氏　　名	Pradeep Lal
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論 文 審 査 委 員	主　査　教　授　岩里　琢治 教　授　広海　健 准教授　小出　剛 准教授　平田　普三 教　授　山本　直之　名古屋大学

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論文内容の要旨

Summary of thesis contents

The study of learning and memory in zebrafish

Learning and memory are important for animals to perform activities skillfully and to cope with the changing environment. Learning is the process of acquisition of new knowledge, and memory is the encoding, storage and retrieval of learned information. Aversive emotion fear is conserved across vertebrate species and fear-motivated learning has been a powerful tool to study the mechanism of learning and memory. In mammals, forebrain structure amygdala plays a central role in mediating fear-motivated learning. Amygdala receives and integrates sensory inputs from brain structures such as hippocampal formation, neocortex and thalamus and hence it is a plausible site for associative emotional learning. Divergent projections from amygdala to hypothalamus and brainstem have been shown to mediate fear response and projection from amygdala to striatum possibly controls operant behavior such as aversive response. These studies have demonstrated the structure-function relationships of mammalian brain for behavioral output during fear-motivated learning but the functional neural circuits modulating aversive learning are still under investigation. Here, I aimed to study neural circuits essential for aversive learning using a model vertebrate zebrafish.

Zebrafish is a freshwater teleost fish and has been used as a model because of high fecundity, rapid growth, transparent body during embryonic stage and the ease of maintenance. Zebrafish exhibits rich repertoire of learning behaviors. The well-developed forward genetic tools make it an attractive vertebrate model to study brain function. Our understanding of the adult zebrafish brain is mainly limited to comparative neuroanatomy, histological and gene expression analysis. The structure-function relationships of fish brain have been described using surgical ablation studies. The surgical ablation studies in goldfish have demonstrated that medial telencephalon is essential for retention of aversive memory and lateral telencephalon is essential for retention of temporal, relational and spatial memory. Thus, fish medial and lateral pallium may correspond to mammalian amygdala and hippocampus, respectively. Recent ablation study using zebrafish demonstrated that the central zone of dorsal telencephalic area (Dc) in zebrafish brain is essential for retrieval of long-term aversive memory and Dc has been suggested to be functional equivalent of mammalian neocortex. The above surgical ablation studies are useful to understand the relationship between the structure and the function of the brain, but to decipher the neural circuits mediating learning, it is important to be able to selectively manipulate specific brain structures or neuronal types. Hence, in this study, I

employed genetic approaches to selectively label specific brain regions and manipulate their function to understand their role in emotional learning.

First, I performed genetic screens using *Tol2* transposon mediated gene trap and enhancer trap methods and obtained transgenic fish lines that expressed Gal4-transactivator in specific tissues. In these lines, the Gal4 expression is visualized with upstream activating sequence (UAS) linked GFP transgene. To identify transgenic lines expressing Gal4 in sub-regions in the adult brain, I observed the heads of adult fish of 349 transgenic fish lines and identified 108 Gal4 lines that had GFP fluorescence inside the head. I isolated the brains of these 108 fish lines and confirmed the GFP expression in the brain in all of these lines. From these Gal4 lines, I selected 77 lines that showed strong and localized GFP expression, and analyzed them by making 100 μm serial coronal slices. I found various unique GFP fluorescence patterns such as lateral telencephalon, medial telencephalon, hypothalamus, habenula, cerebellum, hindbrain and etc. I annotated the Gal4 expressing regions of these 77 transgenic lines and based on Gal4 expression pattern, I classified them into three groups: forebrain, midbrain, and hindbrain groups.

Next, to study emotional learning in zebrafish, I adopted the previously reported active avoidance response system for goldfish and established two-way active avoidance response assay for zebrafish. In this assay, I used a shuttle box divided into two equal compartment by an opaque wedge, green light as conditional stimulus (CS) and electric shock as unconditional stimulus (US). Fish were trained to escape from CS in order to avoid US. My results demonstrated robust avoidance response to CS in zebrafish. Thus, I established a behavioral assay system to study emotional learning in zebrafish.

