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Protein tyrosine phosphatase receptor type Z (Ptprz) is a receptor-type PTP that is expressed predominantly in the CNS as a chondroitin sulfate proteoglycan. Although it is expressed in neurons and astrocytes throughout development and adulthood, *Ptprz*-deficient ($Ptprz^{-/-}$) mice show no obvious anatomical abnormalities in the brain. However, the recent study revealed that adult $Ptprz^{-/-}$ mice exhibit functional impairments in the hippocampus, where Ptprz is highlyl expressed. Adult Ptprz-/- mice show memory deficits in the Morris water maze, and demonstrate enhanced hippocampal LTP in the CA1 region, which is cancelled out by inhibiting ROCK, a major downstream effector of Rho GTPase. In addition, p190 RhoGAP, a GTPase-activating protein (GAP) which potently inhibits RhoGTPase, has been identified as a possible substrate of Ptprz. These findings suggest that the Rho-ROCK pathway is impaired in $Ptprz^{-/-}$ mice, however, little is known about the details of the intracellular signal transduction mechanism of Ptprz.

This thesis investigates the possibility that Ptprz is involved in the molecular mechanisms of memory formation through controlling the activity of p190 RhoGAP using *Ptprz*-deficient ($Ptprz^{-/-}$) mice. First, the hippocampus-dependent learning ability of $Ptprz^{-/-}$ mice is analyzed by fear conditioning, which requires the association of an environment with an aversive electric stimulus. Second, to elucidate the molecular mechanism by which Ptprz controls p190 RhoGAP activity, the site of dephosphorylation of p190 RhoGAP by Ptprz is identified, and the effect of phosphorylation at this site on p190 RhoGAP activity is examined by a Rho activity assay. Finally, the

phosphorylation of p190 RhoGAP in the hippocampus, especially at the site of dephosphorylation by Ptprz, is compared between wild-type and mutant mice after fear conditioning.

To examine the hippocampus-dependent learning ability of *Ptprz*$^{-/-}$ mice in fear conditioning, *Ptprz*$^{-/-}$ mice were tested in two forms of behavioral tasks: contextual fear conditioning and cued fear conditioning. Both are sensitive to amygdala lesions, but only contextual fear conditioning is sensitive to hippocampal lesions. *Ptprz*$^{-/-}$ mice demonstrated marked impairements selectively in contextual fear conditioning.

To further define the specificity of behavioral deficits, *Ptprz*$^{-/-}$ mice were subjected to the elevated plus maze test in which fear or anxiety-related behaviors are analyzed. The maze had two enclosed arms with high walls and two open arms with low rims and was elevated above the floor. As mice usually avoid the open arms of the maze, the extent of anxiety can be evaluated by the time spent in the open arms relative to the closed arms. In addition, the numbers of entries into the arms of the maze likely corresponds to general motor activity or exploratory activity. There were no significant differences between the genotypes.

Ptprz$^{-/-}$ mice exhibited normal vocalizing responses to an incremental series of electric foot shocks and unaltered freezing responses during the conditioning session and cued fear conditioning test, indicating that pain perception or emotional expression is not affected in *Ptprz*$^{-/-}$ mice. Thus, *Ptprz*$^{-/-}$ mice did not show sensory or emotional deficits, but exhibited selective impairments in hippocampus-dependent behavioral tasks.

Second, to elucidate the molecular mechanism by which Ptprz controls p190 RhoGAP activity, the site of dephosphorylation by Ptprz was determined by a mutation study. As Y1087 and Y1105 of p190 RhoGAP are reported to be the sites of tyrosine phosphorylation on p190 RhoGAP, these sites were replaced with phenylalanine. Then, *in vitro* dephosphorylation assays with Ptprz were performed using wild-type p190 RhoGAP and its mutants. The Y1087F mutant as well as wild-type p190 RhoGAP was efficiently dephosphorylated by the whole intracellular region of Ptprz while Y1105F and Y1087/1105F mutants were not, indicating that Y1105 is the site of dephosphorylation by Ptprz.

