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論文の要旨

Emotion are powerful drivers of animal behaviors. Fear is an essential emotion that triggers characteristic defensive behaviors and physiological responses to promote survival in dangerous situations. Fear can be induced by both innate and learned mechanisms. In Pavlovian fear conditioning paradigms, rodents are trained to freeze in response to a conditioned (e.g., olfactory, auditory or visual) stimulus by pairing it with an unconditioned stimulus (e.g., electric foot shock). On the other hand, laboratory rodents are instinctively afraid of snakes or cats despite being isolated from predators for generations. This innate fear represents an evolutionarily conserved and genetically encoded pro-survival mechanism. This study hypothesized that it might be feasible to investigate the molecular mechanism of innate fear using a forward genetics approach.

A highly robust odor-evoked innate fear assay was developed by using 2-methyl-2-thiazoline (2MT), a potent derivative of predator (fox) odorant 2,4,5-trimethyl-3-thiazoline (TMT). A recessive mouse screening platform was designed for instant association of phenotypes with causative mutations induced by the chemical mutagen ethylnitrosourea (ENU) in C57BL/6J background. 14,390 G3 mice (487 pedigrees) for abnormal was screened fear behaviors. In all, 26,584 coding/splicing mutations in 12,512 genes were examined. Approximately 10.2% of all

genes were destroyed or damaged sufficiently to cause a phenotype, with the mutant alleles tested three times or more in the homozygous state.

Among the mutant families identified, one mutant pedigree, termed *fearless (frl)*, contain 4 (of 17 G3 mice) phenovariants showing 20-30% freezing rates upon 2MT exposure. Manhattan plot showed that this "fearless" phenotype was strongly linked ($P = 2.13\times10^{-11}$) to a homozygous mutation in the gene encoding a transient receptor potential A1 (*Trpa1*) ion channel. To confirm that *Trpa1* mutation was the cause of the fearless phenotype, an independently constructed *Trpa1* knockout strain was examined, which removed exons 22-24 encoding the essential pore-forming domain of Trpa1 channel. *Trpa1*$^{-/-}$ mice displayed diminished freezing response to 2MT or TMT as compared to wild-type and heterozygous littermates. Moreover, 2MT exposure resulted in a significant surge in the plasma level of stress hormone corticosterone in *Trpa1*$^{+/-}$ mice, and this response was abolished in *Trpa1*$^{-/-}$ mice.

It has previously been shown that 2MT exposure induces the immediate early gene *c-fos* expression in the central nucleus of amygdala (CeA), which communicates with downstream brain regions, such as the ventral periaqueductal gray (vPAG), to mediate innate and learned freezing behaviors. Accordingly, 2MT-evoked *c-fos* induction was greatly diminished in the CeA, vPAG and the paraventricular nucleus (PVN) of hypothalamus in *Trpa1*$^{-/-}$ brains relative to *Trpa1*$^{+/-}$ brains. The PVN is frequently activated in fearful or stressful situations, as part of the hypothalamic-pituitary-adrenal (HPA) axis, to stimulate stress hormone production. Thus, Trpa1 is required for activation of known fear/stress centers in the mouse brain upon 2MT exposure.

It is plausible that Trpa1 is involved in olfaction because modest Trpa1 expression has been detected in the olfactory epithelium. However, *Trpa1*$^{-/-}$ mice were found not to be anosmic because they were as efficient as *Trpa1*$^{+/+}$ and *Trpa1*$^{+/-}$ littermates in finding the hidden food pellet. Moreover, 2MT induced equivalent levels of *c-fos* expression in the olfactory bulbs (OB) of *Trpa1*$^{+/-}$ and *Trpa1*$^{-/-}$ mice, including the dorsal region of OB that was specifically activated by TMT. Furthermore, habituation-dishabituation test showed that *Trpa1*$^{-/-}$ mice exhibited the same level of detection threshold for 2MT as *Trpa1*$^{+/-}$ mice. Homozygous *Trpa1* mutant mice were proficient in dual odor- and sound-based fear conditioning assays, suggesting that they could distinguish different odors and exhibited normal learned fear responses. Importantly, *Trpa1*$^{-/-}$ mice could be further trained to fear 2MT by pairing it with electric foot shocks. Collectively, these findings suggest that *Trpa1*$^{-/-}$ mice appear to have a normal sense of smell and can learn to fear 2MT.

