Discussion

In the present study, we demonstrated that (1) the treatment of microglia with fibronectin enhanced the expression of functional $P2X_4$ receptors, (2) the spinal fibronectin was upregulated after the peripheral nerve injury, and (3) the fibronectin treatment of microglia lowered the concentration of ATP that was necessary to cause mechanical allodynia by intrathecal transfer. Because the upregulation of $P2X_4$ receptors in spinal microglia is a critical event in the induction of mechanical allodynia after peripheral nerve injury (Tsuda et al. 2005; Tsuda et al. 2003), our present results may partially clarify the mechanism of mechanical allodynia.

The correlation between fibronectin and microglial $P2X_4$ receptor

The $P2X_4$ receptor displays a broad tissue distribution especially in the CNS (Soto et al. 1996). Although the precise physiological roles of $P2X_4$ receptors remain unknown, it has been reported that the upregulation of $P2X_4$ receptors is linked to several pathological conditions such as nerve injury (Tsuda et al. 2003), ischemia (Cavaliere et al. 2003) and muscular dystrophy (Yeung et al. 2004). In the present study, we showed that fibronectin increased the microglial $P2X_4$ expression at the mRNA level by more than two-fold. The elevation was also confirmed at the protein level, and these upregulated $P2X_4$ receptors were shown to be functional by the increase in $[Ca^{2+}]i$ mediated by $P2X_4$ receptors. These findings are of interest because some ECM molecules including fibronectin are known to be upregulated following adult CNS injury (Jones 1996). Fibronectin is involved in neurite growth during development (Matthiessen et al. 1989) and plays an important role in the spinal cord development in human (Krolo et al. 1998). The expression of fibronectin is regionally and developmentally regulated in the brain, and its presence is relatively minor in the normal CNS (Jones 1996). However, once injuries occur, its expression is dramatically increased (Hoke and Silver 1996; Pasinetti et al. 1993). Fibronectin also exists at high concentrations in the blood plasma and a breakdown of the blood-brain barrier (BBB) should result in an increase in its local concentration in the CNS. Thus, it is highly plausible that the increased fibronectin may somehow upregulate $P2X_4$ receptors in microglia. The signaling pathway(s) by which fibronectin promotes $P2X_4$ upregulation is currently under investigation. Microglia possess functional β1 integrin, which is one of the receptors for fibronectin, and they undergo firm adhesion, activation (Milner and Campbell 2002; Milner and Campbell 2003) and proliferation (Nasu-Tada et al. 2005) through this molecule, presumably by regulating intracellular signaling cascades. Thus,

fibronectin-to-integrin-mediated signals may be critical for the $P2X_4$ receptor upregulation. It is interesting that, among various P2 receptors expressed in microglia, i.e., $P2X_7$, $P2Y_2$, $P2Y_6$, and $P2Y_{12}$ receptors, only the $P2X_4$ receptor is upregulated by fibronectin (Figure 1). This result suggests that the $P2X_4$ receptor gene may undergo unique transcriptional regulation by the linkage of fibronectin to integrin.

The correlation between spinal fibronectin and mechanical allodynia in rats

We showed that spinal fibronectin was upregulated after the nerve injury. Several lines of evidence indicate that fibronectin is directly upregulated at the site of injuries in the PNS (Lefcort et al. 1992; Martini 1994; Vogelezang et al. 1999) and CNS (Hoke and Silver 1996; Pasinetti et al. 1993), but to our knowledge, there has been no report that demonstrated that fibronectin is upregulated at a distal region, i.e. in the CNS, after peripheral nerve injury. The clinical signs of allodynia induced in rat in the Chung model become evident by 3-day post operation and the phenotype reaches the maximum by 1-week post operation. The concomitant upregulation of the microglial $P2X_4$ receptors, which is observed only on the ipsilateral side of the spinal cord, follows the same time-course profile (Tsuda et al. 2003). In this study, the expression of the spinal fibronectin was strongly augmented, but again only on the ipsilateral side, during the course of mechanical allodynia and the expression pattern was also similar to the time-course of the above. Therefore, our results strongly suggest that the upregulation of the ipsilateral fibronectin correlates both with the induction of allodynia and with the upregulation of the microglial $P2X_4$ receptor. Since it is usually observed that mechanical allodynia spans several weeks, it is still necessary for us to investigate the relationship between the upregulation of fibronectin and chronic allodynia. Our data yet postulate that fibronectin may be involved in the onset of the disease, most likely by initiating the upregulation of the microglial $P2X_4$ receptor.

