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2. 慢性疼痛の診断

a. はじめに

疼痛は自覚的な症状であり，また個々の患者で痛みへの反応は様々であり，この疼痛を客観的に評価することはなかなか困難なことが多い．特に慢性疼痛では患者は長期にわたり疼痛に苦しめられ，疼痛が患者に及ぼす身体的，心理的，社会的影響は大きく，適切な診断と治療がなされなければ患者の不利益は甚大となる．本項では慢性疼痛，特に神経障害性疼痛の診断について概説する．

b. 慢性疼痛について

慢性疼痛は国際疼痛学会によって「治療を要すると期待される時間の枠組みを越えて持続する痛み，あるいは進行性の非がん性疾患に関連する痛み」とされており[1]，慢性疼痛の多くが外傷や疾病に起因する急性疼痛からの移行した痛みであるが，疼痛を誘発する刺激（侵害刺激）が持続的あるいは断続的に存在するために生じる場合も含まれる．持続時間については一般的に3ヵ月以上持続するものを慢性疼痛とすることが適当とされているが[2]，持続時間についてのコンセンサスは明確でなく，特に急性疼痛からの移行した痛みの場合は，痛みの原因となっている疾患あるいは病態が治癒した後も持続する疼痛を慢性疼痛とすることが妥当とも考えられており，診断における時間の枠は重要でないようである．慢性疼痛の病態は極めて複雑であり，中枢神経系や末梢神経系での疼痛制御の可塑的変化や心理的要因，社会的要因などが病態形成に関与するため治療が難渋することも多い．神経障害性疼痛と心因性疼痛を完全に区別することが困難である場合もあり，両者はむしろ併存するものであるとい

シンポジウム SYMPOSIUM ■四肢のしびれ感

圧迫性頚髄症の痛みとしびれ

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Pain and Numbness in Cervical Stenotic Myelopathy

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Key words：しびれ(numbness)，痛み(pain)，後縦靱帯骨化症(ossification of posterior longitudinal ligament)，頚椎症性脊髄症(cervical spondylotic myelopathy)，患者報告アウトカム(patient reported outcome)

多施設横断研究により圧迫性脊髄症の痛み・しびれに関する調査を行った．後縦靱帯骨化症と頚椎症性脊髄症288名と健常者に患者背景，画像とともにquality of life(QOL)・頚椎関連・心理ストレス・痛みとしびれを調査した．治療内容によらず健常者に比しすべてのアウトカムが低下していた．活動制限を生じる痛みが保存治療で10%，手術治療で15%にみられた．痛みは腰・頚部，しびれは四肢が強く，numerical rating scale(NRS)5以上の頚部痛が36%，上肢しびれが41%にみられた．上肢しびれは四肢・体幹機能や心理ストレスと関連があり，満足度と相関していた．

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はじめに

脊椎外科医は圧迫性脊髄症に対して麻痺の改善を第一の治療目標としてきた．ところが手術後も脊椎の痛みや四肢のしびれがしばしば残存し，麻痺の改善にもかかわらず痛み・しびれを強く訴える患者に遭遇する．しかし過去の研究は運動障害とその回復に関心が寄せられてきたため，こうした痛み・しびれに対して焦点を当てた研究は多くはない．本研究の目的は圧迫性脊髄症患者の実態を把握し，痛み・しびれの患者へのインパクトを解析することである

対象・方法

本研究は厚生労働省難治克服事業脊柱靱帯骨化症の多施設研究として21の大学病院とその関連施設で行われた．調査期間は2006年7月から2007年11月である．対象は頚椎後縦靱帯骨化症(OPLL)と頚椎症性脊髄症(CSM)で，保存治療・手術治療ならびに対照として調査した健常者の計3群を比較した．

調査内容は患者背景と疾患・治療内容，画像情報，患者報告アウトカムの3つである．

患者背景として年齢・性別・感覚障害出現から現在(あるいは手術)までの期間・運動障害出現から現在(あるいは手術)までの期間，手術症例では手術アプローチを，画像情報としてX線像での脊柱管前後径，MRIでのT2高輝度の有無と頭尾側方向の長さである．患者アウトカムとして，

