様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

# 科学研究費助成事業　　研究成果報告書

平成　２９　年　　６　月　１５　日現在

機関番号：　１２１０２
研究種目：　挑戦的萌芽研究
研究期間：　2015～2016
課題番号：　１５Ｋ１４８７４

研究課題名（和文）フォワード・ジェネティックスを用いた先天的恐怖の分子メカニズム解明

研究課題名（英文）Unveiling the molecular mechanism of innate fear using forward genetic screen approach

研究代表者

Ｌｉｕ　Ｑｉｎｇｈｕａ（LIU, Qinghua）

筑波大学・国際統合睡眠医科学研究機構・教授

研究者番号：９０７２３７９２

交付決定額（研究期間全体）：（直接経費）　　3,000,000円

研究成果の概要（和文）：申請者らはマウスにおける捕食者由来の匂い分子を用いた先天的恐怖の誘発法と測定システムを構築し、これらとランダム点突然変異マウスを用いたフォワード・ジェネティックスの手法を組み合わせることで、先天的恐怖反応に異常を示すマウス2系統の樹立に成功した。本研究では、1)遺伝学的連鎖解析と全エクソームシーケンスを駆使して、上記2系統中1系統の原因遺伝子変異（Popcorn突然変異）を同定し、2)突然変異の因果関係を確認するために、CRISPR / Cas9システムによってPopcorn突然変異マウスを作製し、3)Popcorn突然変異マウスのなかに社会的に従順および不安の2つの表現型を同定した。

研究成果の概要（英文）：To understand the molecular mechanism of fear, we developed a robust predator odor-based innate fear assay, and conducted a forward genetic “FEAR” screen in N-ethyl-N-nitrosourea (ENU)-mutagenized mice. To date, we have successfully established two mutant pedigrees with abnormal fear behaviors, and identified the causative mutation in one mutant pedigree named Popcorn. We recreated Popcorn mutant mice by CRISPR/Cas9 system to confirm the causality of the Popcorn mutation. Moreover, we discovered that the Popcorn mice also exhibited socially submissive and anxiety phenotypes.

研究分野：　農学

キーワード：　フォワード・ジェネティックス　先天的恐怖　精神障害　モデルマウス

## １．研究開始当初の背景

Fear is a basic emotion that enhances animal survival by triggering appropriate defensive responses (e.g. freeze, fight or flight) to perceived danger. Uncontrolled fears are linked to a variety of anxiety disorders, such as phobia, obsessive compulsive disorders and post-traumatic stress disorders (PTSD). However, the molecular bases of fear and anxiety disorders are unknown. Fear can be evoked by both innate and learned mechanisms. Whereas learned fear is acquired through experience, innate fear (e.g., predator induced fear) is genetically encoded, Thus, we hypothesized that it might be feasible to study the molecular basis of innate fear using a forward genetic approach.

To identify core fear genes by forward genetics, we used a thiazoline-related fear odor (tFO), a highly potent analog of predator scent TMT, to develop a robust innate fear assay that is suitable for high-throughput mouse screening, Specifically, individual mice were exposed to tFO and video recorded for 20 minutes. Using Freezeframe to quantify freezing as a readout of fear, wild-type mice showed an average 77% freezing time in response to tFO. This assay has a tight relative standard deviation (RSD = ~10%), making it feasible to screen for fear phenodeviants whose freezing rate deviated from the average by at least 3 standard deviations (< 47%). Using this assay, we carried out a forward genetic dominant screen. We have screened 1,596 ENU-mutagenized F1 male mice and established two dominant mutant pedigrees (Fig. 1). Mutants of one pedigree exhibited an unusual phenotype of jumping up to ~2,500 times/20 minutes when exposed to tFO, hence termed Popcorn pedigree. We propose that Popcorn is a putative “fearful” mutant.