Since fibronectin is known to be present at a high concentration in the blood plasma, it is plausible that the peripheral nerve injury caused a local break down of the central BBB, and that the breakdown resulted in a transfer of the blood plasma fibronectin into the corresponding part of the CNS. In a cortical cold-injury model, fibronectin was found to leak from blood vessels (Nag et al. 2001; Nourhaghighi et al. 2003), but in another report such exudation was not seen after spinal cord injury (Farooque et al. 1992). Interestingly, the plasma fibronectin expression is known to be elevated after tissue injury (Thompson et al. 1992). Recently, plasma fibronectin was reported to support neuronal survival and reduce bran injury following transient focal

cerebral ischemia, but it was not essential for skin-wound healing and hemostasis (Sakai et al. 2001). Alternatively, fibronectin may be synthesized by neuronal and glial cells in the CNS. Fibronectin is a vital molecule in neural development and regeneration (Venstrom and Reichardt 1993) and astrocytes are known to synthesize and release fibronectin (Jiang et al. 1994; Matthiessen et al. 1989; Price and Hynes 1985). A recent report by Tom et al revealed that astroglial-associated fibronectin plays a key role in axonal regeneration in the white matter (Tom et al. 2004) and indeed, astrocytes produce and release fibronectin in response to ATP stimulation (unpublished observation). However, so far we have not identified the source of the upregulated fibronectin, and the mechanism by which fibronectin is upregulated after peripheral nerve injury remain to be clarified.

The effect of fibronectin was further highlighted by the experiment involving intrathecal transfer. In our previous study (Tsuda et al. 2003), microglia that were treated with 50 μM ATP could induce mechanical allodynia when they were intrathecally transferred into a normal rat. Microglia that were cultured on fibronectin and treated with 5 μM ATP were capable of inducing mechanical allodynia. In contrast, control microglia were not able to induce mechanical allodynia at that ATP concentration. The result suggests that fibronectin lowered the threshold of pain sensation. Fifty μM of ATP was adequate to cause mechanical allodynia by intrathecal transfer in both groups, suggesting that the effect of microglia in causing allodynia in response to ATP stimulation is saturated at an ATP concentration of 50 μM. Although microglia have other P2 receptors, i.e., $P2X_7$, $P2Y_2$, $P2Y_6$ and $P2Y_{12}$ receptors, only $P2X_4$ receptors are involved in the induction of mechanical allodynia (Tsuda et al. 2003). Interestingly, fibronectin upregulated only $P2X_4$ receptors but downregulated other P2 receptors on microglia at the mRNA level (Figure 1). Thus, the involvement of other microglial P2 receptors in pain sensation, the threshold of which was lowered by fibronectin, would be negligible. Altogether, the results suggest that microglia with upregulated $P2X_4$ receptors by fibronectin treatment were able to transduce signals that lead to allodynia at a lower concentration of ATP.

In summary, we demonstrated that fibronectin induces the upregulation of $P2X_4$ receptors on microglia *in vitro*, and that fibronectin is increased in the spinal cord *in vivo* when mechanical allodynia is induced after peripheral nerve injury. When microglia are intrathecally administered into normal rats, they induce mechanical allodynia only if pre-stimulated with ATP (Tsuda et al. 2003). Our *ex vivo* experiments showed that fibronectin lowers the ATP concentration that is necessary for microglia to induce this pain behavior. Although both the signaling pathway by which fibronectin promotes $P2X_4$ upregulation and the source of increased

fibronectin are currently under investigation, all these findings suggest that the upregulation of spinal fibronectin may be involved in the onset mechanism of mechanical allodynia after nerve injury.

Acknowledgements

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Figure legends

Fig. 1. The effect of fibronectin on the mRNA expression of microglial $P2X_4$.
Fibronectin increased the expression of $P2X_4$ in microglia at the mRNA level. Microglia were cultured on fibronectin for 24 hr at 37°C and the expression of $P2X_4$ was assessed by quantitative RT-PCR. $P2X_4$ was markedly upregulated by fibronectin, whereas the mRNA expressions of $P2X_7$, $P2Y_2$, $P2Y_6$ and $P2Y_{12}$ purinoreceptors were significantly decreased. Data are mean±SE of 3 separate experiments. Asterisks show significant difference from control (*$p<0.05$, **$p<0.01$ vs. control, Student's t test).

Fig. 2. Time-course study of the microglial $P2X_4$ upregulation.
Fibronectin increased the expression of $P2X_4$ in microglia at the protein level. Microglia were cultured on fibronectin for 1, 6, 12, and 24 hr at 37°C and the protein expression of $P2X_4$ receptors was analyzed by Western blotting. The protein expression of $P2X_4$ receptors began to increase after 12 hr of incubation and increased strongly after 24 hr of incubation. The intensity of the bands was quantified with a computing densitometer using NIH ImageJ 1.33u image analysis software. Asterisks show significant difference from control (*$p<0.05$, **$p<0.01$ vs. control, Student's t test).