1) 包括的調査票としてSF-8[3]の8つのドメイン，
2) 頚椎症あるいは頚髄症調査票として日本整形外科学会頚部脊髄症治療成績判定基準JOACMEQ[4]，医師評価である日本整形外科学会頚椎疾患治療成績判定基準(旧JOAスコア)，頚椎症尺度であるneck disability index(NDI)[5]を，
3) 脊柱の可とう性評価として作成し前屈時指床距離(finger floor distance)と相関のあるself-assessment bending scale(SABS)[1]を，
4) 心理評価として日本語POMS短縮版(profile of mood states)[6]を用いた．30の質問からなり，緊張・抑うつ・怒り・活気・疲労・混乱の6因子が同時に測定できる質問票である．

痛みとしびれの評価としては，痛み評価ではchronic pain grade(CPG)と，身体部位別(頚部・頭部・背部・上肢・腰部・下肢の6つ)の11段階による痛みおよびしびれ強度(numerical rating scale：NRS)を調査した．CPGはグレート0から4まであり，高いほど障害が強い．またCPGの質問票から疼痛強度も算出し比較した．

さらに，上肢しびれに関連する因子の検索と，満足度との関連を解析した．

結果

1．アウトカム

調査総数は350名で，内訳はOPLLの保存治療80名，OPLLの手術治療104名，CSMの保存治療53名，CSMの手術治療69名，健常者44名であった．保存治療，手術治療，健常者の特徴を表1に示す．保存治療，手術治療いずれも男性に多く，平均年齢は60歳代であった．手術治療は前方29例，後方138例，その他6例であった．脊柱管前後径は差がなかったが，保存治療群ではMRIのT2高輝度のない症例が多かった．

1) 患者報告アウトカム

SF-8では健常者に比べて保存治療，手術治療ともすべてのドメイン，特に身体機能が低かった(表2)．保存治療，手術治療に有意な差はなかった．JOACMEQでも健常者に比べてすべてのドメインで保存治療，手術治療とも低かったが，頚椎機能は手術治療群が一段と低かった(表2)．旧JOAスコアでは感覚スコアで上肢と体幹で有意差があるものの，保存治療と手術治療の差はほとんどなかった(表3)．NDIでは保存治療群で29.3±17.5，手術治療群で29.8±18.5で同等であったが，健常者5.6±7.5に比べ有意に高かった($p=0.000$)．

SABS(高値ほどやわらかい)でみる体幹のやわ

表1　患者背景と画像情報

	保存治療	手術治療	健常者
総数	133	173	44
男・女	88・45	124・49	29・15
年齢	67.1±10.1	65.8±9.7	63.9±7.9
罹病期間(年)			
しびれ発症から調査まで	7.3±5.5	10.2±7.0**	
しびれ発症から手術まで		3.2±4.4	
麻痺発症から調査まで	5.0±3.6	8.0±6.0**	
麻痺発症から手術まで		0.9±2.1	
画像			
脊柱管前後径(mm)	13.7±1.7	13.8±2.3	
MRI/T2 高輝度あり	47.3%	63.9%*	
T2 高輝度長さ(椎体)	1.8±0.8	1.6±0.8	

*：$p<0.05$
**：$p<0.01$

表2　患者報告アウトカム

	保存治療	手術治療	健常者
SF-8			
全体的健康感	42.3±7.7	45.3±8.1*1	51.1±5.6*2
身体機能	42.7±8.8	43.1±7.9	51.4±3.9*2
日常役割機能(身体)	42.6±9.3	42.7±9.4	51.8±3.6*2
体の痛み	42.4±9.0	43.6±9.4	52.3±6.8*2
活力	46.4±6.7	47.3±7.7	51.9±5.3*2
社会生活機能	43.4±9.4	43.7±9.9	53.0±4.1*2
心の健康	46.3±6.9	47.9±7.1	52.4±4.9*2
日常役割機能(精神)	44.0±10.0	45.6±10.2	52.4±3.0*2
JOACMEQ			
頚椎機能	69.5±28.6	59.2±30.8*3	90.5±17.7*4
上肢機能	83.6±20.0	80.0±19.7	99.0±2.3*4
下肢機能	72.3±27.7	67.2±27.1	97.1±8.2*4
膀胱機能	76.6±19.2	75.6±21.6	91.8±11.3*4
QOL	47.2±18.0	47.8±19.0	68.5±11.6*4

