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論文内容の要旨

1 研究目的

Social stress has deteriorating effects on various psychiatric disorders. In animal models, exposure to socially dominant conspecifics, i.e., social defeat stress, evokes species-specific defeat posture and freezing behavior. Underlying mechanisms are unknown.

Previous studies have suggested that the ventromedial hypothalamus and midbrain periaqueductal gray region have facilitative roles in the control of defensive behaviors in response to various threatening stimuli. However, there are heterogeneous neurons in the ventromedial hypothalamus and periaqueductal gray region. Details of physiological functions of each subtype in the ventromedial hypothalamus and periaqueductal gray region were poorly understood. Furthermore, neural pathways underlying expression of defensive behaviors remain unclear.

Oxytocin neurons are located mainly in the hypothalamic paraventricular nucleus, supraoptic nucleus and the bed nucleus of the stria terminalis. Oxytocin neurons have been shown to be activated by various types of stressful stimuli. Oxytocin plays an important role not only in uterine contraction during parturition and in milk-ejection reflex, but also in the control of stress or anxiety-related responses, social behavior and energy metabolism. Oxytocin administration has been shown to have pro-social, anxiolytic and anorexic actions, although controversial data have also been reported. For examples, oxytocin facilitates aggressive behavior and augments stress responses in some conditions. Underlying mechanisms of actions of oxytocin are largely unclear. Physiological roles of oxytocin during social defeat stress are not known.

I examined in mice whether social defeat stress activates oxytocin neurons and oxytocin receptor-expressing neurons. I also examined projecting fibers of oxytocin neurons of the paraventricular hypothalamic nucleus by using a viral tracer. I then examined roles of the oxytocin receptor during social defeat stress by using oxytocin receptor-deficient mice.

2 研究方法

Animals

Adult male C57BL/6J mice (CLEA Japan, Shiga, Japan), retired CD1 mice (Charles River Laboratories Japan, Kanagawa, Japan), oxytocin receptor-Venus knock-in mice (backcrossed with C57BL/6J mice), oxytocin-Ires Cre knock-in mice (backcrossed with C57BL/6J mice), Rosa-CAG-LSL-tdTomato-WPRE::deltaNeo knock-in reporter mice (C57BL/6J background), and oxytocin receptor-deficient mice (backcrossed with C57BL/6J mice) were used in this study. Oxytocin receptor-Venus knock-in mice express a variant of yellow fluorescent protein (Venus) under the control of the endogenous regulatory region of the oxytocin receptor gene.

The animals were housed in rooms with controlled temperature (20 - 24 °C) and humidity (40 - 70%) under a 12-hour light/dark cycle (lights on at 7:30 AM - 7:30 PM). Food and water were available *ad libitum*. All animal procedures were approved by the Institutional Animal Experiment Committee of Jichi Medical University and were in accordance with the Institutional Regulations for Animal Experiments and Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology.

Social defeat stress

Social defeat stress was applied by 10 min exposures to aggressive CD1 mice. A mouse was placed as the intruder in the chamber of the aggressive resident male CD1 mouse and kept there for 10 min. The behavior of the intruder mouse during 10 min confrontation with the resident aggressor was videotaped. Defensive behaviors during social defeat stress were analyzed. The duration of defeat posture (an upright posture with the belly exposed toward the resident) and duration of freezing behavior (no movements except for respiration with four paws on the ground) were measured.

Immunohistochemical examination

For detection of neurons activated after social defeat stress, mice were exposed to aggressive CD1 mice for 10 min. One hundred minutes after the end of social defeat stress, mice were anesthetized with Avertin (200 mg/kg body weight, i.p.; tribromoethanol) and were perfused transcardially with heparinized saline (20 U/ml) followed by 4% paraformaldehyde. Expression of c-Fos, a marker of neuronal activation, in oxytocin neurons and in oxytocin receptor-expressing neurons was investigated in mice by immunohistochemical examination.

Brain sections were cut coronally at 30 µm intervals with a freezing sledge microtome and processed for immunohistochemical detection of c-Fos and oxytocin or Venus.

