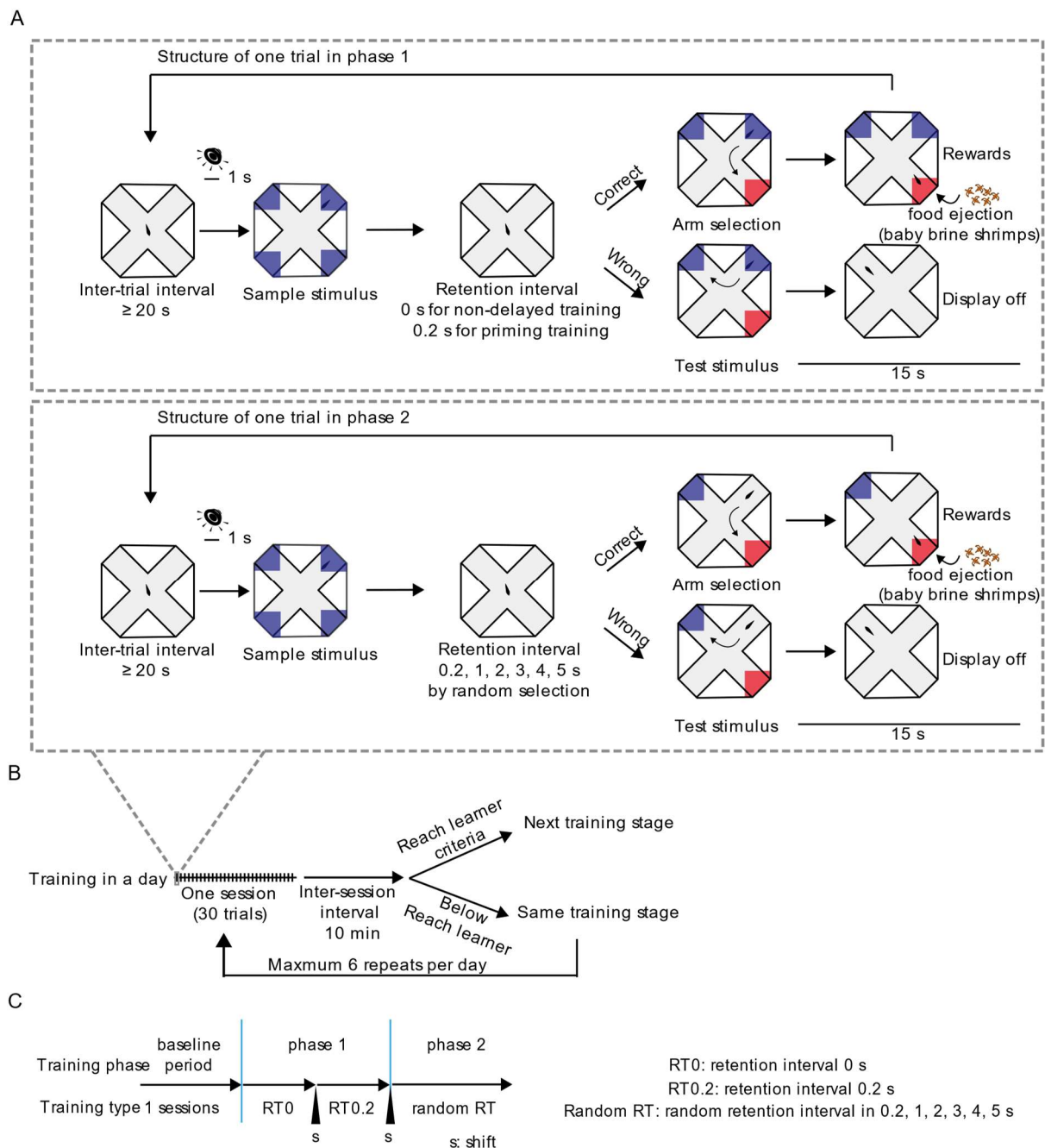
Figure 3-4. Structure of delayed-non-matching-to-sample (DNMTS) task. (A) The training

structure in one trial. A trial of the DNMTS task is composed by the inter-trial interval with

the background bottom color (light gray), the stimulus shows up after starts with a beep

sound (440 Hz) for 1 second, the retention interval, and the 15-second test stimulus with

reward period. Depending on whether the choice was correct or incorrect, the plus-maze

system either ejects food in a designed amount or turns off the display of the color blocks. (B) A maximum of 180 trials over 6 sessions were performed during one training day. The training level is judged by the learner criteria. The training level from 0 s interval was updated to random retention interval. (C) The architecture of the rule-shift task. Three levels of training type plus the one baseline session were designed in DNMTS task. In phase 1 training, the retention interval is from 0 s to 0.2 s depending on whether the success rate reaches the learner criteria. After finishing the phase 1 training, phase 2 is designed to test the working memory span with randomized but defined retention interval. The learner criterion was set to 0.7 success rate within 20-trial moving average (averaged success rate with 10-trial continuous moving window).

Chapter 4. Findings

Zebrafish learned the operant behavior by reward reinforcement training to associate the visual colored cue and positive reinforcer

To make the higher-order cognitive function plausibly be tested, I devised an automated training system, the plus-maze, which flexibly adapts to sophisticated behavioral paradigms during intensive training (Figure 3-1A-E). Although automated aversive training had been developed previously (Amo et al., 2014; Aoki et al., 2015a, 2013), keeping zebrafish in a stressed environment with a negative emotional state could lead to failure of many cognitive tasks. Therefore, I designed the reward-based training in the system.

In an aquatic environment, due to diffusion, it is difficult to control reward delivery. In our model, this was overcome by designing food-dispensing units with valves and a suction pump, which I precisely controlled using a custom-made program that integrated the devices to optimize a combination of parameters including pressure, timing, and duration. The design enabled the precise control of the quantity of liquid flow used to deliver foods or chemicals (Figure 3-1B, 1D). I installed multiple sensory stimulus modules to supply sound and light with pre-designed parameters to execute the experiments under the required conditions. Moreover, the system responds in real-time to the behavior of fish with a feedback loop, which provided the opportunity to apply the paradigms in a dynamic design. This plus-maze system may be used not only to help us to address questions that require precise control of parameters but also to create a technical solution for cognitive function tasks that can be accomplished only by reward-based training.

The plus-maze system was first applied to the reinforcement learning for established the operant behavior by reward-based training, and also to test the baseline learning rate for

zebrafish under the training paradigm. I found that the zebrafish is able to learn the association of reward and color cue within 3 consecutive training days (18 sessions). Among all 6 tested fish, the learning curve fits the linear regression with the averaged coefficient of determination in 0.623 (Figure 4-1). The result indicates that the zebrafish can be trained by reward reinforcer within a short time to elicit the particular operant behavior, which also confirms the previous results that the zebrafish discriminate the different colors and use the feature as the attention dimension with value (Agetsuma et al., 2010; Aoki et al., 2013; Avdesh et al., 2012; Colwill et al., 2005; Levin and Chen, 2004; Parker et al., 2013). This preparatory experiment demonstrated the baseline of the whole study and show that the advanced cognitive function may be tested by applying the parameters.

Strategy for information selection of operant behavior can be examined by internal and external rule-shift tasks

To examine the executive function with sufficient sensitivity of the subcomponent, I measured behavior in the plus-maze system following rule-shift tasks in the present study. Zebrafish were allowed to adopt two different rules that were distinguished by attended information. In rule 1, the reward was associated with an egocentric direction, -for example, turning left at the cross point of the plus-maze, regardless of the goal colors. In contrast, rule 2 required fish to select an allocentric cue to gain the reward, *-for example,* turning toward the arm with the blue target disregarding on which side, the left or right, the blue target was located (Figure 3-3A). Hence, the fish is trained to either use internal information (left or right) based on the awareness of self-direction or external information acquired from the environment (red or blue color). I began the trial with a 1-second sound cue (440 Hz, 74 dB)

and simultaneously showed the triangular color images (color blocks) at the bottom of the ends of the arms right angle to the arm where the fish was initially located. Following the assigned rule, the correct selection resulted in a reward release. In contrast, an incorrect selection immediately turned off the color images and shifted the training step into an inter-trial interval period. Regardless of the rule, the colored target images (color blocks) were always shown at the onset of each trial (Figure 3-3C). The trainee fish, therefore, needed to ignore irrelevant external information during the directional-rule task (Rule 1) but followed external information during the cued-rule task (Rule 2). After the baseline training sessions (30 trials per session) with no reward (two sessions for each rule), the reward-reinforced training started on day 2. In a standard training day, a maximum of 6 sessions, which consisted of 180 trials, were performed. The time of training for a trial is ~1 min including the 20-s inter-trial interval, the variable period of arm selection, and the 15-s reward period. With the 10-min inter-session interval, I gave ~3-h training each day. Once the fish had achieved the learner criterion, I shifted to a different rule in the following session (Figure 1H-1I). Assuming a 0.5 probability of binominal distribution in the target selection with a 95% confidence interval (CI), the learner criterion was set as achieving either the condition of 9 consecutive correct choices in 10 trials (0.9 moving average of success rate: averaged by consecutive frame) or 21 discrete success trials in a single session (30 trials, 0.7 accumulated success rate). I separated training into 2 phases: the introductory period and the rule-shift test period (Figure 3-3E). The introductory session was given to train fish to understand the rules. In the rule-shift test sessions, I examined the ability of fish to adapt to rule-shifts. In the introductory session, I used a stricter standard, in which the fish were required to achieve the learner criterion twice in a training day so that they could be considered a stable learner. I then shifted the rule for the stable learner in the next session. In contrast, in the rule-shift test sessions, once the fish had achieved the learner criterion, I shifted the rule in next session

(Figure 3-3E). By examining the learning process in the rule-shift task, I could clearly monitor the ability of fish to shift the utility of internal (directional) or external (cued) information. In addition, the behavioral flexibility between rules can be evaluated by repeated rule shifts.