SF-8：健康関連 QOL 尺度
JOACMEQ：日本整形外科学会頚部脊髄症治療成績判定基準
*1：保存治療と比較して $p=0.04$
*2：保存治療や手術治療と比較して $p<0.01$
*3：保存治療と比較して $p=0.008$
*4：保存治療や手術治療と比較して $p<0.01$

らかさでは保存治療群で3.8±1.5，手術治療群で3.9±1.6と同等で，健常者4.7±1.5と比べると有意に体幹が硬かった($p=0.004$)．

POMSは健常者と比較すると怒り・敵意を除く5因子でストレスが高いことが示されたが，特に緊張・不安，疲労，混乱での感情ストレスが強いことが示された(表4)．

表3　旧JOA

	保存治療	手術治療
上肢運動機能	3.2±0.9	3.1±1.0
下肢運動機能	2.9±1.1	2.7±1.1*1
上肢知覚機能	1.2±0.5	1.1±0.6
下肢知覚機能	1.4±0.6	1.5±0.6
体幹知覚機能	1.7±0.5	1.6±0.6
膀胱直腸機能	2.6±0.6	2.5±0.7

*1：$p=0.029$

2. 痛みとしびれ

CPGはグレード3以上が保存治療群で約10%，手術治療群で15%を占めており，治療内容によらず活動制限を生じている割合が少なくないことを示す(表5)．疼痛強度は健常者と比べて保存治療，手術治療群ともに有意に大きかった．

身体別疼痛では頚椎疾患患者への調査にもかかわらず，腰部が保存治療群で3.7±3.1，手術治療群で3.8±2.9と最も高く，次いで頚部(保存治療3.5±2.9，手術治療3.2±2.8)と下肢(保存治療3.3±3.2，手術治療3.3±3.1)が強かった．頚部の痛みはNRS5以上の患者が36.1%であった．また頭痛が健常者と比べ両群とも強かった(保存治療1.6±2.2，手術治療1.5±1.5，健常者0.6±

表4　Profile of Mood Status(POMS)短縮版

	保存治療	手術治療	健常者
(T得点)			
緊張・不安	48.2±9.6	47.5±10.0	43.2±8.8*3
抑うつ・落ち込み	49.4±9.6	50.1±9.5	45.3±6.8*2
怒り・敵意	47.3±9.0	48.1±9.5	44.9±6.7
活気*1：	41.2±10.1	42.1±9.2	46.2±8.8*2
疲労	48.2±8.7	47.7±9.1	44.0±6.9*3
混乱	51.9±9.3	52.1±9.9	45.8±7.6*3

*1：活気のみ高いスコアほどよい
*2：保存治療や手術治療と比較してp<0.05
*3：保存治療や手術治療と比較してp<0.01

表5　Chronic Pain Grade(CPG)のグレードと疼痛強度

	保存治療	手術治療	健常者
グレード0	7.5%	11.6%	30.6%
グレード1	45.3%	39.5%	61.1%
グレード2	36.8%	34.0%	8.3%
グレード3以上	10.4%	15.0%	0%
疼痛強度	40.0±29.0	43.4±30.0	18.7±19.0*1

*1：保存治療や手術治療と比較してp<0.01

1.2)．

身体別しびれでは上肢しびれ(保存治療4.4±3.1，手術治療3.9±3.2)とともに，下肢しびれ(保存治療4.1±3.2，手術治療4.2±3.2)が次に強かった．上肢しびれはNRS5以上の患者が41.4%であった．

3．上肢しびれと関連する因子

上肢しびれと関連する因子を調べると，保存治療群では上肢機能・下肢機能・頚椎機能，さらに各ストレス因子，SF-8の各因子，SABSと相関があり，手術治療群ではさらに年齢，体重，MRIでのT2強調画像での高輝度の有無と相関があった(表6)．また満足度との関連では手術群で頚部痛みと相関があった．(Spearman相関係数0.48，$p<0.001$)