Finally, I selected 48 Gal4 lines that showed strong Gal4 expression in the forebrain for behavioral analysis. I crossed these lines with the UAS:BoTx-GFP effector fish and inhibited the function of Gal4 expressing neurons by expressing botulinum neurotoxin. Out of these 48 Gal4 double transgenic fish, 9 fish lines could not survive to adulthood. I selected 30 double transgenic fish lines out of 39 lines that showed strong GFP fluorescence in the forebrain and analyzed them for active avoidance learning and found that 18 lines exhibited abnormalities in the active avoidance response. Most of these fish showed Gal4 expression in multiple brain regions including forebrain, midbrain and hindbrain structure. By observing the Gal4 expression in these 18 lines, I identified two transgenic lines, named gt-70A and gt-120A, that had Gal4 expression specific to a subpopulation of neurons in the

medial zone of dorsal telencephalic area or dorsomedial telencephalon (Dm) in the brain suggesting that these Gal4 expression neurons in Dm are essential for this behavior. In mammals, forebrain structure amygdala mediates active avoidance response behavior. From thisThe study and previous anatomical and developmental studies, teleost Dm may be functional equivalent of mammalian amygdala.

Furthermore, I analyzed the connectivity of the Gal4 expressing neurons in these two transgenic lines and found that Dm neurons have well-organized connections with dorsal nucleus of ventral telencephalic areas (Vd), entopeduncular nucleus (EN) and hypothalamus. Zebrafish Vd has been proposed to be putative striatum. In mammals, amygdala-striatum neural circuit possibly mediates operant behavior such as escape response from harmful stimulus and hence Dm-Vd neural circuit in zebrafish may have similar role. In mammals, hypothalamus is involved in fear response including modulation of heart rate, blood pressure and release of glucocorticoids. Hence, the neural circuit between Dm and hypothalamus may modulate these behaviors during avoidance response behavior. In zebrafish, entopeduncular nucleus has axonal projection to habenula (Hb) and habenula has been shown to mediate experience dependent modification of fear response. Hence, the neural circuit involving Dm, EN and Hb may play a crucial role in fear response during active avoidance response. Thus, I have identified possible functional neural circuits mediating active avoidance response behavior in zebrafish.

Active avoidance response behavior involves learned aversion to conditional stimulus (CS). In mammals, amygdala mediates both learned and innate fear. To analyze the role of neurons in Dm in innate fear, I studied alarm response behavior using skin extract of zebrafish and found that gt-70A;UASBoTx-GFP and gt-120A;UASBoTx-GFP fish showed normal alarm response behavior. This suggests that Gal4 expressing neurons in these lines may not be involved in regulating innate fear response only mediate learned fear response.

Mammalian amygdala has also been shown to mediate anxiety. In zebrafish, dark-light preference assay has been used to measure anxiety-like behavior. Wild type adult zebrafish has innate preference to darker region. I analyzed the gt-70A;UASBoTx-GFP and gt-120A;UASBoTx-GFP fish in dark-light preference assay and found that these fish show no change in preference compared to wild type fish. Hence, the Gal4 expressing neurons in Dm of these lines may not modulate anxiety-like behavior and are specifically involved in aversive learning.

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In summary, by employing a genetic approach, I created a repertoire of Gal4 transgenic lines that express Gal4 in specific brain regions and thus successfully subdivided the zebrafish brain into various Gal4 expressing regions. Using the isolated transgenic lines and the active avoidance response assay, I identified a subpopulation of neurons in dorsomedial telencephalon (Dm) essential for avoidance response behavior. Previous surgical ablation study has shown that Dc was essential for retrieval of long-term active avoidance memory and Dc was suggested to be functional equivalent of mammalian neocortex. Our finding show that Dm is essential for acquisition of aversive memory and Dm may have similar function as that of mammalian amygdala. Furthermore, Dm has well-organized projections to other forebrain structures such as dorsal nucleus of ventral telencephalic areas (Vd), entopeduncular nucleus (EN) and hypothalamus and these neural circuits may play crucial role in mediating aversive learning in zebrafish.