Zebrafish can use both internal and external information for operant behavior learning and flexibly shift attended information

To examine whether zebrafish is capable of adopting the directional rule (internal information) and the cued rule (external information), I imposed the rule-shift task on adult zebrafish. I isolated fish with two-week starvation for starting the training. To simplify the test, I started the trials when the fish was positioned in arm 1 (rule-shift task with fixed initial position, Figure 3-3A). The results showed that the fish performed the baseline sessions at a low success rate (0.184 ± 0.033, mean ± standard error of mean [SEM], n=14, Figure 4-2A). However, performance improved to chance levels (approximately 0.5 success rate in 10-trial moving average) with a concomitant decrease in the time spent until selecting color blocks, once I initiated the reward reinforcement (Figure 4-2B). With extended training, performance further improved gradually to the level of the stable learner criterion, indicating that the fish experienced two steps of learning (Figure 4-3A). In the first step of the introductory session, fish learned that once triggered by the sound cue, they must turn to either arm with different colors or directions (red or blue, left or right). This step of learning, that is, necessity-to-turn learning, was evidenced by the increased rate of a continuous selection toward either lateral arm for 10-times over 11 trials (Figure 2A, necessity-to-turn learning). I compared the selection percentage in the sessions before and after the fish reached the criteria of necessity-to-turn learning, and confirmed that fish reduced their probability of remaining at

the arms with no color (Supplemental figure 1C-1D). In the second step of the introductory

session, fish learned to apply rules to reach the correct target arm and to achieve the stable

learner criterion (Figure 4-3A, rule learning). As the training proceeded, I changed the rules

repeatedly in the subsequent sessions. The results showed that the zebrafish could

continuously change the selected information after each rule shift (Figure 2B). Furthermore,

zebrafish could adapt to shifted rules with the gradually decreased number of trials as they

experienced the repeated rule shifts (Figure 4-3C and 4-3D). The results demonstrated that

zebrafish can exploit both internal (directional) and external (cued) information in operant

learning. Furthermore, the retrieval of the rule memory became quicker as fish experienced

repeated rule shifts.

Silencing the lateral subregion of the dorsal habenula impairs directional rule learning

As mentioned in chapter two, the habenula-IPN circuit is a great candidate for regulating the

various sensory information. Therefore, I asked whether the dorsal habenula-

interpeduncular nucleus plays an important role in internal sensory information utilization

and selection. The dHbL-silenced transgenic line, *Tg(narp:GAL4VP16); Tg(UAS: tetanus

neurotoxin light chain (TeTxLC))* in which the dHbL-d/iIPN pathway was silenced by expression

of tetanus toxin light chain (TeTxLC) in the dHbL neurons, was used in rule-shift tasks

(Agetsuma et al., 2010). Although all the dHbL-silenced fish could learn the rule eventually, I

found that the dHbL-silenced fish significantly required additional trials than the wild-type

fish to learn the directional rule (Figure 4-4A (D1, Wilcoxon rank sum test, p=0.011, statistic

power close to 1) and Figure 4-5A-C). In contrast, in the cued rule, these transgenic fish

required a fewer number of trials to achieve the same learner criterion than the wild-type fish (Figure 4-4B and Supplemental figure 4-5D-F). I asked whether the results could be caused by a general learning deficit *per se*. However, the necessity-to-turn learning, in which fish learned to access either the left or right arm regardless of the rule (Figure 4-3A), was equally performed by the dHbL-silenced transgenic fish and wild-type fish (Figure 4-2E). This suggested that general learning abilities remained intact even after the inactivation of the dHbL-d/iIPN circuit.

Measuring from the session (R1 in Figure 4-3A, B) immediately after the successful learning of the necessity to turn, either left or right, I then imposed the directional rule (wild-type, Left, n=5, Right, n=9; transgenic, Left, n=2, Right, n=2). Both transgenic fish and wild-type fish showed the same levels of innate preferences for left or right turning and no bias for either red or blue (Figure 4-2C and D). This result excluded the possibility that a low learning rate in the dHbL-silenced transgenic fish was caused by an abnormally augmented biased innate preference. I further examined locomotion activity and eye-body movement coordination to clarify whether the dHbL-silenced fish showed any further deficits in motor response or body movement coordination. I measured locomotion activity by the swimming distance during the trials and eye-body movement coordination by detecting the eye and body directions over 200 milliseconds and calculating the correlation between them. I found that the wild-type fish and the dHbL transgenic fish showed similar levels of locomotion activity (Figure 4-4C) and no differences in coordinated eye-body movements (Figure 4-4D and Figure 4-5 A-C, see methodology section). The results demonstrated that the impairment of directional rule learning might not be caused simply by a functional loss in motor ability. I, therefore, hypothesized that the lower learning rate observed in the directional rule could result from an impairment in the use of directional information through the learning process.

The rule-shift task with randomized initial position confirmed that the impairment of directional rule adoption could result from the loss of capacity in utilizing directional information

The dHbL-silenced fish could eventually become learners in the rule-shift task using the fixed starting arm. I suspected that this could result from contextual learning due to the subtle differences in the textures of different arms at the choice point. To confirm the hypothesis that the dHbL is essential in the utilization of directional information, I varied the initial starting arm in the rule-shift task, to exclude the possibility that fish could use the contextual information (subtle texture difference of different arms) at the turning point (rule-shift task with randomized starting arms) (Figure 3-3B). Since the initial position was variable, the fish had little chance to achieve the directional rule learning by directly referring to the environmental context. Besides expressing TeTxLC for silencing dHbL neurons, I additionally used transgenic line, *Tg(narp:GAL4VP16); Tg(UAS:nfsB-mCherry)*, that expresses nitroreductase-mCherry fusion protein (Agetsuma et al., 2010). I ablated the dHbL neurons by administrating metronidazole (Mtz) to silence the dHbL-d/iIPN pathway to examine whether the similar effect could be observed as seen in the TeTxLC-expressing transgenic line. I observed the consistent results from both approaches (Figure 4-6E). Since all non-silenced fish could become learners within 7 days (42 sessions), I classified the learner or the non-learner according to the learner criteria on the 7th day. By combining the data in both approaches, approximately half (43.75%, 7 of 16 training fish) of the dHbL-silenced fish could not become learners in the directional rule in the rule-shift task with randomized initial starting arms. In contrast, the non-silenced sibling performed with an intact learner ratio (100%, n=11) (Figure 4-4E, left panel (D1, Fisher's exact test, p=0.022, statistic power close to

0.91) and Figure 4-6D, left panel). The remaining 9 dHbL-silenced fish could become learners within 7 days and showed consistent learning performance as the non-silenced sibling (Figure 4-6F), probably due to insufficient silencing of the dHbL neurons in these fish (Figure 4-7). Nevertheless, in the cued rule, both the dHbL-silenced and the non-silenced fish showed identical performance (Figure 4-4E, middle panel, Figure 4-6D, right panel). I gave the extended training for the non-learner of the dHbL-silenced fish (n=7) after the learner criteria judgment on the 7th day and found that 6 of 7 fish could not learn the directional rule even with such extra training trials (Figure 3H, blue box). Similarly, the dHbL-silenced fish showed no abnormality in the locomotor activity during training (Figure 4-4F), behavioral flexibility (Figure 4-6G), and even better performance in necessity-to-turn learning (Figure 4-6H). These results confirmed that the lower learning rate in the dHbL-silenced fish in the rule-shift task was caused by an impairment of directional information utilization, whereas the dHbL-silenced fish could eventually become learners in the initial-position-fixed condition most likely because the fish learned to distinguish subtle differences between the testing arms. The results provided further evidence that the dHbL is essential in the utilization of egocentric directional information.

Delayed-non-matching-to-sample task failed to detect the working memory span in zebrafish

In the rule-shift task, the results demonstrated that the fish is capable of implementing the higher-order cognitive function. By the definition of the subcomponents of executive function, the fish showed that their behaviors matched the realms of the rule engagement, flexible behavior with extra-dimensional attention shift, and behavioral inhibition. However, the

working memory, which is postulated as the essential function to guide these behaviors, is only implicitly suggested. In the rule-shift task, to repeatedly achieve the shifted goal by exploiting two types of information: the internal-directional information and external-cued information, fish needs to hold the abstractive rule memory. However, this type of memory did not directly be manipulated in the rule-shift task, which lacks the specific property in the rigorous definition of working memory. The specifically defined working memory hard to be explicitly detected quantitatively. Thus, the delayed-non-matching-to-sample (DNMTS) task was designed here to interrogate the existence of visual-spatial working in zebrafish. As the methodological details I have described in Chapter 3, the DNMTS task requires the zebrafish to select the different stimulus in the same stimulus dimension (e.g. color) between sample stimulus and test stimulus with a short retention interval in between the stimuli. The length of the retention interval provides the quantitative result of the span of the working memory.

The DNMTS task showed that the zebrafish was hard to engage the DNMTS rule in the first training phase, which is designed for introducing the rule. Although compared to the baseline the success rate was increased after I started the reward delivery, the success rate was still approximately in chance level (after the necessary-to-turn training, the chance level of success rate is 0.5) and showed statistical non-significant in the binomial test. As a result, the DNMTS is too difficult for zebrafish in terms of reasoning of the rule for further working memory test (Figure 4-8A). Nonetheless, I had tried to reduce the learner criteria to 0.7 success rate by calculating the moving averaged performance of 20 trials to force the task entered into the next testing phase. With the different retention intervals, the results showed even consequence among all of six different retention length (one-way ANOVA, p=0.927) (Figure 4-8B). The results indicated that the DNMTS task is above the ability for zebrafish in reasoning the rule in the first place and led to the failure of the test in visual-spatial working memory. A recent work used delayed-matching-to-sample (DMTS) task by comparing two