表6　上肢しびれと相関のある因子

	保存治療	手術治療
年齢	ns	.185*
体重	ns	−.195*
T2高輝度	ns	.240**
JOA上肢運動機能	−.327**	−.477**
下肢運動機能	−.397**	−.235**
SABS*1	−.316**	−.301**
JOACMEQ頚椎機能	−.395**	−.267**
上肢機能	−.511**	−.438**
下肢機能	−.505**	−.316**
膀胱機能	−.387**	−.212*
QOL	−.528**	−.480**
POMS緊張・不安	.411**	.316**
抑うつ・落ち込み	.260*	.226*
怒り・敵意	.237*	.266**
活気	ns	−.257**
疲労	.475**	.327**
混乱	.299**	.279**
SF−8全体的健康感	−.593**	−.395**
身体機能	−.554**	−.324**
日常役割機能(身体)	−.556**	−.439**
活力	−.444**	−.316**
社会生活機能	−.591**	−.374**
心の健康	−.435**	−.389**
日常役割機能(精神)	−.569**	−.362**

*1：Self-Assessment Bending Scale，
*：$p<0.05$，**：$p<0.01$(Pearson　相関係数)，ns：not significant

考察・結語

たとえ早期に治療を行った場合でも感覚神経障害，すなわち痛みやしびれが改善しないことは少なくない．とくに後頚部から肩甲部にかけての痛みと上肢のしびれに対する術後の訴えは多い．今回の結果では治療の内容によらず，同程度の頚部の痛みと上肢しびれがあり，NRS5以上の強度のある患者が頚部痛みで1/3以上，上肢しびれで約4割もいた．

手術症例に関しては，後方手術という間接的除圧が多いことや，神経根への対処が不十分な可能性はある．しかし前方手術による直接除圧でもかなりの患者でしびれを訴え，QOLを悪化させるような感覚神経障害が残ることがある．これらは難治性の術後疼痛やしびれとして扱われてきたが，近年は神経障害疼痛あるいは脊髄障害性疼痛と呼ばれるようになってきた．神経障害性疼痛とは病変あるいは疾患が感覚神経への直接的影響により生じる疼痛，と定義されている[6]．その本態は

maladaptive plasticity，すなわち障害に対する知覚神経の誤った可塑反応と考えられており[2]，障害原因がない状態や生理的な軽度の刺激でもQOL障害につながる痛みが起きている病態である．神経障害性疼痛は整形外科医が得意な侵害受容性疼痛とは異なる病態であり，非ステロイド性抗炎症薬の効果も限定的であると言われている．神経障害性疼痛には異なった治療戦略で取り組む姿勢が求められるであろう．

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Comparison Between a Real Sequential Finger and Imagery Movements: An fMRI Study Revisited

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Abstract Recently, much discussion has been centered on the brain networks of recall, memory, and execution. This study utilized functional magnetic resonance imaging to compare activation between a simple sequential finger movement (real task) and recalling the same task (imagery task) in 15 right-handed normal subjects. The results demonstrated a greater activation in the contralateral motor and somatosensory cortex during the real task, and a higher activation in the contralateral inferior frontal cortex, ipsilateral motor, somatosensory cortex, and midbrain during the imagery task. These real task-specific areas and imagery-specific areas, including the ipsilateral motor and somatosensory cortex, are consistent with recent studies. However, this is the first report to demonstrate that the imagery-specific regions involve the ipsilateral inferior frontal cortex and midbrain. Directly comparing the activation between real and imagery tasks demonstrated the inferior frontal cortex and midbrain to therefore play important roles in cognitive feedback.

Keywords fMRI · Finger movements · Imagery · Cognitive feedback · Recall

Introduction

The human primary motor cortex is the motor system region that provides signals to muscles, and the supplemental motor area is related to the preparation of motor tasks. Studies in monkeys have also revealed the brain networks related to

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motor tasks, such as the primary motor cortex, to be active, not only during execution, but also during the preparation of motor tasks (Alexander and Crutcher 1990). Since reports first demonstrated that blood oxygen concentrations could influence the T2* results in the animal cortex, functional MRI has now become a commonly used modality for measuring activation in the human brain (Ogawa et al. 1990). The supplemental motor area and primary motor cortex have also been shown, through fMRI methodology, to be activated during both the preparation and execution phases of sequential movements (Richter et al. 1997). The supplemental motor area is the center for the planning, preparation, and execution of sequential movements (Mushiake et al. 1991; Shima and Tanji 1998). The supplemental motor area is activated during not only real motor task preparation, but also during imagery motor tasks (Gerardin et al. 2000; Hanakawa et al. 2003, 2008). They used simple finger tapping tasks. On the other hand, an old study done by Roland et al. (1980) which used a relatively complex task using SPECT (single photon emission computed tomography) revealed a large area, including the SMA (supplemental motor area) and primary motor area, to be activated. At that time, the functional MRI (fMRI) method was not available. Regarding the spatial resolution, the fMRI method is considered to have some advantages over SPECT.