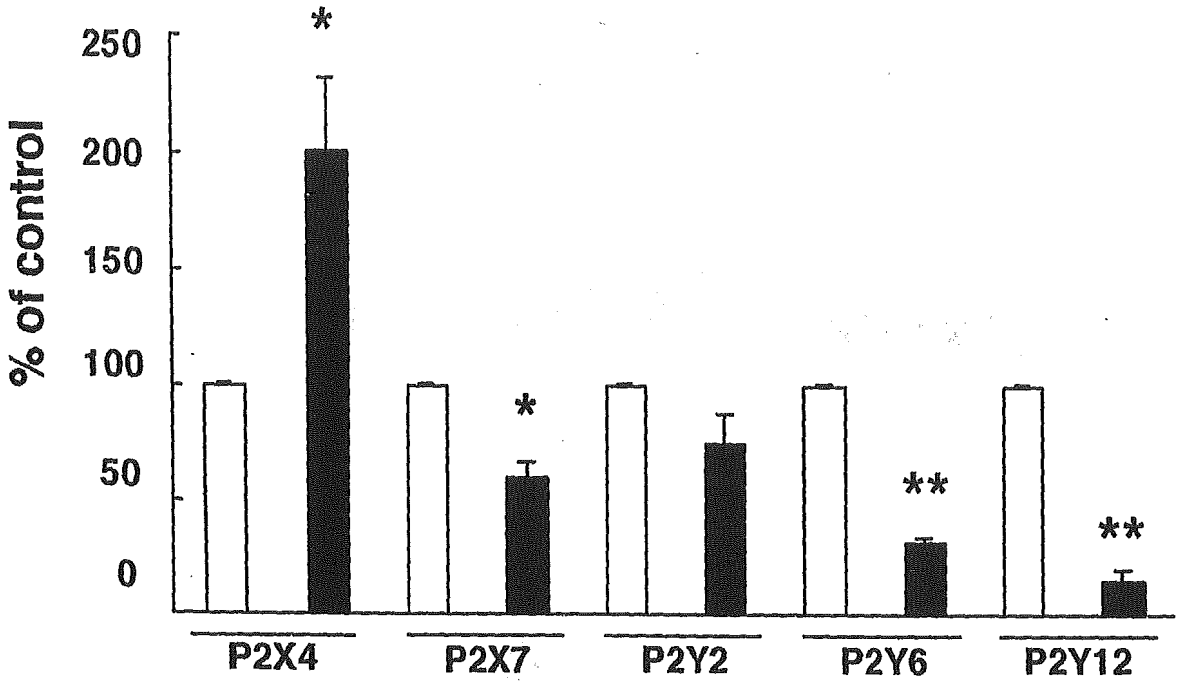
Fig. 3. Enhancement by fibronectin of the function of $P2X_4$ receptors in microglia.
The function of microglial $P2X_4$ was assessed by fura-2 based $[Ca^{2+}]i$ imaging (ratio of F340/F380). Microglia cultured on fibronectin showed an increase in the Ca^{2+} response to stimulation with ATP 50 μM. Microglia were cultured for 24 hr on fibronectin or on a control, and pretreated with TNP-ATP (100 μM) or PPADS (10 μM) for 2 min where required. Flexiperm cover glass (i.e. non-coated), and the ATP (50 μM)-evoked increase in $[Ca^{2+}]i$ was monitored. 0 Ca^{2+} indicates removal of Ca^{2+} from the extracellular medium. Asterisks and #s show significant difference from non-coated control and ATP alone, respectively (*$p<0.05$, **$p<0.01$ vs. control; #$p<0.05$, ##$p<0.01$ vs. ATP alone, Student's t test).

Fig. 4. Time-course study of fibronectin expression in the allodynia rat spinal cord.
The rat spinal nerve on the left side was exposed, tightly ligated with a silk suture and its peripheral side was completely transected. On Day 1, 3 and 7 post-operation, L5 spinal cords from control and allodynia rats were collected and the tissues were subjected to homogenization

and Western blotting. Anti-fibronectin (Dako, 1:100 dilution) antibody and HRP-conjugated anti-rabbit IgG (Amersham) antibody were used for the detection. The data represent 3 independent experiments.

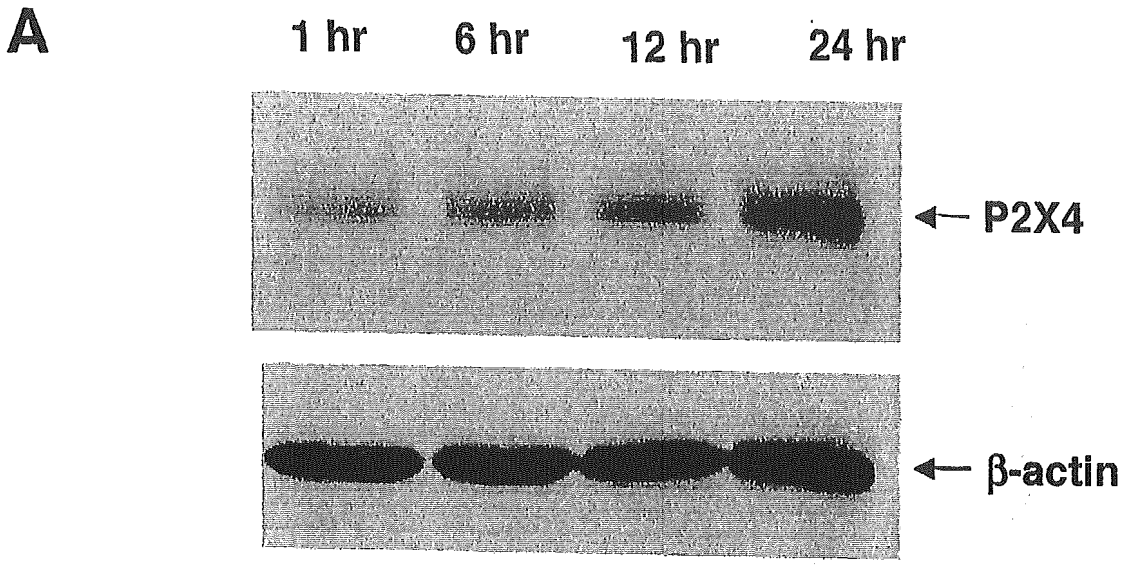
Fig. 5. Changes in nociceptive response after intrathecal transfer of microglia with elevated expression of $P2X_4$ receptors.