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博士論文の審査結果の要旨

Summary of the results of the doctoral thesis screening

嫌悪学習は動物の生存に必須の行動であり、これまでマウスなど哺乳類をモデルとして精力的に解析されてきたが、その基盤となる神経回路の理解はまだ十分でない。Lal 君は、神経科学研究において大きな将来性をもつ新しいモデル生物であるゼブラフィッシュを用いて嫌悪学習にかかわる神経回路の研究を行った。

研究の歴史の浅いゼブラフィッシュでは、マウス等と比較すると、現時点では様々な周辺リソースの整備が不十分である。そこで Lal 君は、キンギョなどでの先行研究を参考にして、嫌悪学習に基づく実験系の確立を試みた。その結果、電気ショック（非条件刺激）と光刺激（条件刺激）を組み合わせて与えることで光刺激のみでゼブラフィッシュが回避行動をとるようになる「能動的回避行動（active avoidance behavior）」が成立する条件をみつけ、その回避行動の程度を定量的に解析するシステムの構築を行った。

次にその回避行動に責任のある脳の領域を同定するために Lal 君は、所属研究室で独自の Tol2 トランスポゾン法を用いて開発された、多様な Gal4 トランスジェニックフィッシュ系統を活用することを考えた。Lal 君はまず、349 系統のトランスジェニックフィッシュの脳における Gal4 発現の領域特異性を、UAS-GFP レポーターと交配することにより網羅的に調べた。その中から成魚の脳で Gal4 が強く発現する 48 系統を選んだ。次いで、それらの系統を用いてボツリヌストキシンを発現させ、Gal4 発現領域から投射される軸索終末でのシナプス放出を抑制した。それらのゼブラフィッシュで上述の能動的回避行動実験を行い、18 系統で回避行動に異常が起きることを示唆する結果を得た。Lal 君は 18 系統の中から Gal4 の発現パターンが脳の終脳背側野内側部（dorsomedial telencephalon）に限局する 2 系統に特に注目し、これらの系統はどちらも終脳背側野内側部特異的遺伝子である Emx3 領域にトランスポゾンが挿入されていることを明らかにした。以上の結果は嫌悪学習行動の一つである能動的回避行動における背内側終脳の重要性を示唆するものである。さらに Lal 君は Gal4 発現細胞由来の軸索を GFP で可視化することによって、終脳背側野内側部の神経細胞が脚内核（entopeduncular nucleus）と視床下部（hypothalamus）に投射することを見いだした。さらに、終脳背側野内側部の神経細胞から終脳腹側野背側部（dorsal zone of ventral telencephalon）へプロセスが伸びていることも見いだし、これらの神経核間の回路の存在を示唆した。マウスなど哺乳類では嫌悪学習において扁桃体が中心的な役割を担うことが知られているが、Lal 君はゼブラフィッシュでは背内側終脳がマウス等の扁桃体に相当すると考えている。

ゼブラフィッシュは哺乳類であるマウスと比較すると圧倒的にシンプルな脳をもっている。また、近年飛躍的に進歩しているイメージング技術と組み合わせることも容易で、特定行動の基盤となる神経回路の全体像を理解する上で、マウスなど哺乳類と比べて大きなアドバンテージをもっており、大きな将来性を有する。Lal君の研究は、ゼブラフィッシュで特定の行動を定量的に調べる実験系の構築を行ったこと、および、ゼブラフィッシュ遺伝学のメリットを最大限に活用して能動的回避行動を特定の脳部

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位と関連づけたこと、さらに将来に向けて嫌悪学習の神経回路を理解するための実験基盤をつくったという観点で先導的であり重要と考えられる。以上の理由から、Pradeep Lal君の申請論文は博士号授与の要件を満たすと審査員全員一致で判断した。

学位申請論文の研究内容に関する公開発表において、Lal君は、明解な発表を行い質問に対して的確な応答を行った。また、その後引き続き行われた非公開審査においても同様に的確な質疑応答を行った。これらの内容から、Lal君が自分自身の研究分野および周辺分野に関する十分な知識を有し、主体的かつ意欲的に研究を遂行してきたことが明らかであり、Lal君の研究者としての優れた素質が示された。また、Lal君は日常的に英語を用いて研究を行っており、学位申請論文も明解な英語で書かれている。公開発表および非公開審査の発表と質疑応答のすべては英語で行われており、英語能力に関しては申し分ない。

以上のことを総合的に鑑みて、Pradeep Lal君が学位を取得するに十分な水準に達していると、審査員全員一致で判断した。