The current study used fMRI with a relatively more complex task, to show detailed locations with a difference between a real and imaginary task. The difference between the real and imagery task is a feedback system such that the real task has sensory feedback and the imagery task has cognitive feedback. Complex finger movement requires more cognitive function than a simple task which can cause a more sensible fMRI signal, similar to the findings reported in the study by Roland et al. The complex finger movement task was expected to distinguish the difference between the real and imaginary task. The hypothesis is that, using fMRI, a relatively complex finger movement task, which could activate prominent and wider brain regions than simple finger tapping, would thus reveal a more accurate location, such as the brain stem or mid brain structure than using SPECT, and more accurate differences between real and imagery tasks. Therefore, functional MRI, as well as real and imagery motor tasks which were similar to those described in previous reports (Roland et al. 1980), were utilized for the present study, and several new findings were thus observed.

Materials and methods

Subjects

Fifteen right-handed, normal subjects (12 males, 3 females, mean age 30.9±7.9) were studied. The subjects were not on any forms of medication, and had no history of psychosis/mental illnesses or drug abuse. All participants had >12 years of education. Written consent was obtained from all participants. This study was approved by the Kurume University IRB committee.

Task design

The activation task utilized for this study was similar to previously published methods (Roland et al. 1980). All participants were instructed to move their right fingers in the order from their thumb to little fingers and then back again (Fig. 1, 3 Hz self-paced movement). The rest task required the subject to remain still and attempt to keep the mind free of thoughts. The finger movement task was administered during the activation periods of each block, every 51 s. Three activation blocks and three rest blocks, including a starting rest period took a total of 6 min and 6 s.

The same design was used with the imagery task; the participants were instructed to think about the movement they had just performed previously. Following the imagery

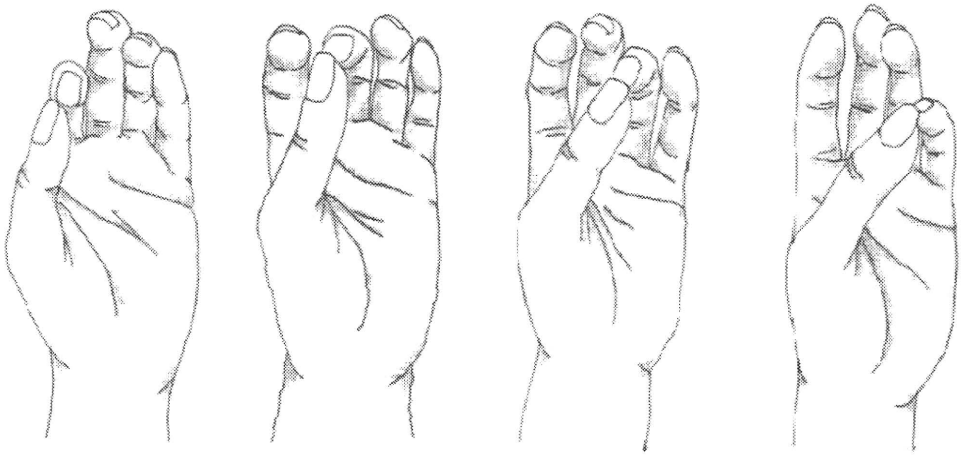

Fig. 1 Sequential finger movement task. Real task required real movement from thumb to little finger, and back again, with 3 Hz self-paced movement. Imagery task required thinking of the same task, without any real movement

Table 1 Locating and p-values (the real movements specific areas)

	T (voxel)	p-value	x (mm)	y (mm)	z (mm)	p (cluster)	Cluster-size
Left precentral gyrus	5.23	0.000064	−32	−18	60	0.00065	129
	3.90	0.000807	−32	−30	50		
Left postcentral gyrus	4.86	0.000126	−50	−22	58	0.01111	62
	4.12	0.000519	−46	−20	48		

task, the participants were asked to estimate how much they could recall the task, with a score ranging between 0 and 10. During the imagery task, the movements were monitored with a camera in order to confirm whether or not the subjects stayed still.