Brain sections were incubated with a rabbit polyclonal antibody against the N-terminal region (4–17) of the c-Fos peptide at 4 °C for 2 days and with peroxidase-labeled goat anti-rabbit IgG at 4°C overnight. c-Fos immunoreactivity was visualized by incubation with 3,3'-diaminobenzidine tetrahydrochloride, nickel sulphate, glucose and glucose oxidase.

Sections for immunohistochemical detection of oxytocin were incubated with guinea pig anti-oxytocin antibody at 4°C for 2 days, biotinylated anti-guinea pig IgG at room

temperature for 2 h, and avidin biotinylated horseradish peroxidase complex at room temperature for 30 min. Oxytocin immunoreactivity was visualized as a brown cytoplasmic precipitate with 3,3'-diaminobenzidine tetrahydrochloride or as a black precipitate with 3,3'-diaminobenzidine tetrahydrochloride and nickel sulphate.

Sections for immunohistochemical detection of Venus were incubated with rat monoclonal anti-green fluorescent protein antibody at 4°C for 2 days and a peroxidase-labeled polymer solution containing an anti-rat Fab' fragment, amino-acid polymer and peroxidase at room temperature for 2 h. Immunoreactivity of green fluorescent protein was visualized as a pink cytoplasmic precipitate with 0.01% Tris-aminophenylmethane and 0.07% p-cresol.

Anterograde tracing

The projection of oxytocin neurons was examined with an anterograde viral tracer, which induces selective expression of membrane-targeted palmitoylated green fluorescent protein in oxytocin neurons. Green fluorescent protein tagged with a palmitoylation signal has been shown to be sorted to the plasma membrane and has been used to trace neuronal fibers efficiently. Adeno-associated virus vectors were applied into oxytocin-Cre mice in order to label oxytocin fibers by selective expression of palmitoylated green fluorescent protein in oxytocin neurons of the paraventricular hypothalamic nucleus. The adeno-associated virus vectors contained a sequence for expressing palmitoylated green fluorescent protein under the control of the CAG promoter selectively in oxytocin cells expressing Cre recombinase activity.

Data Analysis

Data are expressed as means \pm SEM. Data were analyzed by the Mann-Whitney *U* test, repeated-measures two-way ANOVA or *t*-test. $P < 0.05$ was considered statistically significant.

3 研究成果

During exposures to aggressive CD1 mice, mice showed defeat posture and freezing behavior, suggesting that that exposure to the aggressor induced social defeat stress.

Following social defeat stress, expression of c-Fos protein was increased in oxytocin neurons of the bed nucleus of the stria terminalis, supraoptic nucleus, and paraventricular hypothalamic nucleus. Expression of c-Fos protein was also increased in oxytocin receptor-expressing neurons of the insular cortex, lateral septum, amygdala, thalamic nuclei, the ventrolateral part of the ventromedial hypothalamus and midbrain periaqueductal gray.

Projecting fibers from oxytocin neurons of the paraventricular hypothalamic nucleus were found in the ventrolateral part of the ventromedial hypothalamus and in the ventrolateral periaqueductal gray.

Oxytocin receptor-deficient mice showed reduced defeat posture during social defeat stress.

4 考察

Social defeat stress induces species-specific defeat posture in animals. Concerning neural

mechanisms of social defeat, social defeat stress has been shown to activate the ventrolateral part of the ventromedial hypothalamus and ventrolateral periaqueductal gray. On the other hand, oxytocin neurons in the hypothalamus are activated by a variety of stressful stimuli. Here, we suggest that oxytocin neurons in the paraventricular hypothalamic nucleus innervate the ventrolateral part of the ventromedial hypothalamus and ventrolateral periaqueductal gray, and that social defeat stress activates oxytocin neurons in the paraventricular hypothalamic nucleus and oxytocin receptor-expressing neurons in the ventrolateral part of the ventromedial hypothalamus and ventrolateral periaqueductal gray. Oxytocin receptor-deficient mice showed reduction of social defeat posture during social defeat stress. Our results suggest that social defeat stress activates oxytocin-oxytocin receptor system in the brain and that activation of oxytocin-oxytocin receptor system facilitates social defeat posture during social defeat stress.