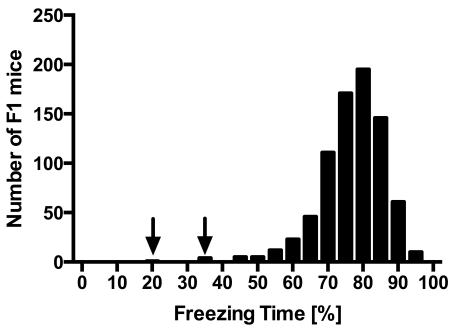


**Fig. 1.** Freezing rate distribution of F1 mice screened. Arrows indicate the phenovariant founders of two mutant pedigrees.

## ２．研究の目的

In this study, we planned to primarily focus on achieving three objectives: (1) identifying the *Popcorn* mutant gene by genetic mapping and whole-exome sequencing; (2) verifying the causality of the mutation by generating the same mutation in wild-type mice using CRISPR technology; (3) determining if Popcorn mutant ($Pop^{m/+}$) mice are truly fearful mutants by a host of neuroscience and behavior assays. These studies will demonstrate the proof-of-principle for our FEAR screen, which represents the first forward genetic screen on emotion.

## ３．研究の方法

(1) Identifying the *Popcorn* mutation by Linkage analysis and exome sequencing.

The F1 $Pop^{m/+}$ male (C57BL/6J/N) was mated with wild-type C57BL/6N females to produce N2 and N3 generations. The C57BL//6J (JAX) and C57BL/6N (NIH) strains differ by ~1,000 single nucleotide polymorphisms (SNPs). A set of 96 SNP markers was used to map the causative mutation of *Popcorn* to a small critical region on chromosome one by QTL analysis. Subsequently, whole-exome sequencing was performed to identify the causative *Popcorn* mutation in this critical region.

(2) Verifying causality of *Popcorn* mutation by recreating $Pop^{m/+}$ mice by CRISPR/Cas9

To produce $Pop^{m/+}$ knock-in mice, the mixture of Cas9 mRNA, sgRNA and a single-stranded donor oligonuceotide containing *Popcorn* point mutation were injected into the pronuclei of one-cell-stage embryos. The injected embryos were then transferred into pseudopregnant mice. F0 mice were genotyped for the presence of the *Popcorn* point mutation by sequencing. F0 mice containing the *Popcorn* mutation were further examined for the presence of the Cas9 transgene and off-target effects.

(3) Determining if *Popcorn* is a *bona fide* fearful mutant by behavior tests

The $Pop^{m/+}$ knock-in mutant mice and their wild-type littermates received a host of behavior tests at 12-18 weeks of age, including social dominance, open field, forced swim, tail suspension and sucrose preference tests.

## ４．研究成果

(1) Identification of the *Popcorn* gene mutation in the *Popcorn* pedigree

Linkage analysis in the N2 and N3 generations, and subsequent whole-exome sequencing of *Pop*$^{m/+}$ mutants identified a heterozygous single nucleotide substitution at the 3' splice acceptor site for an intron of *Popcorn* gene on chromosome 1, an uncharacterized novel gene that is conserved in human, chimpanzee, rat, and zebrafish. The identified mutation is predicted to result in a mis-spliced transcript encoding a mutant protein.

(2) Verification of the causality of the *Popcorn* mutation by its fearful phenotype

For confirming the causal relationship of the *Popcorn* gene mutation to the fearful phenotype, we introduced the same point mutation in wild-type mice using the CRISPR/Cas9 system. Several F0 mice possessing the *Popcorn* mutation were obtained, and mated with wild-type mice to generate F1 mice. The CRISPR *Pop*$^{m/+}$ mutant mice of N2-N3 generation will be examined in our innate fear assay to confirm whether the Popcorn mutation is the sole cause of the fearful phenotype.

(3) Behavior analysis

The behavior tests showed that the *Pop*$^{m/+}$ mice are socially submissive as judged by the classical "tube test" (Fig. 2) and by ultrasonic vocal sound in response to female mice. In addition, we found that the *Pop*$^{m/+}$ mutant mice showed a significant reduced body weight comparing with their wild-type littermates, which is in line with their social subordinate status. Other tests suggested that the *Pop*$^{m/+}$ mice also exhibited anxiety phenotypes.