Data acquisition

Magnetic resonance imaging was performed on a SIEMENS 1.5 T scanner. The T2*-weighted images, which covered the entire brain, were acquired from each subject using an EPI (Echo Planer Imaging) pulse sequence (TE 70 ms, TR 5.1 s, flip angle 90°, FOV 225*225*148 mm^3, voxel dimension 64*64*40, slice thickness 3.5 mm). This series was designed as a block task, namely 10 volumes served as the control resting state and 10 volumes served as activation periods. One series included 60 volumes of Echo Planer images, which captured the three periods of activation tasks and three periods of rest.

Data pre-processing

All images were acquired from the scanner in the DICOM format and then were subsequently converted to the Analyze format through the DICOM toolbox in the SPM5 software package (Wellcome Department of Cognitive Neurology, London, UK) included in MATLAB (Mathworks Inc., Natick, MA). The first five scans were excluded from the series, due to signal instability. The SPM5 software program allowed for data realignment to correct for movement. Subjects whose movements were within 2 mm for axis and less than 1° of rotation were selected. The Montreal Neurological Institute EPI reference brain, provided by SPM, was utilized to normalize each EPI image with $2\times2\times2$ mm^3 voxel resampling. Lastly, a Gaussian spatial filter was employed to smooth the images to a final smoothness of 5 mm.

Statistical analysis

Data were analyzed on an individual (subject per subject, and task per task) basis, as well as across subjects (group analysis) using subject variance (random effect model). For individual analysis, data from each run were modeled using the general linear model, with separate functions modeling the hemodynamic response for each experimental epoch. Covariates were used to model long-term signal variations (temporal cut-off of 128 s), overall differences between runs, and sex. One contrast (real finger movement task or imagery versus rest) was defined for each series (per subject). Subsequent to attaining contrast images, repeated measures ANOVA (analysis of one variance) with sphericity correction (Greenhouse-Geiser) was applied to the data to determine whether more activated areas existed in one task over another (real>imagery, or imagery>real). The F maps were thresholded to $F=17.14$ ($p<0.001$) for the main task effect. Lastly, a post-hoc *t*-test was applied to the areas where differences existed between the tasks with $T=3.79$ ($p<0.001$) as the threshold. In these thresholded maps, activated clusters were considered significant if their spatial extent was >34, corresponding to a risk factor (type I error) of $p<0.05$ (corrected).

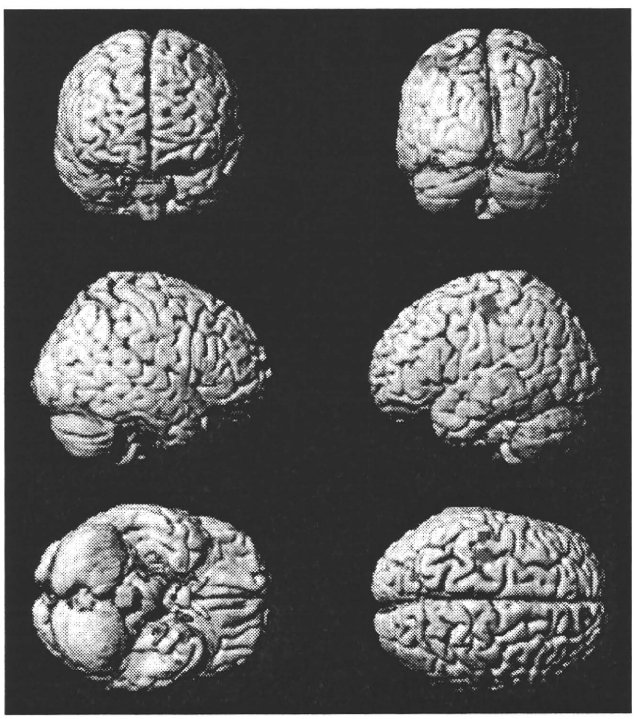

Fig. 2 Real sequential finger movement resulted in greater activation, in comparison to the imagery task. A difference was detected in the contralateral (*left*), precentral gyrus, including the supplemental motor cortex, as well as the motor cortex and post central gyrus, which included the somatosensory cortex