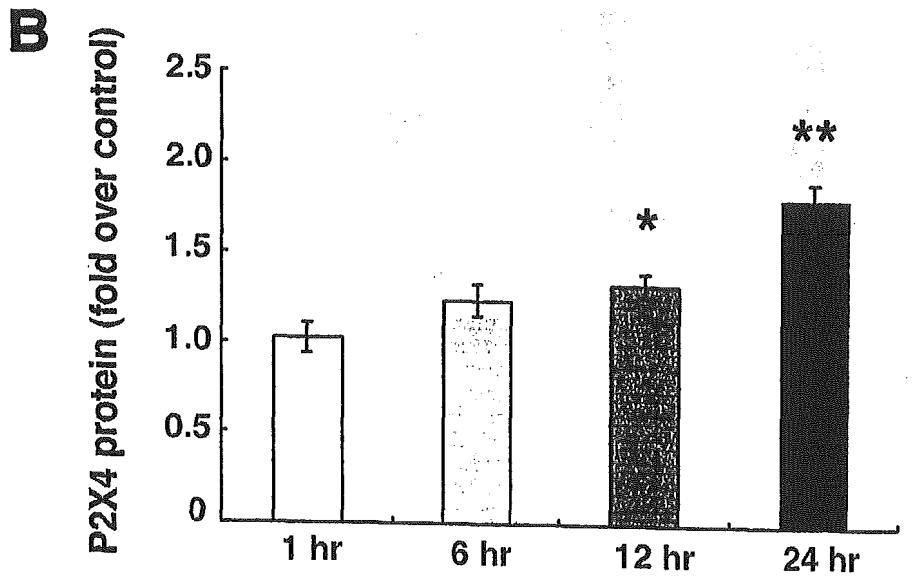
Microglia were cultured either on fibronectin (red circle) or on control plastic (white circle) for 24 hr at 37 °C and both groups were subsequently stimulated with ATP at 0 (control), 0.5 and 5 μM for 1 hr at 37 °C. Without intrathecal microinjection of microglia, no rats showed pain behavior. Then the cells were intrathecally transferred to the lumbosacral spinal cord of a normal rat. Five hr after the microinjection, nociceptive responses were evaluated by measuring the 50% paw withdrawal threshold to mechanical stimuli. Six rats were used in each group for this study. Mann-Whitney U-test was performed and statistical significance was set at $p<0.05$ [*$p<0.05$ vs. control (before microglial injection); #$p<0.05$ vs. non-coated microglia; $$p<0.05$ vs. ATP-untreated microglial injection].



*p<0.05, **p<0.01 vs. Control

Figure 1
Nasu-Tada et al.