Next, the effect of the phosphorylation at this site on p190 RhoGAP activity was examined using wild-type p190 RhoGAP and Y1105F mutant. GAP activity was indirectly observed by the Rho activity assay, which detects the active form of Rho. Cotransfection of wild-type p190 RhoGAP and v-src in HEK293T cells led to an increase in p190 RhoGAP tyrosine phosphorylation along with further inhibition of Rho, indicating that increased tyrosine phosphorylation of p190 RhoGAP enhances its GAP activity. On the other hand, Y1105F p190 RhoGAP, a mutant that can not be phosphorylated at Y1105, inhibited Rho to a comparable extent to wild-type p190 RhoGAP. However, v-src did not increase the tyrosine phosphorylation of the Y1105F mutant, and further inhibition of Rho was not observed, indicating that phosphorylation at Y1105 is critical for the regulation of p190 RhoGAP activity. Thus, tyrosine phosphorylation at Y1105 positively controls the activity of p190 RhoGAP, which indicates that p190 RhoGAP activity may be suppressed when p190 RhoGAP is

dephosphorylated at Y1105 by Ptprz.

Finally, the phosphorylation of p190 RhoGAP in the hippocampus was compared between wild-type and mutant mice after fear conditioning. To analyze the phosphorylation of p190 RhoGAP especially at the site of dephosphorylation by Ptprz, a phospho-specific antibody (anti-pY1105 p190 RhoGAP) directed against Y1105-phosphorylated p190 RhoGAP was generated by immunizing rabbits with the tyrosine-phosphorylated peptide. Then, hippocampal homogenates were prepared from animals after conditioning, and the effects of *Ptprz* knock-out on tyrosine phosphorylation of hippocampal proteins were examined.

There were no significant differences in overall tyrosine phosphorylation patterns of the hippocampal homogenates among the four groups. However, conditioned $Ptprz^{+/+}$ mice showed significantly reduced p190 RhoGAP tyrosine phosphorylation compared with sham-conditioned $Ptprz^{+/+}$ mice. In contrast, conditioned $Ptprz^{-/-}$ mice exhibited similar levels of tyrosine phosphorylation to sham-conditioned $Ptprz^{-/-}$ mice. Assessment by immunoblotting with anti-pY1105 p190 RhoGAP antibody revealed that the level of p190 RhoGAP phosphorylated at Y1105 was significantly lower in conditioned $Ptprz^{+/+}$ mice than in sham-conditioned $Ptprz^{+/+}$ mice, and almost identical between conditioned and sham-conditioned $Ptprz^{+/+}$ mice. These results can be explained as indicating that only wild-type mice show dephosphorylation of p190 RhoGAP after fear conditioning, suggesting that p190 RhoGAP is dephosphorylated by Ptprz after fear memory formation.

Moreover, the phosphorylation of p190 RhoGAP at Y1105 in the hippocampus was

examined by immunohistochemistry using anti-pY1105 p190 RhoGAP antibody. A comparable level of p190 RhoGAP immunolabeling was confirmed among the four groups by staining with anti-p190 RhoGAP antibody. However, only sham-conditioned *Ptprz*$^{+/+}$ mice showed significantly enhanced staining with anti-pY1105 p190 RhoGAP antibody. The immunostaining in sham-conditioned *Ptprz*$^{+/+}$ mice was observed in the stratum oriens as well as the stratum radiatum, in which Ptprz is prominently distributed. These results indicate that dephosphorylation of p190 RhoGAP by Ptprz is involved in fear memory formation.

The present study demonstrated that the phosphorylation of p190 RhoGAP was aberrantly regulated in *Ptprz*$^{-/-}$ mice after fear conditioning. The level of phosphorylation at Y1105 was decreased in conditioned *Ptprz*$^{+/+}$ mice compared with sham-conditioned *Ptprz*$^{+/+}$ mice whereas no change was observed in *Ptprz*$^{-/-}$ mice. These results indicate that p190 RhoGAP activity is suppressed and consequently Rho GTPase is activated after fear conditioning in wild-type mice, however, p190 RhoGAP activity is maintained and subsequent Rho GTPase activation after learning does not occur in mutant mice. The lack of Rho GTPase activity after learning is thus likely responsible for the learning defects in Ptprz-deficient mice, which is consistent with a previous study in which post-training infusion of Y-27632 into the hippocampus impaired spatial memory.