different colors and reinforced with the matching stimulus selection to test the visual-spatial working memory in zebrafish (Bloch et al., 2019). Similar to our design but manually implement every step in the experiment. Incongruent to my result, they showed that the zebrafish easily engaged in the DMTS rule within 15 sessions (150 trials) and hold the visual-spatial working memory at least for 3-4 seconds. Although it is still unknown why the results are dramatically different, their data indeed bring the possibility that the visual-spatial working memory exists in zebrafish.
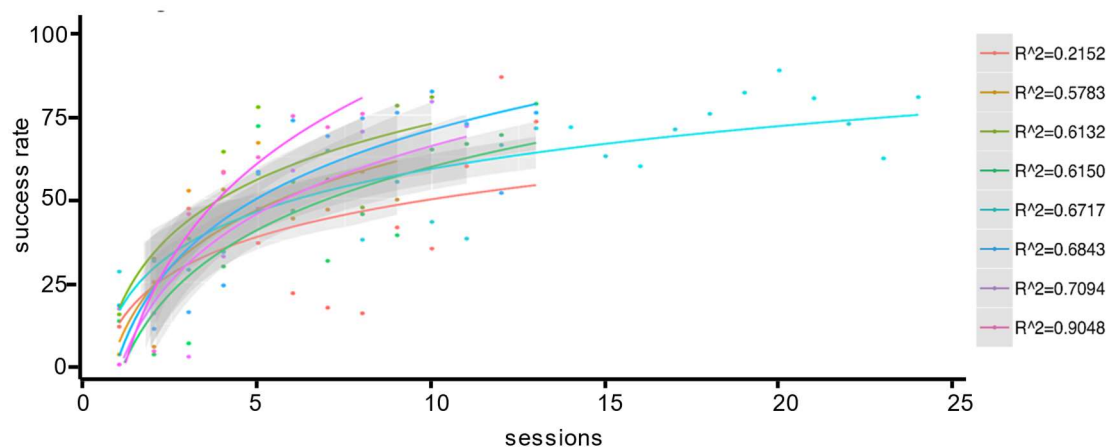
Figure 4-1. Zebrafish learn the operant behavior in a short period by reward reinforcement training. Zebrafish was reinforced with reward to associate with the specific colored cue. The plot demonstrates the training results of 8 fish. 7 of 8 fish could reach the learner criteria within 3 days (18 trials). The right panel showed the linear regress coefficient of determination (R square).
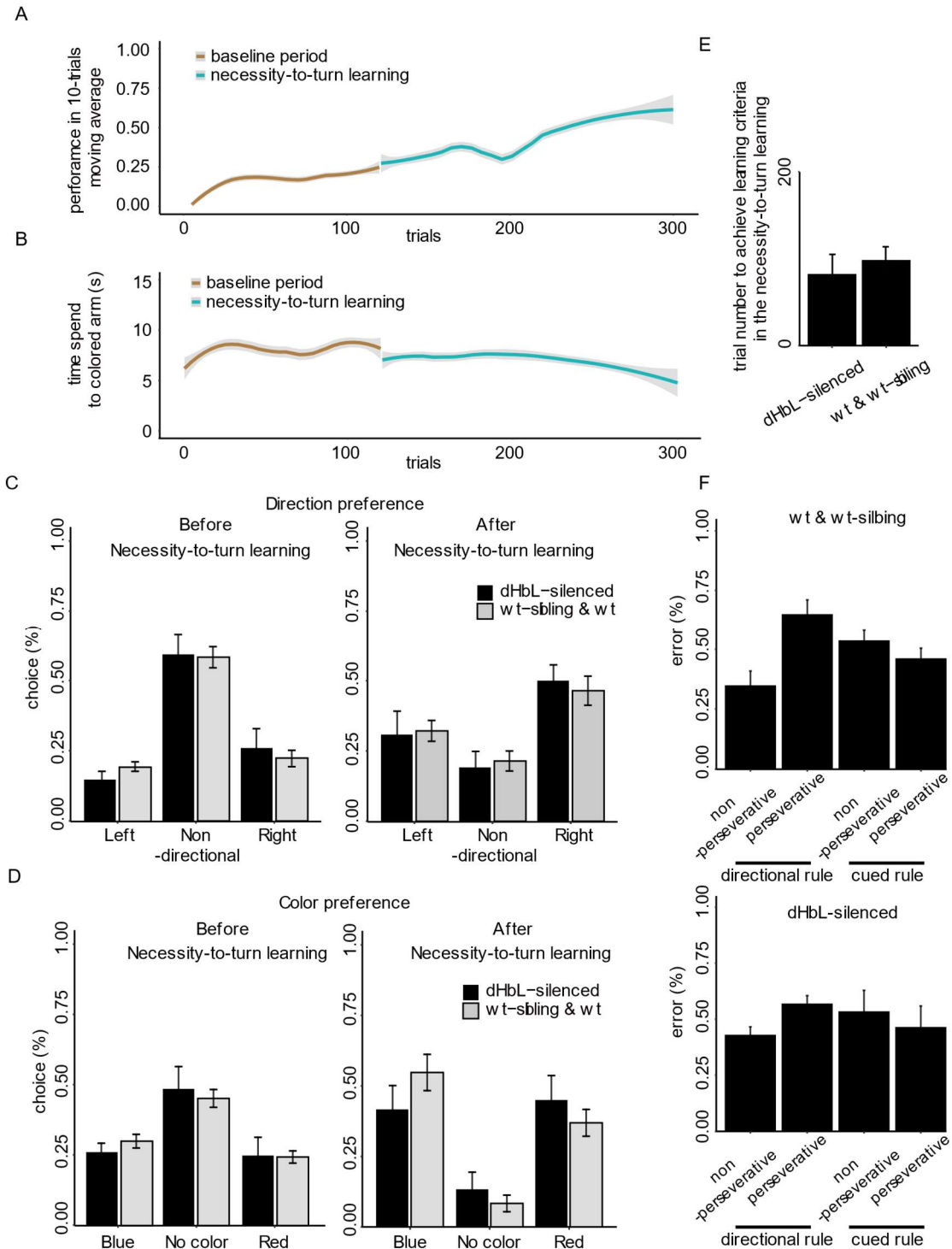
Figure 4-2. The dHbL-silenced and wild-type fish showed the same level of general learning ability, equal preference in choosing direction and color, and similar flexibility in rule-shift task with fixed initial position

(A,B) The necessity-to-turn learning of wild-type zebrafish (A) Performance shown by the 10-trials moving averages as a function of the trial numbers. (B) The time spent to reach the colored blocks from the initiation of the trials. The brown line indicates the performance and time spent in the baseline period without a reward. T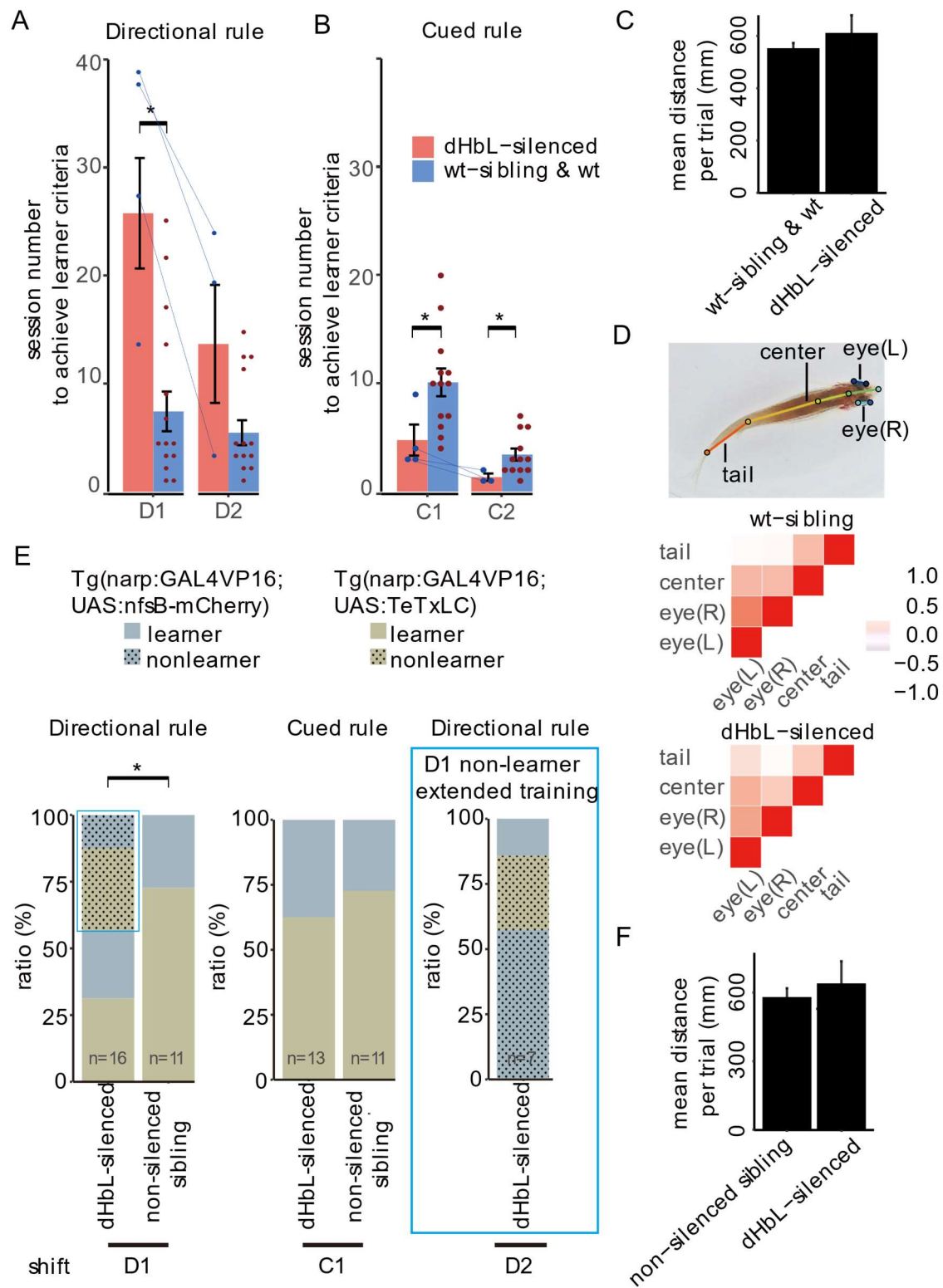he green line shows learning in the part of the introductive period (necessity-to-turn learning) after the rewards were supplied. Both curves were fitted and smoothened by linear local regression in 30 data points (degree of local polynomial, 1; confidence interval, 0.95). (C, D) The preference of colors and directions before and after fish reached the learning criterion in the necessity-to-turn learning. The non-directional choice and non-color choice were counted if fish stayed in the non-colored arm without left or right arm choice (see Figure 2A, before, e.g. N2; after, R1). (C) Preference of direction choice (mean ± SEM; wild-type and wild-type sibling, n=14, dHbL-silenced, n=4; Wilcoxon rank sum test, not significant). (D) Preference of color choice (mean ± SEM; wild-type and wild-type sibling, n=14, dHbL-silenced, n=4; Wilcoxon rank sum test, not significant).