During acute social defeat stress, animals show species-specific submissive posture, defeat postures. Expression of submissive posture can induce suppression of aggressive behaviors by the dominant conspecific animal. It is interesting to speculate that oxytocin facilitates submissive defeat posture in an imminent confrontation with dominant conspecifics to reduce aggression of dominant conspecifics and as a result increases adaptive values in group-forming animals.

5 結論

Social defeat stress activates oxytocin-oxytocin receptor system, and activation of the oxytocin receptor possibly in the ventrolateral part of the ventromedial hypothalamus or the ventrolateral periaqueductal gray facilitates social defeat behavior. This newly defined oxytocin pathway may lead to treatments for stress-related disorders.

論文審査の結果の要旨

申請者の論文は、マウスにおける社会的敗北ストレスが分界条床核、視索上核、視床下部室傍核のオキシトシン神経細胞の Fos 蛋白質発現を亢進させること、Fos 蛋白質発現はオキシトシン受容体を発現する島皮質、外側中隔、扁桃核、視床核、視床下部腹内側核、中脳水道周囲灰白質の神経細胞でも亢進すること、視床下部室傍核のオキシトシン神経は視床下部腹内側核、中脳水道周囲灰白質に投射すること、オキシトシン受容体欠損マウスでは社会的敗北ストレスによる敗北姿勢が限弱するが不動は変化しないことを示した。これらの研究結果から申請者は、社会的敗北ストレスによる敗北姿勢は視床下部腹内側核および中脳水道周囲灰白質に投射する視床下部室傍核オキシトシン神経の活性化により生じることを示唆するとともに、敗北姿勢の種族保存上の意義、オキシトシン神経系のストレス関連障害の病態解明における重要性を指摘している。本論文は社会的敗北ストレスによるオキシトシン神経系の活性化、オキシトシン神経の投射する経路を明らかにし、敗北姿勢におけるオキシトシン神経・受容体の関与の可能性を示した点に新規性がある。

論文は研究背景、研究方法、結果および討論が明瞭簡潔に記載されており、図も明解である。一部の用語で統一がなされていない点、精神医学用語の使用法の誤り、スペース・改行のズレ、図の解像度の低さが指摘されたが、これらは試問後に適切に修正された。

論文は全体として明確に論理立てて記述され、新規の発見を含んでおり、学位論文として十分にふさわしいと満場一致で判断された

最終試験の結果の要旨

発表は、マウスにおける社会的敗北ストレスとオキシトシン神経系についてのこれまでの研究成果の解説から始まり、次いで社会的敗北ストレスによる分界条床核、視索上核、視床下部室傍核のオキシトシン神経細胞および島皮質、外側中隔、扁桃核、視床核、視床下部腹内側核、中脳水道周囲灰白質のオキシトシン受容体発現細胞の Fos 蛋白質発現亢進、視床下部室傍核から視床下部腹内側核、中脳水道周囲灰白質へのオキシトシン神経の投射、オキシトシン受容体欠損マウスでの社会的敗北ストレスによる敗北姿勢の限弱が明解かつ論理的な研究結果により示された。

引き続き、オキシトシン神経細胞の活動性の性差および定常状態での活動性、Fos 蛋白質発現細胞の定量法、社会的敗北ストレスにより Fos 蛋白質発現が亢進する神経細胞の分布、社会的敗北ストレスにおける敗北姿勢の恐怖記憶との関連および種族保存上の意義、社会的敗北ストレスモデルのヒトにおける疾患との関連性、今後の研究計画についての質疑応答がなされた。候補者はこれらの質問に真摯な姿勢で適切に回答した。

候補者は関連領域にわたり幅広い知識と教養を持ち合わせており、博士（医学）の学位に相応しい研究能力と思考力を有していると満場一致で判断された。