Table 2 Locating and p-values (the imagery movements specific areas)

	T(voxel)	p-value	x (mm)	y (mm)	z (mm)	p (cluster)	Cluster-size
Left inferior frontal gyrus	6.44	0.000008	−40	46	6	0.00023	157
Left inferior frontal gyrus	5.11	0.000079	−38	40	0		
Right middle frontal gyrus	6.02	0.000016	26	6	44	0.04362	36
Right middle frontal gyrus	4.64	0.000192	28	12	52		
Right precentral gyrus	5.68	0.000028	46	−12	44	0.00387	85
Right precentral gyrus	4.44	0.000282	44	−4	50		
Middle cerebellar peduncle	5.35	0.000052	6	−30	−38	0.03888	38
Middle cerebellar peduncle	4.83	0.000132	10	−36	−26		

Results

Real finger movement demonstrated significantly greater activation in the contralateral (left), precentral gyrus (MNI; x=−32, y=−18, z=60, T=3.83, p=0.000064) including the supplemental motor cortex, as well as the motor cortex and post central gyrus (MNI; x=−50, y=−22, z=58, T=4.86, p=0.000126), which included the somatosensory cortex in comparison to imagery finger movement (Table 1, Fig. 2).

Significantly greater activation was demonstrated with the imagery finger movement in the left inferior frontal gyrus (MNI; x=−40, y=46, z=6, T=6.44, p=0.000008), right middle frontal gyrus (MNI; x=26, y=6, z=44, T=6.02, p=0.000016), right precentral gyrus (MNI; x=46, y=−12, z=44, T=5.68, p=0.000028), and midbrain (MNI; x=6, y=−30, Z=−38, T=5.35, p=0.000052) in comparison to real finger movement, (Table 2, Fig. 3).

The ability of the participants to recall the real task presented with a rating score of 7.53±1.16.

Discussion

During real motor tasks, significantly greater activation was recorded in the supplemental motor area, motor cortex, and contralateral somatosensory cortex, compared with the imagery task. This activation was considered to be due to real motor activity. A number of studies have suggested that these areas play a fundamental role in the recognition of tactile stimuli and motions (Donchin et al. 1998; Penfield 1950; Roland et al. 1980; Sawamura et al. 2002). The present study demonstrated a similar impact from real motor movement. The activation of real movement was compared to imagery; namely, areas such as the supplemental motor area (SMA), primary motor area (PMA), and contralateral somatosensory area (S1) were greatly activated in the real task of finger movement, rather than imagery finger movement. These areas were thought to be solely related to executive functions. fMRI results have been previously compared between real and imagery finger tasks. (Gerardin et al. 2000; Sirigu et al. 1996), thus demonstrating bilateral activations for the real task. However, the current study showed an activation difference only in the contralateral hemisphere, not in the bilateral areas. Penfield and Welch illustrated the homunculus through the employment of electrical stimulation (Penfield and Welch 1949). A recent study, utilizing an independent component analysis, revealed contralateral activation due to finger movement (Kansaku et al. 2005). In addition, a detailed visual cued fMRI study also revealed contralateral activation (Hanakawa et al. 2008). Contralateral SMA, PMA, and S1 should therefore be regarded as the execution of specific areas. The BOLD (blood oxygen