(E) General learning ability described by the number of trials to achieve the necessity-to-turn learning criterion. The learner criterion of the necessity-to-turn learning was set to a 0.9 choice rate of either left or right arm turning across 11 consecutive trials. (mean ± SEM, wild-type & wild-type-sibling, n=14; dHbL-silenced, n=4; Wilcoxon rank sum test, not significant).

(F) Error analysis from the rule shift test period. The committed errors were divided into non-perseverative error (the wrong choice independent of the information in the previous rule) and perseverative error (the wrong choice dependent on the information in the previous rule). Percentage of error in the condition with fixed initial position (mean ± SEM, wild-type & wild-type-sibling, n=14; dHbL-silenced, n=4).
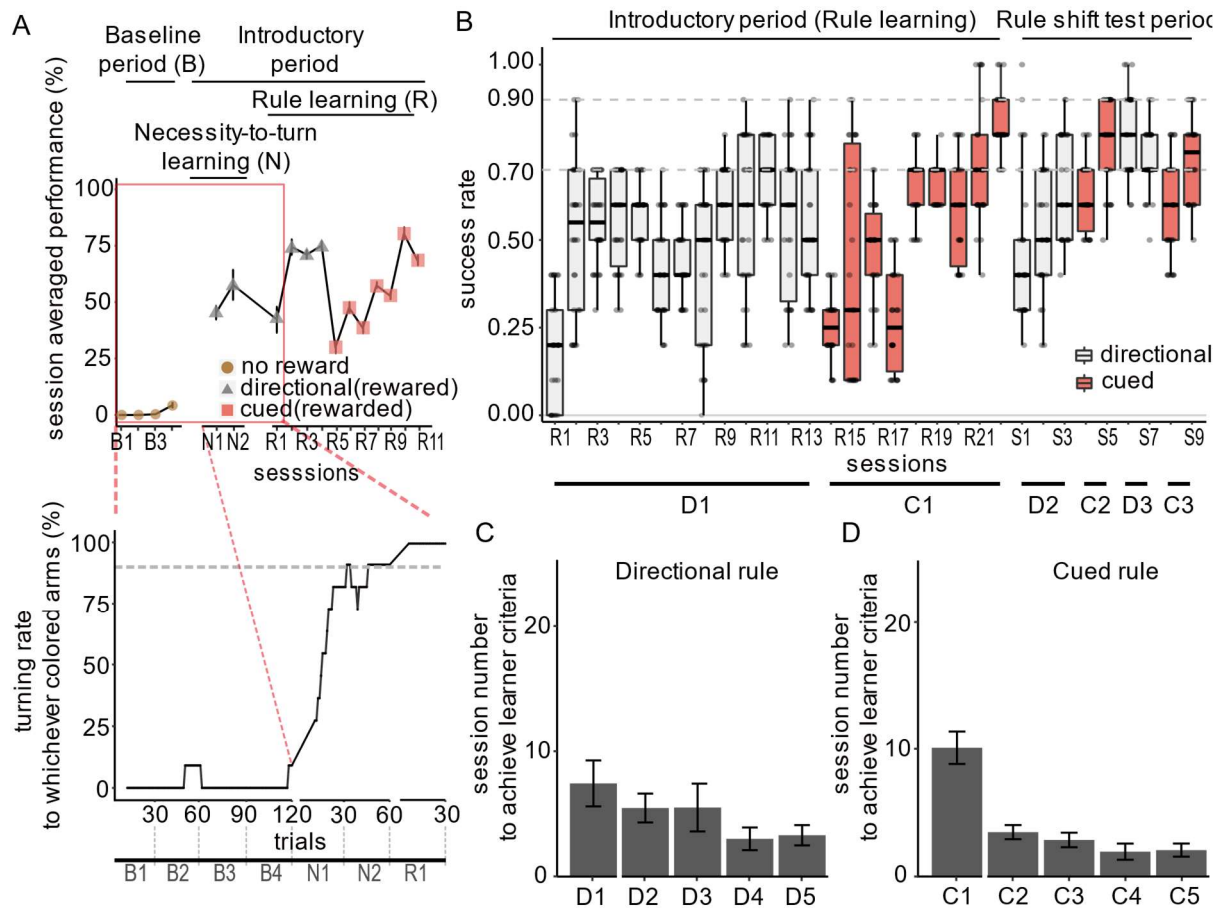
Figure 4-3. Operant learning can be achieved both by selecting directional information and by selecting cued information in wild-type zebrafish

(A) An example of performance of one fish in the baseline period and introductory period. The performance was first calculated by 10-trial moving average (10-trial average with continuous moving windows) and the 10-trial moving average data points were further averaged as a session mean performance (mean ± SEM). The introductory period could be further subdivided into two steps before or after the fish achieved the necessity-to-turn learning by counting the turning rate towards either colored arms (>0.9) (bottom panel). (B) A box plot showing the performance of one fish after learning the necessity to turn in the 10-trial moving average. The upper and lower boundary of the box indicates the 75th and 25th percentiles. The black line in the middle of the box represents the median. Upper broken line,

0.9 success rate; lower broken line, 0.7 success rate. Gray box, session in the directional rule. Red box, session in the cued rule. (C-D) The session numbers until the achievement of the learner criterion in the directional rule and cued rule of wild-type fish (mean ± SEM; n=14). C1 and D1 indicate the introductory period. C2-C5 and D2-D5 indicate the rule-shift test period.

A Directional rule

B Cued rule

C

D

center eye(L)

eye(R)

tail

wt-sibling

dHbL-silenced

E

Tg(narp:GAL4VP16;
UAS:nfsB-mCherry)
learner
nonlearner

Tg(narp:GAL4VP16;
UAS:TeTxLC)
learner
nonlearner

Directional rule

Cued rule

Directional rule
D1 non-learner
extended training

shift D1 C1 D2

F

Figure 4-4. The capability of directional rule learning is specifically impaired in the dHbL-silenced transgenic fish

(A, B) Comparison of session numbers required to achieve the learner criterion in the directional rule and the cued rule with fixed initial position between the dHbL-silenced transgenic and wild-type fish. The wild-type group included the wild-type and the dHbL-silenced wild-type siblings (mean ± SEM; wild-type, n=14, dHbL-silenced, n=4; D1, p=0.011, statistic power close to 1; D2, n.s; C1; p=0.023; C2; p=0.045; Wilcoxon rank sum test, *p⩽0.05, n.s. not significant). (C) Locomotor activity measured by averaged swimming distance per trial in rule-shift task with the fixed initial position (mean ± SEM; wild-type, n=14, dHbL-silenced, n=4; Wilcoxon rank sum test. not significant) (D) The body-eye coordination analysis. Free moving fish were annotated using DeepLabCut (Nath et al., 2019). Points were used to calculate the directional vectors and to further calculate their angles. The angle changes were computed by the angle difference between two consecutive frames (200 milliseconds). The Spearman correlation coefficient was computed to show the correlation between different angle changes of different body parts (wild-type sibling, n=3; dHbL-silenced, n=3). (E) The ratio of nonlearners in the rule-shift task with randomized initial position (directional rule, non-silenced sibling, n=11; dHbL-silenced, n=16; Fisher's exact test, p=0.022, , statistic power close to 0.91, *P⩽0.05; cued rule, non-silenced sibling, n=11; dHbL-silenced, n=13). Left, directional rule (D1); Middle, cued rule (C1); Blue box, the extended directional rule training for the fish that were categorized as nonlearner. (F) Locomotor activity of the dHbL-silenced nonlearner versus non-silenced sibling with randomized initial position rule-shift task measured by averaged swimming distance per trial (mean ± SEM; non-silenced sibling, n=11, dHbL-silenced, n=7; Wilcoxon rank sum test. not significant).
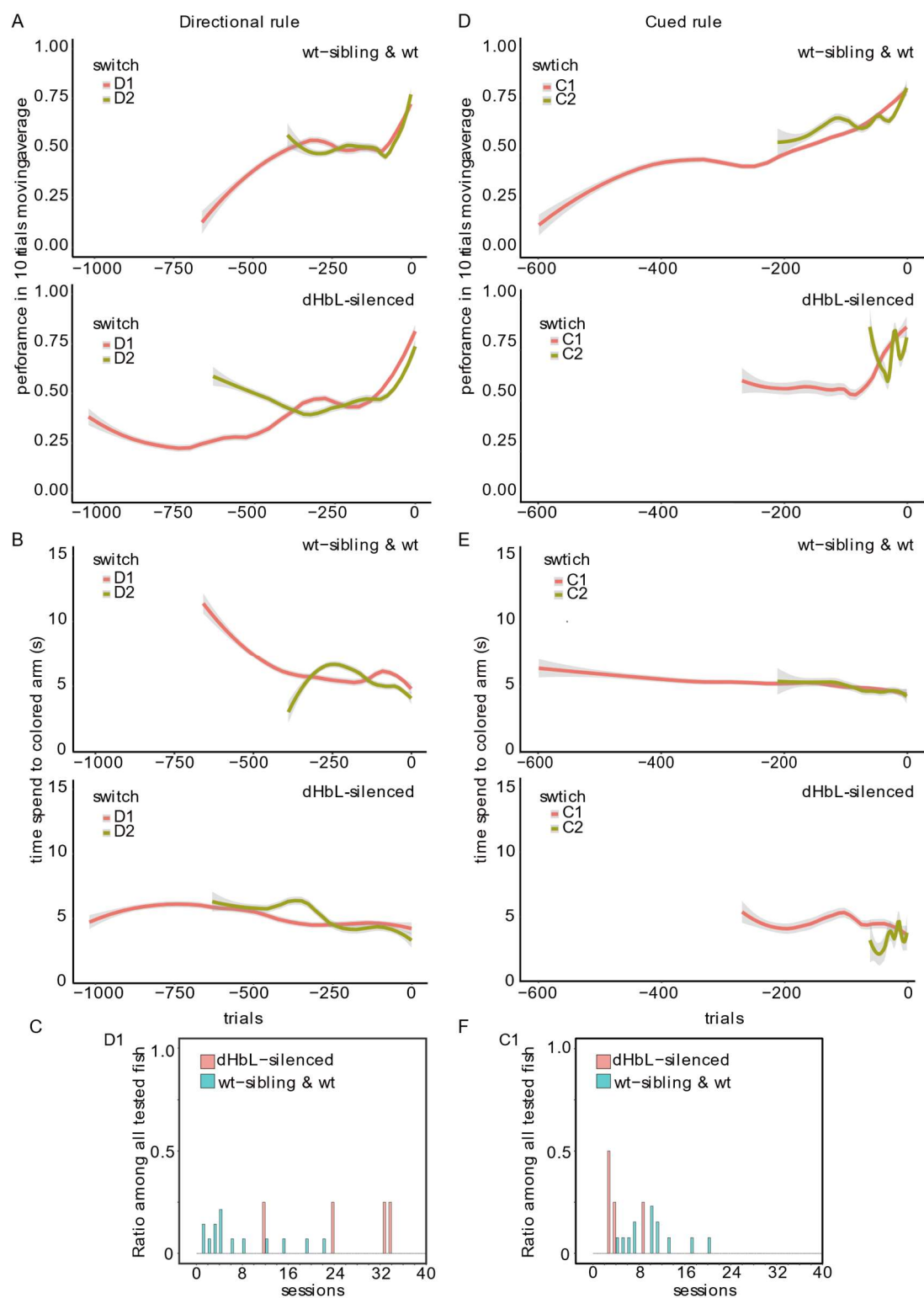
Figure 4-5. Learning performance and time spent to reach colored block from the start of trial with rule switches under the condition with fixed initial position