*p<0.05, **p<0.01 vs. Control

Figure 2
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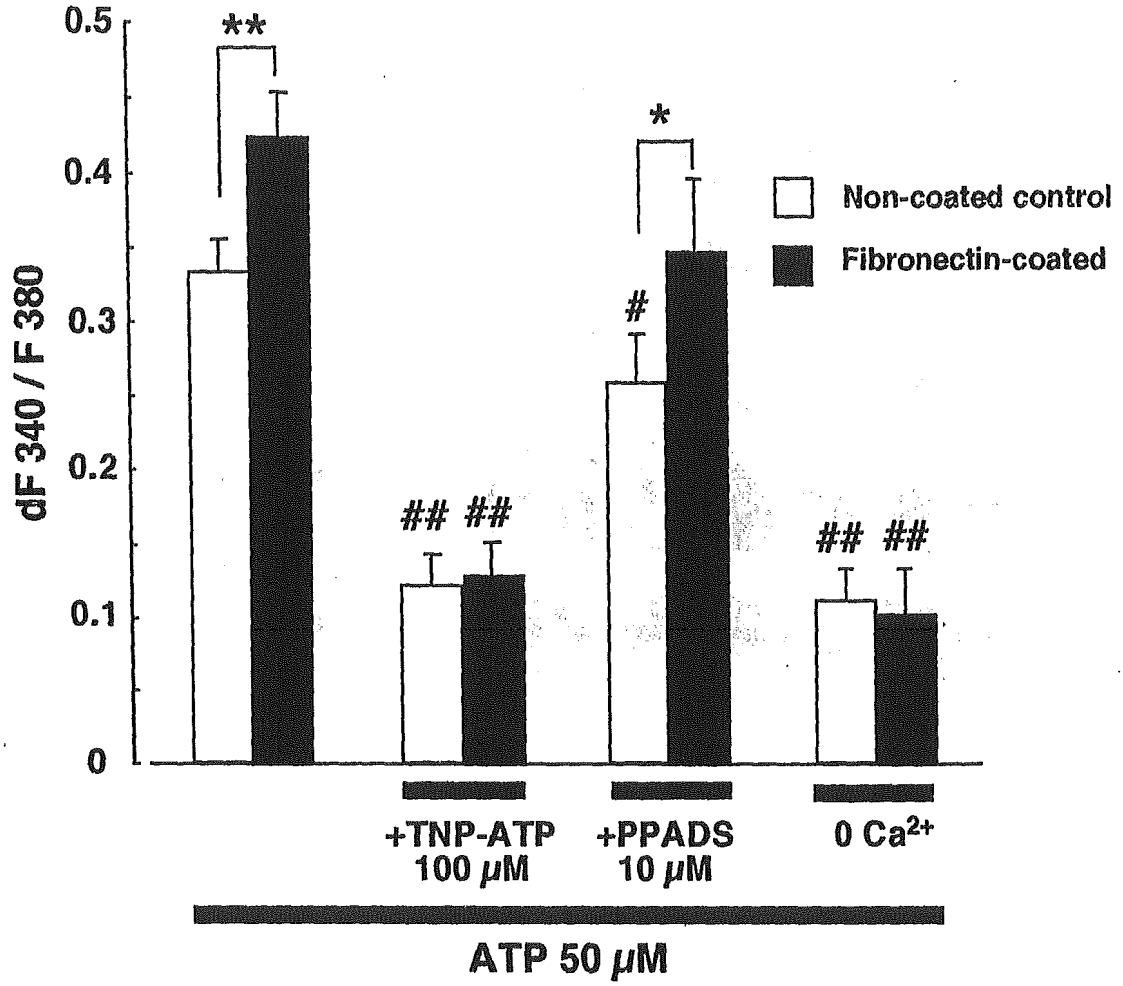

*p<0.05, **p<0.01 vs. non-coated control

#p<0.05, ##p<0.01 vs. ATP alone

Figure 3
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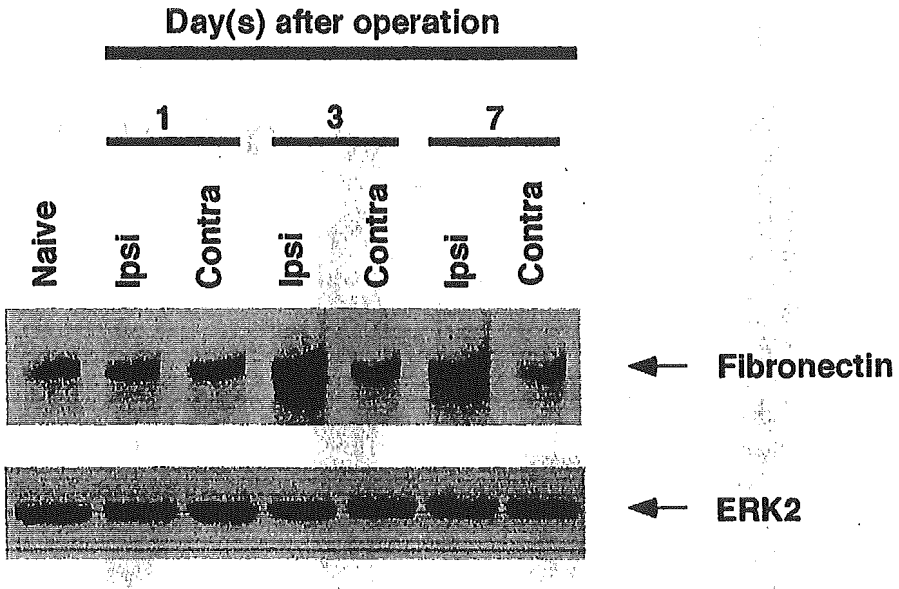