In conclusion, Y1105 is the site of p190 RhoGAP dephosphorylation by Ptprz. Ptprz inhibits p190 RhoGAP through dephosphorylation at this site, and consequently activates Rho GTPase. This regulation of p190 RhoGAP by Ptprz plays crucial roles in

the molecular mechanisms underlying hippocampus-dependent memory formation.

（論文審査結果）

本学位論文はProtein tyrosine phosphatase receptor type Z (Ptprz)の海馬学習における役割とその分子メカニズムを*Ptprz*遺伝子ノックアウト(*Ptprz*-KO)マウスを用いて研究したものである。Ptprzは受容体型チロシンホスファターゼの1つであり、コンドロイチン硫酸プロテオグリカンとして中枢神経系に発現している。成体の*Ptprz*-KOマウスは、モリス型水迷路における空間学習に障害があり、海馬CA1領域において長期増強(LTP)の亢進を示す。このときRhoの下流で働くROCKを阻害すると、海馬での長期増強の亢進は消失する。また、Ptprzの基質分子として、Rhoの不活化を担うGTPアーゼ活性化タンパク質(GAP)である、p190 RhoGAPが同定された。このように、*Ptprz* KOマウスではRhoシグナルの制御に異常があることが示唆されていた。まず第1章において、研究の目的が、海馬依存的学習においてPtprzの果たしている役割を明らかにすることであることを示している。第2章では*Ptprz*-KOマウスの海馬依存的学習障害を評価した。恐怖条件付け学習における文脈条件付け記憶と音条件付け記憶において、扁桃体は両記憶の成立に関与するのに対し、海馬は前者のみに関与することが知られている。ノックアウトマウスは海馬依存的な文脈条件付け記憶に選択的な異常を示すことが判明した。また、情動を調べる高架式十字型テストでは異常は見られなかった。このように、*Ptprz-KO*マウスは海馬機能に選択的に異常があることが示された。第3章では、Ptprzによるp190 RhoGAPの脱リン酸化部位の同定を行った。p190 RhoGAPのチロシンリン酸化部位として1105番及び1087番チロシンが知られているが、*in vitro*の脱リン酸化実験によりPtprzによる脱リン酸化部位として1105番チロシンを同定した。さらに、1105番チロシンのリン酸化のp190 RhoGAPのGAP活性に与える影響をv-srcを共発現させることによって評価した。その結果、1105番チロシンのリン酸化によりp190RhoGAPのGAP活性は正に制御されており、Ptprzによる脱リン酸化によってGAP活性は抑制されると考えられた。第4章では、恐怖条件付けを行ったマウス（条件付けマウス）の、海馬におけるp190 RhoGAPのチロシンリン酸化レベルを解析した。コントロールとして、恐怖条件付けの際、電気ショックを与えないマウス（非条件付けマウス）を用いた。1105番チロシンがリン酸化された状態のp190 RhoGAPに対する特異抗体を作成し、海馬抽出液から免疫沈降したp190 RhoGAPに対して抗リン酸化抗体でウェスタン解析したところ、野生型マウスでは条件付けマウスは非条件付けマウスと比べ有意に低いチロシンリン酸化レベルを示すこと、ノックアウトマウスでは非条件付けマウスと条件付けマウスで同程度のチロシンリン酸化レベルを示すことが判明した。免疫染色においても*Ptprz*の発現する海馬神経層において同様の結果を得た。以上の結果は、恐怖条件付け後の野生型マウスの海馬ではPtprzによるp190 RhoGAPの1105番チロシンの脱リン酸化が起こるのに対し、*Ptprz-KO*マウスではそれが起こらないことを示唆している。

このように、海馬依存的な学習の成立には、Ptprzがp190 RhoGAPの1105番チロシンを脱リン酸化することによってGAP活性を抑制し、Rhoの活性化を導くことが必要であることが明らかになった。本研究は、海馬学習におけるRhoシグナル伝達系の関与を示す新たな知見を提供するとともに、その経路へのPtprzの関与のメカニズムを明確に明らかにしたものであり、博士（理学）に十分に値するものであると判定した。なお本学位申請論文の内容に関しては、2編の原著論文（内1編は申請者が筆頭著者）として、国際誌に発表済及び掲載予定である。