(A, B) directional rule (D1-D2, Figure 4-4A). (D, E) cued rule (C1-C2, Figure 4-4B) Performance

is calculated by the 10-trials moving average. Data were aligned on the trial that the fish reached the learner criterion. (A, D) The learning performance separated by the number of rule shifts. (B, E) The time spent to reach the colored blocks from the start of the trials divided by the number of rule shifts. Curves were fitted and smoothened by linear local regression of 30 data points (degree of local polynomial, 1; confidence interval, 0.95). (C, F) Distribution of learning time until reaching the learner criteria counted by session number. (C) directional rule (D1, Figure 4-4A). (F) cued rule (C1, Figure 4-4B).
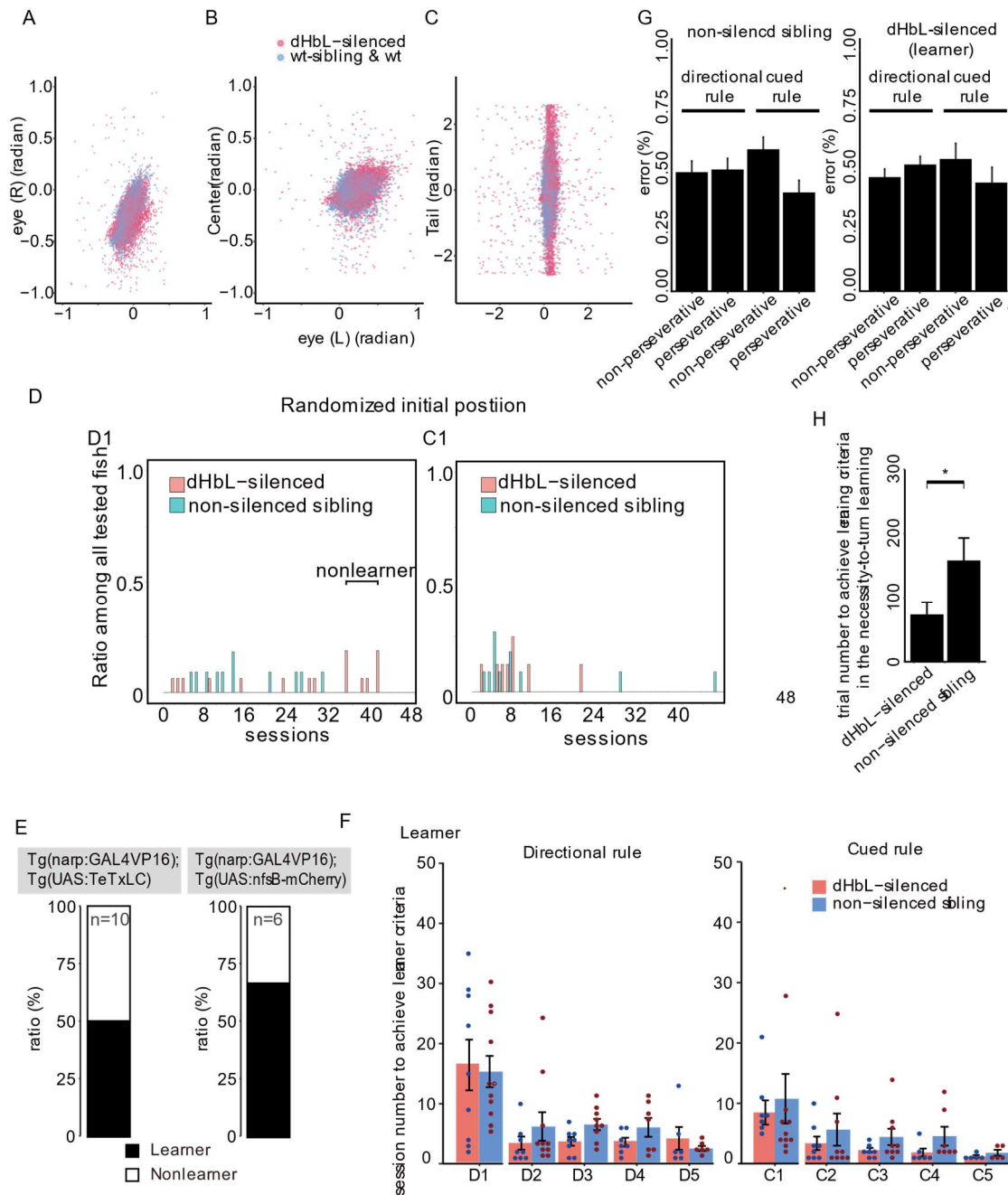
Figure 4-6. The capability of directional rule learning is specifically impaired in the dHbL-silenced transgenic fish

(A-C) Angle changes correlation of body proportion in free-moving zebrafish. The angular changes in 200 milliseconds were calculated to determine the body-eye movement coordination (wild-type & wild-type-sibling, n=3; dHbL-silenced, n=3). (A) The angle changes of the right eye and left eye directional vector (correlation coefficient, wild-type & wild-type-

sibling, r=0.62; dHbL-silenced, r=0.45; Z-test, not significant). (B) The angular changes of the center and left eye directional vector (correlation coefficient, wild-type & wild-type-sibling, r=0.35; dHbL-silenced, r=0.38; Z-test, not significant). (C) The angle changes of the tail and left eye directional vector (correlation coefficient, wild-type & wild-type-sibling, r=0.22; dHbL-silenced, r=0.15; Z-test not significant). (D) Distribution of learning time shown by the number of trained sessions in the directional rule (D1) and the cued rule (C1) in the rule-shift tasks with the randomized initial position. Data for both learners and nonlearners were mixed for calculation. The nonlearners showed variable training time because the number of the sessions that these fish could receive within the 7-day training period varied depending on how many sessions were initially spent for them to establish the necessity-to-turn learning. (E) The proportion of nonlearner of the dHbL-silenced fish separated by different silencing approaches (*Tg(narp:GAL4VP16);Tg(UAS:nFsB-mCherry)*, n=6; *Tg(narp:GAL4VP16);Tg(UAS:TeTxLC)*, n=10). (F) Comparison of the session number to achieve learner criteria. The learners of the dHbL-silenced fish versus the non-silenced sibling (mean ± SEM, non-silenced sibling, n=11; learner of dHbL-silenced fish, n=9; Wilcoxon rank sum test. not significant). (H) Error analysis from the rule shift test period in the condition with randomized initial position (mean ± SEM, non-silenced sibling, n=11; dHbL-silenced, n=9). (G) General learning ability described by the number of trials to achieve the necessity-to-turn learning criterion. The learner criterion of the necessity-to-turn learning was set to a 0.9 choice rate of either left or right arm turning across 11 consecutive trials (mean ± SEM; non-silenced sibling, n=11; dHbL-silenced, n=16; Wilcoxon rank sum test, p=0.047, *p$\leqslant$0.05).
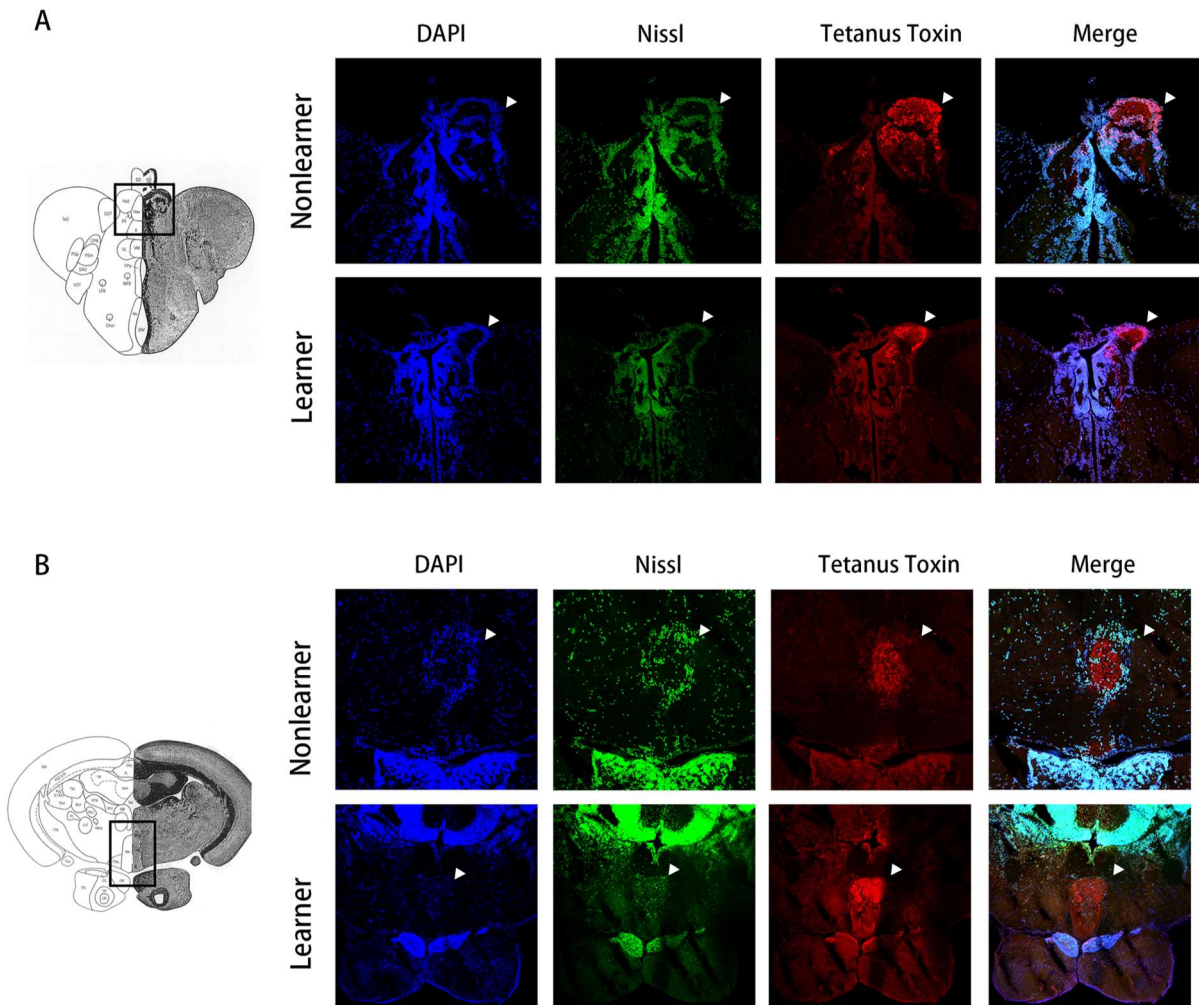
Figure 4-7. The learner fish in directional rule with randomized condition could result from the insufficient tetanus toxin (TeNT) expression. (A) Example of TeNT expression in dorsal habenula in learner and nonlearner fish detected by IHC staining. The white arrowhead indicates the left dorsal habenula. (B) The TeNT expression in dorsal IPN. The example is the same sample as shown in (A). The white arrowhead indicates the dorsal IPN.