Figure 4
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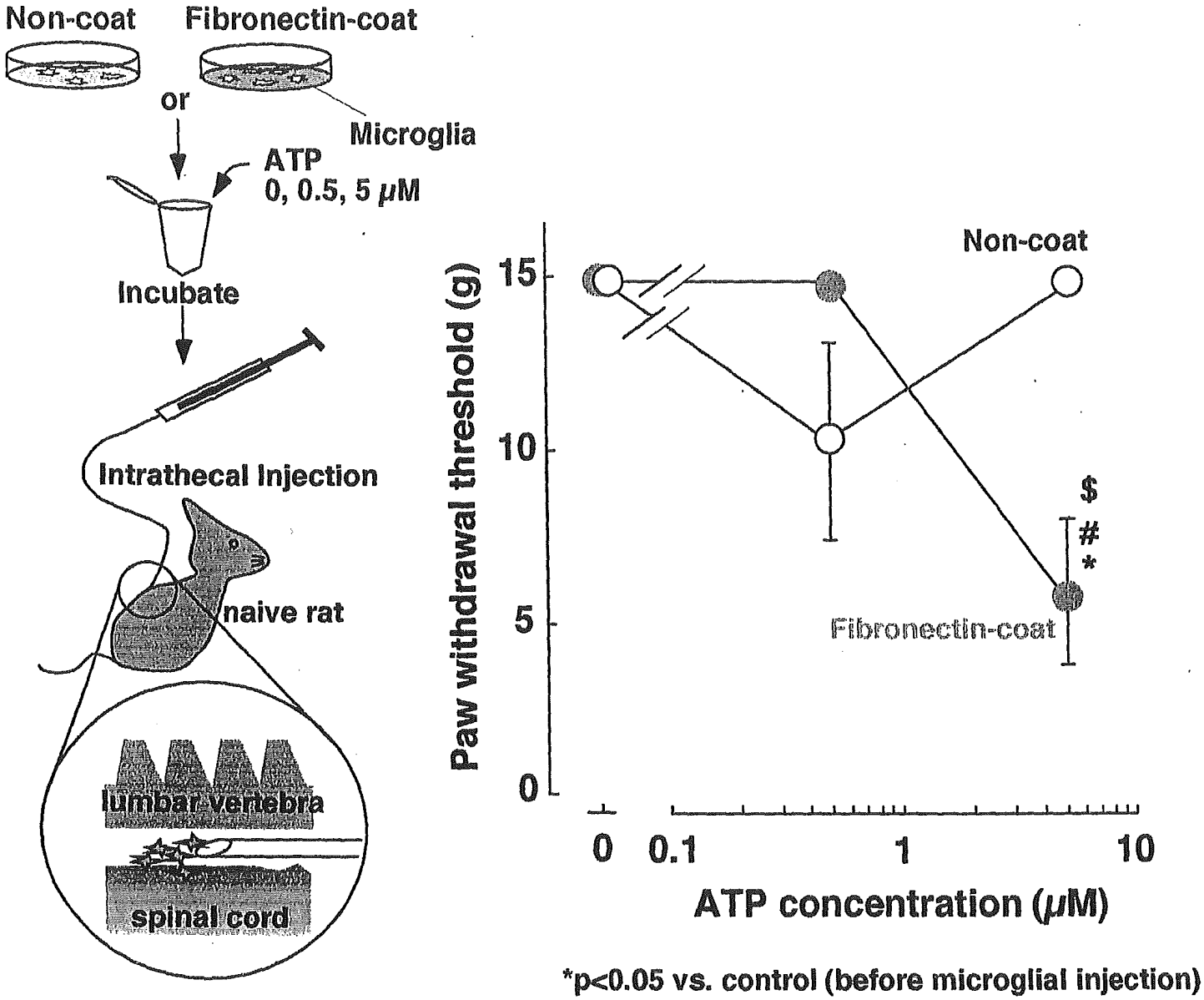

*p<0.05 vs. control (before microglial injection)

#p<0.05 vs. non-coated microglia

$p<0.05 vs. ATP-untreated microglia

Figure 5
Nasu-Tada et al.

Pain Clinic 印刷中

『Gタンパク共役型ATP受容体と痛み』

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『G-protein coupled –P2Y receptors and Pain』

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要旨

エネルギーの通貨であるATPは、細胞外に放出されて情報伝達物質としても機能する。このATP及びその受容体P2受容体を介する情報伝達は、知覚情報伝達と強くリンクしており、特にイオンチャネル型P2X受容体は、末梢及び中枢の痛覚情報伝達との強い関連性から特に注目されている。一次求心性神経にはG蛋白共役型ATP受容体であるP2Y受容体も存在しているが、P2Y受容体と痛覚伝達に関しては情報が少ない。本稿では痛覚伝達とATPに関し、特にP2Y受容体に注目して最近の知見を報告する。