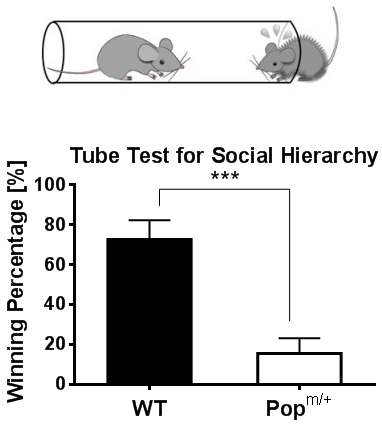


**Fig. 2.** Tube test for social dominance

This is the first successful forward genetic screen on emotions. The *Popcorn* mutant mice will be a valuable model for studying fear-related mental disorders and for developing mechanism-based medicines and therapeutic interventions for anxiety disorders as well as for social stress-related mental and physical health problems.

Our future work will focus on: ①To verify the causality of *Popcorn* mutation by examining the phenotypes of CRISPR *Pop*$^{m/+}$ mice; ②To examine the expression pattern of *Popcorn* gene in mouse brain by in situ hybridization; ③To perform whole brain *c-fos* activity mapping to study where Popcorn protein may act in the brain; ④ To determine the molecular and neural basis of the fearful phenotypes of *Popcorn* mice by multi-disciplinary research.

## ５．主な発表論文等

**（研究代表者、研究分担者及び連携研究者には下線）**

〔雑誌論文〕（計 2 件）


① Funato H, et al., Liu Q, Kume K, Wakana S, Takahashi JS, Yanagiaswa M. Forward-genetic analysis of sleep randomly mutagenized mice. Nature 539:378-83 (2016) （査読あり） Doi:10.1038/nature20142

② Isosaka T, et al., Kobayakawa R, Kobayakawa K. Htr2a-Expressing Cells in the Central Amygdala Control the Hierarchy between Innate and Learned Fear. Cell 163:1153-64 (2015) （査読あり） Doi: 10.1016/j.cell.2015.10.047


〔学会発表〕（計 3 件）

① 2016.12.12 Liqin Cao. "Molecular basis of innate fear: from fear genes to fear/anxiety disorders" The 5th Annual IIIS Symposium and The 32nd Wako Workshop. Tokyo Conference Center Shinagawa (Minato-ku, Tokyo)

② 2016.2.26 Qinghua Liu. "A fear screen to uncover the molecular bases of fear and fear/anxiety disorders" The 4th Annual IIIS Symposium. IIIS Building, University of Tsukuba (Tsukuba, Ibaraki)

③ 2016.2.26 Liqin Cao. "Understanding the molecular basis of fear and the role of emotion in sleep regulation" The 4th Annual IIIS Symposium. IIIS Building, University of Tsukuba (Tsukuba, Ibaraki)

〔その他〕

ホームページ等
http://liu.wpi-iiis.tsukuba.ac.jp/

６．研究組織

(1)研究代表者
Liu Qinghua（LIU, Qinghua）
筑波大学・国際統合睡眠医科学研究機構・教授
研究者番号：90723792

(2)研究分担者
曹　麗琴（CAO, Liqin）
筑波大学・国際統合睡眠医科学研究機構・助教
研究者番号：60399475

(3)研究分担者
佐藤　牧人（SATO, Makito）
筑波大学・国際統合睡眠医科学研究機構・研究員
研究者番号：70743699

(４)研究分担者
クレウエネベニウス　ダニエラ
（KLEWE-NEBENIUS, Daniela）
筑波大学・国際統合睡眠医科学研究機構・研究員
研究者番号：60737667

(５)連携研究者
小早川　令子（KOBAYAKAWA, Reiko）
公益財団法人大阪バイオサイエンス研究所・神経機能学部門・研究部長
研究者番号：40372411

(６)連携研究者
柳沢　正史（YANAGISAWA, Masashi）
筑波大学・国際統合睡眠医科学研究機構・教授
研究者番号：20202369