A
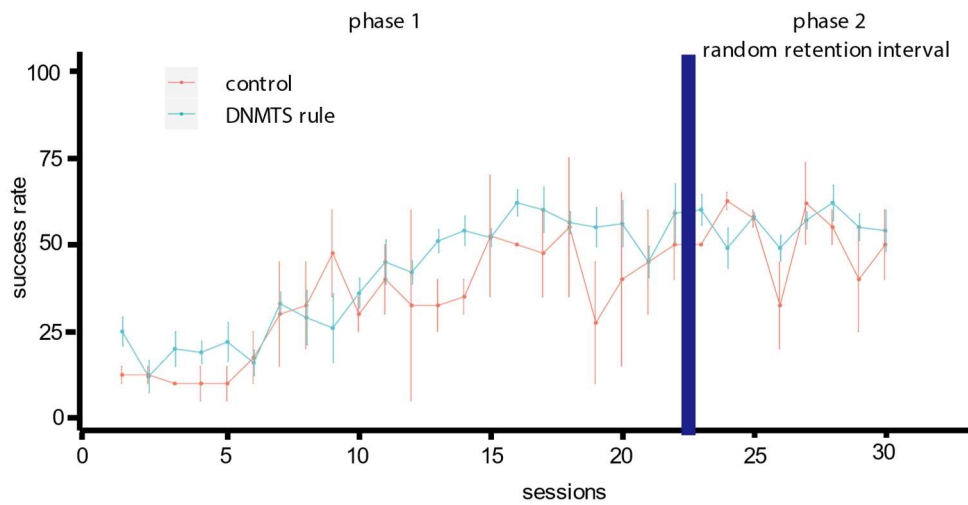
B
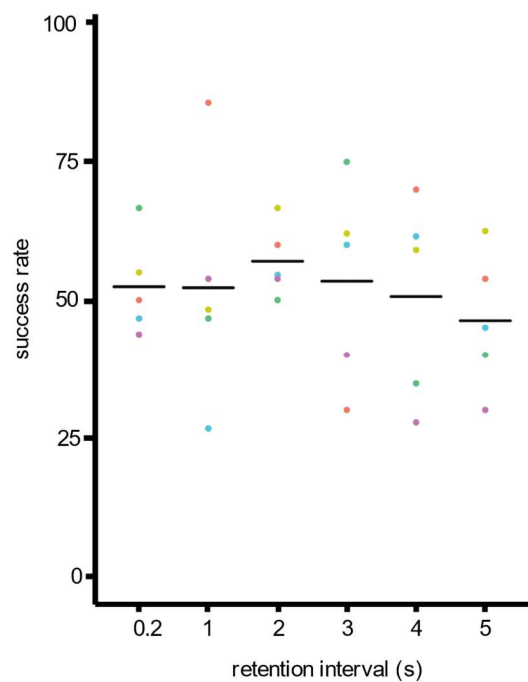
Figure 4-8. The visual-spatial working memory is untestable by DNMTS task for zebrafish.

(A) Performance in DNMTS task was calculated by 10-trial moving average. Due to the different lengths of training in different samples, the results from the phase 1 training were aligned backward from the last trial in phase 1. In phase 2, the data were aligned from the first trial of phase 2 with the minimum of trial number commonly across all samples. Dark

blue vertical line illustrates the session to separate the phase 1 and phase 2 training. Control group applied the same experimental setting as DNMTS task but without fixed rule (randomly select the matching/nonmatching rule). (B) The results demonstrate the performance in DNMTS phase 2 training with different retention intervals from 0.2 s to 5 s tested randomly. Colored dot represents the results from each sample. The average success rate is shown by the horizontal bar. The data indicate that the success rate is even among different retention intervals (one-way ANOVA test, P=0.927).

Chapter 5. Summary, Conclusion, and Discussion

Summary

Inspired by the T-maze (Thompson et al., 1980), Y-maze (Aoki et al., 2015a), starmaze (Rondi-Reig et al., 2006), and cross-maze (Packard and McGaugh, 1996; Ragozzino, 2002a; Tolman et al., 1946, 1947), I designed the plus-maze system. This system was firstly proved to be capable of establishing the operant behavior efficiently and lately applied to rule-shift task and delayed-non-matching-to-sample task in this study. I found that zebrafish quickly learned to adapt to the alternation of rules, indicating the capacity of retrieving the rule memory to prioritize information selection. It has been reported in primate studies that a proportion of cells in the prefrontal cortex are modulated by rules in the Wisconsin card sorting test (Mansouri et al., 2006). A lesion study in non-human primates revealed that the principal sulcus is more specifically involved in the maintenance of abstract rules in working memory (Buckley et al., 2009). Although it is unclear whether zebrafish have a homologous brain structure to the prefrontal cortex, we have observed a functional analogy based on the behavioral results. Our results imply that functional modules in zebrafish may exist to regulate higher-order cognitive function.

The zebrafish dHb responds to multiple sensory stimuli, such as odors (Dreosti et al., 2014a; Jetti et al., 2014a; Krishnan et al., 2014; Miyasaka et al., 2009a) and lights (Zhang et al., 2017a). In the rat nucleus homologous to the zebrafish dHb, the medial habenula (MHb), neurons showed responses to illumination to the retina (Zhao and Rusak, 2005). Furthermore, rats with the IPN lesions destabilized the heading response of head direction cells in the anterior dorsal thalamus, which suggested that the habenula-IPN circuit may be involved in generating an anticipatory internal motor cue for future head directing (Clark et al., 2009).

Therefore, the dHb-IPN circuit is a strong candidate engaged in directional information selection. Conversely, behavioral flexibility was reduced when the rodents shifted from directional navigation to cued navigation by the inhibition of dopamine D1 receptor neurons in the prelimbic-infralimbic area (Ragozzino, 2002b), the putative downstream pathway of the medial habenula-IPN circuit (Cornwall et al., 1990). Our task was also designed to test whether the dHb-IPN circuit was involved in flexibility control. We showed a similar error level in selecting the last trained rule in a new assigned rule (perseverative error) between the dHbL-silenced fish and wild-type (Figure 4-2F and 4-6G). The fish maintained the flexibility to adapt to the changed rule and repeatedly improved performance after the rule shifts. Intriguingly, the dHbL-silenced fish had a significantly slower directional rule learning speed under the condition of fixed initial position compared to wild-type fish (Figure 4-4A) and even lost the capability to become learners when we tested with randomized initial positions (Figure 4-4E). In conclusion, our findings showed that silencing the dHbL-d/iIPN circuit specifically impaired directional (left-right) related information processing during decision-making.

Although the delayed-non-matching-to-sample (DNMTS) task and the delayed-matching-to-sample task has been widely used across human and animal models to test the capacity of working memory (Cardoso-Cruz et al., 2019, 2019; Galizio et al., 2020; Goonawardena et al., 2019; Karakuyu et al., 2007; Lind et al., 2015; Stout and Griffin, 2020), It had rarely been applied in teleost. Applying the plus-maze with modified conditions, I established the DNMTS task with non-biased training (Figure 3-4). It showed that zebrafish had difficulties to adopt the rule in DNMTS task with unsupervised reinforcement training. As a result, visual-spatial working is untestable in this behavior paradigm for zebrafish.

Conclusion

Based on the experimental results, I conclude that the higher-order cognitive function is partially conserved in zebrafish, specifically in flexible learning, attention selection, and rule engagement. However, the existence of visual-spatial working memory is still inconclusive due to the incapability of reasoning for the complex rule in DNMTS task. Furthermore, I conclude that the dHbL-d/iIPN circuit is essential for directional (left-right) related information processing during decision-making.

Discussion

The IPN may combine the directional information with valence for operant behavior learning

Using a brain-wide functional screen, a recent study revealed that the IPN circuit may encode the left-right turning rate in zebrafish larvae under perceptual decision-making tasks (Dragomir et al., 2019). The authors showed that larvae zebrafish reacted to the left- or right-ward moving dots projected on the bottom of a screen with varying directional coherence. Fish used their observation of moving dots in the time scale of seconds to make judgments and revealed directional neural activities were determined by the IPN. Thus, the authors predicted that this functional unit given its extended stability could be also engaged in other behavioral circumstances such as operant conditioning. Our study provides experimental evidence supporting that the dHbL-d/iIPN pathway indeed plays an essential role in operant conditioning learning by using directional information. Thus, we show that the IPN may not only translate directional information into innate behaviors and motor response but may also

associate this with valence for adaptive behavior.