キーワード：ATP、P2Y受容体、皮膚-知覚神経連関

はじめに

ATP（アデノシン三リン酸）はあらゆる細胞に存在するエネルギーの通貨であるが、1993 年に最初の ATP 受容体（P2 受容体）cDNA がクローニングされて以来、その細胞間情報伝達物質として役割が注目されている。P2 受容体は、イオンチャネル型 P2X 受容体と G 蛋白共役型 P2Y 受容体に大別され、それぞれが 7 及び 8 種類のサブタイプに分類されている（図 1）。これらは中枢・末梢を問わず、生体のあらゆる部分に多様に発現しており、そこでの種々の生理反応と密接にリンクしていると考えられている。ATP/P2 受容体が担う生理的役割に関してはまだまだ不明な点が多いが、現在最も解明が進んでいる分野の一つが、痛覚情報伝達との関連性である。1995 年にイオンチャネル型 P2X 受容体サブクラスの $P2X_3$ 受容体が末梢一次求心性感覚神経に局在していることが明らかとされたのに端を発し [1,2]、$P2X_3$ 受容体 [1-3] さらには $P2X_{2/3}$ 受容体 [4] と、特に急性期の疼痛に関しての多くの知見が集積された。また、神経因性疼痛等の慢性疼痛の基礎的研究では、末梢一次求心性感覚神経である坐骨神経や脊髄神経などを人為的に損傷させるモデルがよく用いられるが、このような病態モデル動物で誘発される異痛症（アロディニア）の発生及び維持には、脊髄後角ミクログリアの $P2X_4$ 受容体の発現亢進が中心的な役割を果たしていることが明らかとなった [5,6]。このように P2X 受容体系は、末梢の痛み発生・調節に大きく影響を与えるだけでなく、脊髄における知覚情報の伝達の制御と深く関連し、慢性疼痛発生・維持と強く関わっている重要な分子である。一方、一次求心性神経及びその周辺細胞には G 蛋白質共役型 P2Y 受容体も存在していることが明らかとなったが、P2Y 受容体と痛覚伝達に関する報告は少ない。本報告では、一次求心性神経の痛覚伝達と ATP との関連性について、特に P2Y 受容体に注目して最近の知見を報告する。

1. DRG の P2Y 受容体

一次求心性神経には P2Y 受容体も発現している。P2Y 受容体と知覚情報の伝達に関する最初の知見は、Nakamura と Strittmatter[7] の報告であり、彼らは $P2Y_1$ 受容体 mRNA が大型後根神経節細胞（dorsal root ganglion; DRG）神経細胞のマーカーである RT-97 に陽性の細胞に発現していることを見出した。さらに、$P2Y_1$ 受容体を強制発現させたアフリカツメガエル卵母細胞では、触刺激により惹起される内向電流を低濃度 ATP（1 μM 未満）が強く増強する。また，カエル下肢皮膚の触刺激による感覚神経の興奮も ATP で顕著に増大し、ATP 受容体拮抗薬（suramin，pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS)）および ATP 分解酵素 apyrase により消失する。またマウスの急性単離 DRG 細胞においても、比較的大型の DRG 神経細胞で ATP 刺激により細胞内カルシウム濃度上昇が認められ、$InsP_3$ 受容体拮抗薬および小胞体 Ca^{2+}-ATPase 阻害剤により消失する。当研究室においても同様の結果を得ており、これらの知見は，$P2Y_1$ 受容体が Aβ 繊維由来の非侵

害性刺激（触刺激）の伝達制御に深く関与している可能性を示唆している。

前述したように、$P2X_3$受容体は小型DRG神経細胞[1,2]に、$P2X_2$及び$P2X_3$受容体は中型のDRG神経細胞[4]に強く発現している。痛覚を伝えるこれらの小型DRG神経細胞にP2Y受容体は発現しているのだろうか？ATPで小型及び中型DRG神経細胞を刺激して細胞内Ca^{2+}濃度（$[Ca^{2+}]i$）変化を観察すると、80 %程度のDRG神経細胞で$[Ca^{2+}]i$上昇が観察される。興味深い事に、これらの$[Ca^{2+}]i$応答は細胞外Ca^{2+}を除いても観察され、またphospholipase C (PLC)阻害薬U73122、小胞体Ca^{2+}-ATPase阻害剤cyclopiazonic acid(CPA)及びP2受容体拮抗薬suraminで抑制された。さらにuridine 5' -diphosphate (UTP)によってもほぼ同様の$[Ca^{2+}]i$上昇が認められた（図２）。従って、小型DRG神経細胞には$PLC/InsP_3$系と共役したUTP感受性のP2Y受容体、つまり$P2Y_2$ ($P2Y_4$)受容体が共発現している（図２Ｂ）。$P2Y_2$受容体は、ATP及びUTPによりほぼ同程度の強さで活性化される。これは、小型のDRG神経細胞でUTPが細胞内Ca^{2+}動員を惹起するという報告によっても支持される[8) 9)]。興味深い事は、UTP応答DRG神経細胞の多くがTRPV1受容体作用薬カプサイシンにも応答したことである。こられの知見は、P2X受容体系だけでなく、P2Y受容体、特に$P2Y_2$受容体が痛覚伝達の制御と関係していることを強く示唆するものである。