Because TMT is a pungent odor, it was hypothesized that Trpa1, a well-known pungency/irritancy receptor, function as a chemosensor for TMT-like thiazolines. To test this hypothesis, Ca^{2+} imaging was performed in HEK293T cells that were transiently transfected with the mCherry or Trpa1-P2A-mCherry constructs. Both TMT and 2MT, but not 2-methyl-2-oxazoline (2MO), a structurally related non-fear inducing odorant, evoked Ca^{2+} transients in the Trpa1-expressing cells in a dose-dependent manner. Inhibition of Trpa1 by HC-030031 treatment abolished 2MT-evoked Ca^{2+} transients in transfected HEK293T cells. Furthermore, twelve mammalian Trp channels belonging to all four subfamilies, Trpa, Trpc, Trpm, and Trpv1, were compared for 2MT/TMT-evoked Ca^{2+} responses. Only Trpa1, but not other Trp channels, could be specifically activated by 2MT/TMT.

Multiple pungent chemicals as well as oxygen could directly activate human TRPA1 channel by covalent modification of critical cysteine residues within its cytoplasmic domain. Indeed, substitution of Cys66, Cys415, Cys422, Cys622, Cys666, Cys714 or Cys859 in TRPA1 with Ser abolished or reduced 2MT/TMT-evoked

Ca^{2+} responses. Moreover, alkynyl-TMT was synthesized by adding an alkynyl moiety to the C2 position of TMT. If alkynyl-TMT could covalently label HA-TRPA1 ectopically expressed in HEK293T cells, one should be able to precipitate modified Trpa1 proteins by biotin conjugation through click chemistry and streptavidin pull-down. Accordingly, alkynyl-TMT-treated, but not TMT-treated Trpa1 was precipitated from transfected HEK293T cells by streptavidin beads. C415S mutant Trpa1 was precipitated less efficiently than wild-type Trpa1 from alkynyl-TMT-treated HEK293T cells. These results suggest that Trpa1 may act as a direct chemosensor for TMT/2MT through covalent modifications of critical Cys residues of the receptor.

This study developed a highly robust 2MT-evoked innate fear assay and established a forward genetics screen to identify randomly mutagenized mice with abnormal fear responses. The unbiased fear screen identified that inactivation of Trpa1, a pungency/irritancy receptor, diminished 2MT/TMT and snake skin-evoked innate fear/defensive behaviors. Accordingly, 2MT exposure failed to efficiently activate known fear/stress centers in the brain of *Trpa1*$^{-/-}$ mice, despite their apparent ability to smell and learn to fear 2MT. Furthermore, Trpa1 could function as a chemosensor for 2MT/TMT and was absolutely essential for 2MT sensing by a subset of TG neurons. It was shown that Trpa1-expressing TG neurons contributed critically to 2MT-evoked innate freezing behavior. These results suggest that the Trpa1-dependent somatosensory system plays a prominent role in mediating predator odor-evoked innate fear/defensive responses.

審査の要旨

【批評 Review】

Chia-Ying (Jessica) Lee made three major contributions to this comprehensive study. First, she performed a series of beautiful experiments to unequivocally show that TRPA1 is essential for 2MT sensing by TRPA1-expressing trigeminal ganglion neurons. Second, she performed technically very difficult AAV rescue experiments to show that ectopic expression of TRPA1 in trigeminal ganglion partially rescued 2MT-evoked freezing response in *Trpa1*$^{-/-}$ mice. She was able to perform challenging experiments and deliver high quality data for the original publication and for her dissertation.

【最終試験の結果 Result】

The final examination committee conducted a meeting as a final examination on May 31, 2019. The applicant provided an overview of dissertation, addressed questions and comments raised during Q&A session. All of the committee members reached a final decision that the applicant has passed the final examination.

【結論 Conclusion】

Therefore, the final examination committee approved that the applicant is qualified to be awarded a Doctor of Philosophy in Human Biology.