The morphological similarity of the ellipsoid body in Drosophila and the IPN in zebrafish suggests a functional homology in direction information processing

Turning decisions found in the IPN correlating with 6-slices functional wedges (Dragomir et al., 2019) is reminiscent of the heading-sensitive cells in the Drosophila central complex (Green and Maimon, 2018). The central complex is composed of the ellipsoid body and the protocerebral bridge, which respectively consist of 8 ring-structured wedges and 18 glomeruli arranged linearly. As a fly turns left or right, peak activity shifts follow in the opposite direction in the ellipsoid body and the protocerebral bridge. The ellipsoid body and the protocerebral bridge have been suggested to be the structures responsible for transmitting neural signals reciprocally at both the neuronal and synaptic levels. A similar anatomical structure has been identified in zebrafish, as the axon from the dHb surrounds the IPN forming a ring structure (Aizawa et al., 2007) (Figure 2-8C).

The attention shift between self and others may be strongly linked to the consequences of social conflicts

The subregions of the dHbL have been reported to regulate the consequences of dyadic social fighting (Chou et al., 2016b). The dHbL-d/iIPN neural transmission is potentiated in winner fish (the winner pathway), while, the dHbm-i/vIPN circuit is potentiated in the loser fish (the loser pathway) (Chou et al., 2016b). Research in mice has indicated that interbrain synchrony could reflect the social hierarchy. In other words, neurons in the prefrontal cortex in winners respond better to their own behavior compared to the loser animals. In contrast, the losers' neurons respond more to the opponent winners' behavior (Kingsbury et al., 2019). This suggests that the attention shift between self and others may be strongly linked to the

consequences of social conflict. Therefore, we speculate that the dHb may share a dual function both in controlling social behavior and in controlling internal (self-centered)/external (other-directed) selection of information. Taken together, the winning response in dyadic fighting could synergistically elicit a weight shift of information selection from other-directed to the self-centered selection, concomitantly with the brain state changing towards being more egocentric.

The left and right asymmetry of the dHb could be associated with the function of the dHb in the utilization of directional information

Previous work (Aizawa et al., 2005b; Gamse et al., 2005b) revealed the asymmetrical projection in the habenulo-interpeduncular circuit, *i.e.* the left and right dorsal habenular neurons project mainly to the dorsal IPN and the ventral IPN respectively and this was caused by the size asymmetry of the dHbL and dHbM. In addition, the functional asymmetry of the dHb has been found previously, *i.e.* the right habenula neurons receive direct input from the olfactory bulb and respond to olfactory stimuli (Dreosti et al., 2014b; Jetti et al., 2014b; Miyasaka et al., 2009b). In contrast, the dorsal left habenular neurons receive the visual projections and show the response to light illumination (Dreosti et al., 2014b; Zhang et al., 2017b). However, it remains to be studied whether such anatomical asymmetric projection of the habenulo-interpeduncular circuit could be associated with the processing of directional information.

Future works

The results of behavior examination in this study founded the essential knowledge for further investigation of the neural architecture and computation essential for higher-order brain function. Based on the results and inspired by the study of neural basis in directional information processing, I postulated two computational models as follows. To test the model and address how the elements in the model work systematically, will be my future project for this research.

Model for the directional information encoding and the involvement of the dHb-IPN circuit for top-down learning

The evidence showed that the dHbL-d/iIPN and the dHbm-i/vIPN circuits are engaged in the dual function in the determination of social conflict for the winner or loser (Chou et al., 2016a) and information selection between internal/external information shifts (Cherng et al., 2020). Considering a model that matches the current findings, I propose the hypothesis that the dHb-IPN circuit serves as a primary classifier to enhance the sets of stimuli-value association.

The model is separated into two parts to postulate the possible mechanisms from two viewpoints: 1. What is the neural framework to construct the rule and facilitate the specific behavior depending on the selected information? 2. What is the neural design to compute the directional information?

I defined the internal cue as the integrated information including the self-generated signal such as motor-command plus vestibular sensation. On the contrary, the external cue is the integrated information that excludes the self-generated signals which are purely elicited

by the external stimulus, including vision, olfaction, somatosensory, and interoception. The distinct types of information are conveyed through the IPN to the different downstream nuclei where the d/iIPN transmits the internal information to the dorsal tegmental area (DTA) (the putative homologous nucleus of periaqueductal gray (PAG) in mammal) (Agetsuma et al., 2010; Chou et al., 2016b) whereas the i/vIPN carries the external information through medial raphe to the ventral entopeduncular nucleus (vEN)(Amo et al., 2014) (Figure 5-1A). The PAG and the vEN may play a role as the regions which learned the basic distinction of internal information or external information respectively through reinforcement learning (primary rule, Figure 5-1). The PAG and the EN have been demonstrated as the neural substrate in reinforcement learning (Johansen et al., 2010; Li et al.). These two brain nuclei have been revealed with strong anatomical interactions to the thalamus, the frontal cortex, and the striatum (An et al., 1998; Javed and Cascella, 2020; Johnson, 1961; Mantyh, 1983; Omelchenko and Sesack, 2010; Wu et al., 2014; Zheng and Monti, 2019). Thus, the classified and weighted information could be conveyed into the cortico-striato-thalamo-cortical loop. Within the loop, the information is dissected with specific features and undergoes the reinforcement learning again with the specific features (Figure 5-1). This part of reinforcement learning could be similar to the theory proposed by Sutton and Barto, the complete serial compound stimulus, which is the hypothetical elements reflecting the sensory input with serial time points, sends input into the ventral tegmental area (VTA) for reinforcement learning (Sutton and Barto, 1987, Schultz et al., 1997). Additionally, it is also reminiscent of the attention filter model that selects the attended information with hierarchy (Treisman, 1960). As a result, the multiple features weighted with value are formed in the loop. It is worth mentioning that the dopamine activity is well known to be related to the expectation error in model-free reinforcement learning. However, recent evidence showed that dopamine expectation error can be extended to more dimensions rather than scaler

value (Langdon et al., 2018; Wunderlich et al., 2012). This brings the hypothetical explanation that the multiple features at a certain layer of the hierarchical classifier are associated with the dopamine neuron for expectation error calculation for updating the feature weights in learning. Finally, I propose that this model can be used to facilitate information utilization by regulating the plasticity in the dHb-IPN circuit (Top-down pathway) (Figure 5-1, dark blue arrow). An alternative version of this model is that the information from DTA does not directly send to the cortico-striao-thalamus loop. Instead, it is conveyed into the cerebellum. Therefore, the internal information and external information is calculated in the different downstream loop (Figure 5-2). Considering the connectivity between cerebellum and cortex, the cerebello-cortical loop and the cortical-striato-thalamo-cortical loop might function competitively to facilitate the plasticity change in the dHbL-d/iIPN and dHbm-i/vIPN information classification (Brissenden; Gao et al., 2018). The idea of the model is reminiscent of the dynamic filter theory proposed by Arthur Shimamura (Shimamura, 2000) (Figure 2-7B). However, instead of the PFC-posterior associate cortex, my model proposes the cortical-striato-thalamo-cortical loop filters the bottom-up information from the IPN circuit.

Now, I would like to focus on the IPN circuit to postulate the possible mechanism in explaining the neural design to establish the information with the direction feature. In the model, the central pattern generator (CPG) axis is proposed to spontaneously generate the signal that controls the left and right tail flip (Berg et al., 2018; Thiele et al., 2014). Because of the mutual inhibition connection in the left unit (Figure 5-3, CPG blue ball) and right unit (Figure 5-3, CPF red ball), with the same strength of the initial activity, the left and right CPG would be activated with repeated rhythm. The CPG axis is suggested as the final module to receive both the signal from the d/iIPN and the v/iIPN respectively.

The dHbm-i/vIPN is assumed to convey the external information to CPG. When one side of specific sensory information such as color is stronger than the other side, the stronger

input is conveyed into one side of CPG, e.g. right side, the inclined right activity would therefore increase the activity of right tail flipping stronger than the left tail flipping. As a result, the fish moves toward the asymmetrical sensory input toward one side of the body axis. However, this is not yet the direction information, nevertheless, it can control the turning. On the other hand, the integrated internal signal which incorporated the self-generated commands is conveyed into the dHbL-d/iIPN circuit (Figure 5-3A, colored in green; left internal information input is represented by the blue line; right internal information input is represented by the red line). I propose an architecture of the directional sensing unit in the d/iIPN circuit with parallel aligned directional sensing unit (Figure 5-3A, direction sensing unit 1-6). The directional sensing unit is composed of an interneuron that inhibits the excitatory neuron, which sends the information to CPG. The directional sensing units symmetrically project to the left and right CPG with an equal number (Figure 5-3A, directional sensing units). When the input strength or timing is equal from the left and right side, each direction sensing unit gets an equal amount of stimulus by the summation of left and right inputs (Figure 5-3B, top row, symmetrical LR input). However, once the balance is disrupted by the different propagation time or strengths, the unbalanced left and right input skews the summation and causes a specific direction sensing unit with less inhibition (Figure 5-3B, bottom row, asymmetrical LR input). As a result, the excitatory neuron of the same direction sensing unit is activated more than other units. Because the neuron is topologically aligned, this activated direction sensing unit can represent the specific angle of body direction, and the directional information is therefore generated.