2. 痛みとP2Y受容体

DRG神経細胞の$P2Y_1$受容体を刺激すると、N型電位依存性Ca^{2+} channel[10]及び$P2X_3$受容体[11]が抑制され、知覚情報の伝達が阻害される。それでは，UTP刺激によって$P2Y_2$受容体を活性化すると、知覚情報はどの様に制御されるのだろうか？痛みは出るのであろうか。最近我々は，ラットの後肢足底部にUTPを投与し，太さの異なるvon Frey filamentで一次求心性神経を刺激することで、触刺激に対する痛み応答、アロディニアが発現することを見出した（図３）。同様のアロディニアは、中型DRG神経細胞に存在する$P2X_{2/3}$受容体を刺激した場合にも惹起されるが[4]、UTPにより惹起されるアロディニアは同程度かそれ以上の強さであり、またその持続時間はより長かった。UTPによるアロディニアは、P2受容体拮抗薬PPADSにより消失し，さらにPLC阻害剤U73122およびカルシウムキレート剤BAPTA-AMによっても抑制される。これらの阻害様式は，DRG神経細胞でのUTPによる細胞内Ca^{2+}動員の阻害様式と一致しており，両者が同じP2Y受容体を介している可能性が示唆される。また，カプサイシン感受性神経破壊ラットでは、UTPによるアロディニアが抑制されることからも、カプサイシン感受性一次求心性神経（C繊維）がUTPにより興奮しアロディニアを惹起しているものと考えられる。この点も、*in vitro*の結果と一致している。候補として挙げられる責任受容体は，UTPに感受性の$P2Y_2$，$P2Y_4$，$P2Y_6$受容体である。しかし，$P2Y_6$受容体作用薬UDPはアロディニアを誘発しないことから、$P2Y_6$受容体の関与は考えにくい。さらに，$P2Y_2$受容体を減少させるためにラットの脊髄くも膜下腔内へ$P2Y_2$アンチセンスオリゴを投与すると、UTPによるアロディニアはほぼ完全に抑制される。一方、$P2Y_4$アンチセンスオリゴでは全く影響がない。した

がって、UTPはC繊維の$P2Y_2$受容体を介して，アロディニアを発現していることが示された.

3. 痛み伝達のメカニズム

$P2Y_2$活性化が如何にしてアロディニアを引き起こすかに関しては不明である。先行する報告で，TominagaらはP2Y受容体（$P2Y_1$あるいは$P2Y_2$）刺激によりTRPV1の応答の感受性が亢進すること[12]、また熱刺激に対する痛み行動が亢進すること[13]を報告している。つまり、P2Y受容体はTRPV1をエフェクターとして痛み情報を伝えているのである。P2Y受容体刺激によるTRPV1応答増強反応にはprotein kinase C(PKC)の活性化が必要だが，UTPによるアロディニアはPKC阻害薬に全く影響されず、さらにTRPV1阻害薬による影響も受けない。またDRG神経細胞におけるカプサイシンによる$[Ca^{2+}]i$上昇もUTPで変化がないことから、UTPによるアロディニアにはTRPV1は関与していないと推察される（図５右図参照)。従って、メカニカルアロディニア発現にはTRPV1感受性亢進とは別のメカニズムが関係している可能性が考えられる。またMolliverらは、$P2Y_2$受容体刺激により小型DRG神経細胞が脱分極し活動電位を発生すること[14]、つまり$P2Y_2$受容体刺激が痛み伝達に関わる小型DRG神経細胞を直接興奮させるというものである。交感神経節細胞の$P2Y_2$受容体でも同様のメカニズムが提唱されている。さらに前述したように、大型DRG神経細胞には$P2Y_1$受容体が発現しており、この$P2Y_1$受容体が触刺激の応答亢進とリンクしている可能性がアフリカツメガエル卵母細胞や、カエル下肢皮膚-感覚神経系で明らかにされている[7]。小型DRG神経細胞にも種々の機械応答センサーが存在していることから、同様のメカニズムが小型DRG神経細胞の$P2Y_2$受容体を介するシグナル経路に存在している可能性も考えられる。また、UTPは$P2Y_2$受容体を介して小型のDRG神経細胞で転写因子のCREB（cyclic AMP response element binding protein)を活性化する。これらの知見は、$P2Y_2$受容体が、転写レベルでも痛みの伝達を制御している可能性を示唆するもので非常に興味深い。このように、小型のDRG神経細胞に存在しているP2Y受容体は、痛み伝達の調節に重要な役割を果たしているようであるが、その痛み伝達の調節メカニズムの詳細は、まだ不明な点も多く残されている。

一方、UTPおよびUDPは神経因性疼痛に対して抑制効果も有している。Okadaらは、UTPやUDPを神経因性疼痛モデルラットの脊髄くも膜下腔内へ投与することで、神経損傷によるアロディニアが抑制されることを報告している[15]。また，UTPおよびUDPは正常動物でも鎮痛効果を出す。このUTPとUDPの標的P2Y受容体が同一かどうかは不明だが、同じP2Y受容体系でも、脊髄$P2Y_6$受容体はむしろ鎮痛作用とリンクした分子である可能性が強い。