Compare to the vIPN framework mentioned above, because the integrated internal signal consists of self-generated commands, it can work without environmental input. In other words, even with a simple movement, the animal can sense the directional information. This model may match the result in the study, silencing the dHbL-d/iIPN leads to the

impairment of directional-information-associated learning.
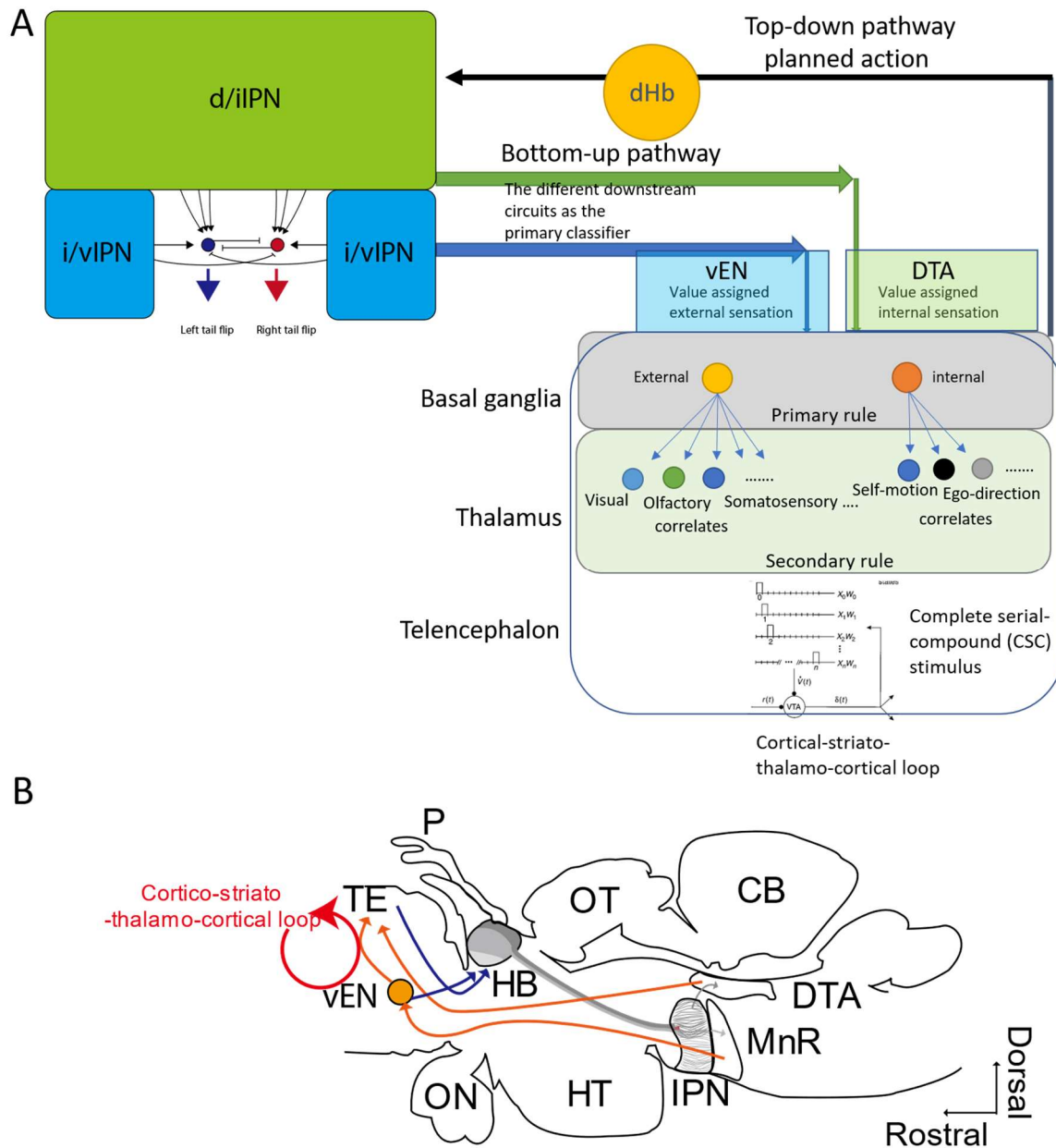
Figure 5-1. The model illustrates the involvement of the dHb-IPN circuit for top-down learning. (A) The dHbL-d/iIPN sends information to the cortico-striato-thalamo-cortical loop through the dorsal tegmental area (Green arrow, bottom-up pathway) whereas the dHbm-i/vIPN sends information to the cortico-striato-thalamo-cortical loop (Blue arrow, bottom-up pathway) through the ventral entopeduncular nucleus. Dark yellow circle, neurons receiving internal information for the primary rule. Orange circle, neurons receiving external

information for the primary rule. (B) The anatomical illustration for bottom-up pathway and top-down pathway. Orange arrow, the bottom-up pathway. Dark blue arrow, the top-down pathway. Red loop, cortico-striato-thalamo-cortical loop. TE, telencephalon. OT, optical tectum. CB: cerebellum. DTA, dorsal tegmental area. ON, optic nerve. HT, hypothalamus. HB, habenula. IPN, interpeduncular nucleus. vEN, ventral entopeduncular nucleus.

Figure 5-2. The alternative model illustrates top-down learning with the interaction of dHb-IPN and cerebellum. (A) The dHbL-d/iIPN sends internal information to the cortico-striato-thalamo-cortical loop through the dorsal tegmental area (Green arrow, bottom-up pathway) whereas the dHbm-i/vIPN sends external information to the cortico-cerebella-cortical loop

(Blue arrow, bottom-up pathway) through the ventral entopeduncular nucleus. Dark yellow circle, neurons receiving internal information for the primary rule. Orange circle, neurons receiving external information for the primary rule. (B) The anatomical illustration for bottom-up pathway and top-down pathway. Orange arrow, the bottom-up pathway. Dark blue arrow, the top-down pathway. Red loop, cortico-striato-thalamo-cortical loop. Green loop, cortico-cerebella-cortical loop. TE, telencephalon. OT, optical tectum. CB: cerebellum. DTA, dorsal tegmental area. ON, optic nerve. HT, hypothalamus. HB, habenula. IPN, interpeduncular nucleus. vEN, ventral entopeduncular nucleus.
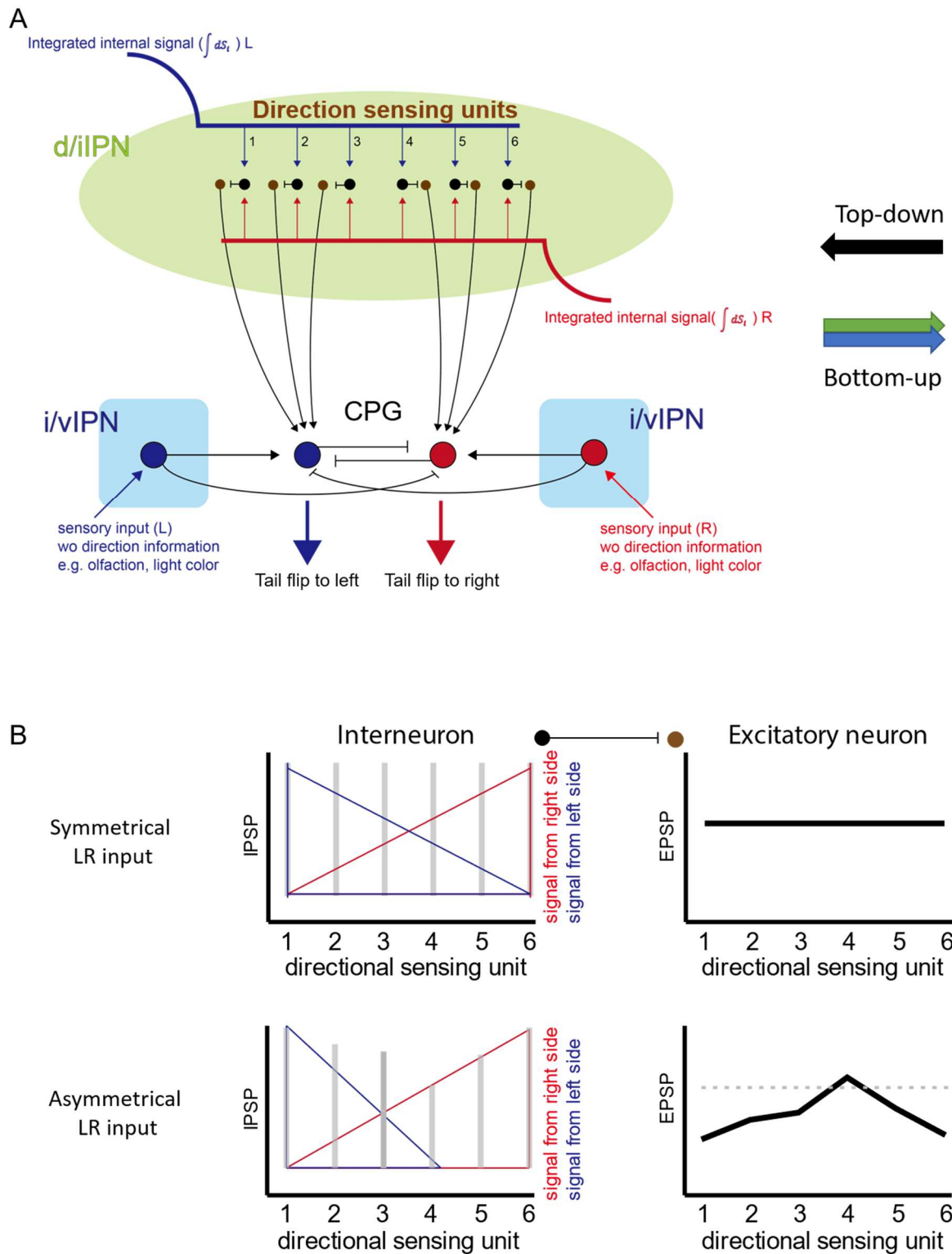
Figure 5-3. The model of neural encoding for directional information. (A) The green region

and blue region represent the d/iIPN and the i/vIPN respectively. The central pattern

generator (CPG) is suggested as the final receiver to control the left tail flipping and right tail flipping. Two parts of the i/vIPN separately receive the sensory input from the left side or right side and relay the signal to the CPG (external information). The neurons in the d/iIPN are aligned to receive the integrated internal signal (internal information) from the left and the right body side (direction sensing units 1-6). A direction sensing unit consists of an interneuron (black circle) that receives the internal information input and inhibits the excitatory neuron (blown circle) that propagates the neural signal to CPG. (B) The scheme illustrates the putative activities of the interneurons and excitatory neurons in the direction sensing units. The upper panel demonstrates the balanced sensory input from the left and right sides (symmetrical LR input). The bottom panel demonstrates the biased input that the right side receives the stronger or earlier signals than the left side. Left column: gray bar, the interneuron activities; red line, right sensory input; blue line, left sensory input. Right column: the excitatory neuron activities; gray broken line, the threshold for the action potential.

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