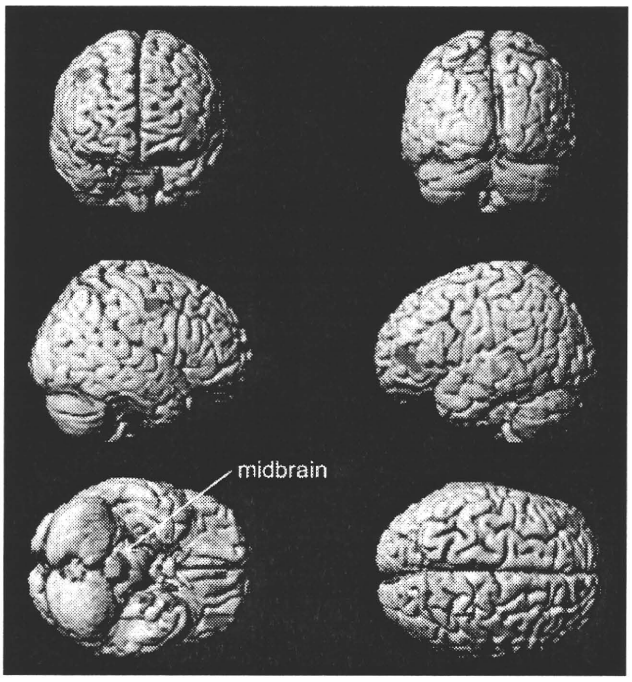

Fig. 3 The imagery sequential finger movement resulted in greater activation, in comparison to the real task. A difference was detected in the left inferior frontal gyrus, the right middle frontal gyrus, the right precentral gyrus, and the midbrain. The small area in the midbrain is indicated with an *arrow*

dependent signal) might therefore be more sensitive to the real motor function on the contralateral side.

In contrast, the imagery finger movement task stimulated a greater activation in the ipsilateral gray matter, such as the motor and somatosensory cortices, in comparison to the real finger movement task. For this study, the participants were asked to recall the task that they had just previously performed. The hypothesis was that the memory of performing a task, even if a lateral task had been demanded, could involve the ipsilateral post- and pre-central gyrus. Hanakawa and colleagues reported that the imagery-specific regions involved the frontal-parietal areas, as well as the cerebellum (Hanakawa et al. 2003). Recently, the same group demonstrated that the superior frontal sulcus serves as an imagery-specific region (Hanakawa et al. 2008). However, the results from the present study suggested that ipsilateral activation activated the front-parietal area, which thus serves as an imagery-specific area.

In addition, the present study revealed midbrain activation. The activated area in the midbrain was located in the middle cerebellar peduncle during the imagery task. A previously published study demonstrated that the pedunculopontine nucleus region induced impaired conditioned responses, absent of global motor impairment, to salient stimuli (Florio et al. 2001). This area might therefore play an important role in learning, memory, or the recollection of motor tasks.

The left ventrolateral prefrontal cortex has previously been shown to be related to the cognitive control of memory (Badre and Wagner 2007). The movement task employed for the present study might thus require the activation of the left ventrolateral prefrontal cortex in order to recall a task and control motor task memory. Recently, the mirror neuron system theory supported the hypothesis that this region could be involved in task coding (Lotze et al. 2006; Rizzolatti and Craighero 2004). This area has also been reported to play an important role in the rehearsal of a given movement in the context of working memory theory (Wagner et al. 2001). If the left ventrolateral prefrontal cortex is only involved in the mirror neuron system, then no activation difference could be detected between the real and imagery tasks, because both tasks require movement coding. By comparing real activation to imagery, the present results demonstrated that this area should thus be regarded as more than just a part of the mirror neuron system. The difference between the two tasks was due to information feedback. While the real task included physiological (sensory) feedback, the imagery task contained no feedback from the finger, only cognitive feedback. One hypothesis is that this area is used to control memory through cognitive feedback, rather than through the sensory feedback system. In addition, the mirror neuron system served as an inferior neural network, which thus coded for movement, and was utilized for both real and imagery tasks. In contrast, the cognitive feedback system might be superior to the mirror neuron system.

This study did not use electromyograms because of the problem of the equipment environment during the scan. Although the movements were carefully checked by camera and the participants stayed still, there is a possibility that accurate movements, for example the subtle muscle movements, could still be detected. This is one limitation of this study.

Conclusion

In summary, the present study demonstrated motor-specific activation in the contralateral supplemental motor area, motor area, and somatosensory motor area (consistent with recent studies). Imagery-specific activation stimulated the ipsilateral front-parietal area (consistent with other fMRI studies), midbrain, and contralateral (left) inferior frontal cortex (the first report using fMRI). Imagery-specific areas might therefore play an important role in cognitive feedback.

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Leukotriene Synthases and the Receptors Induced by Peripheral Nerve Injury in the Spinal Cord Contribute to the Generation of Neuropathic Pain

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KEY WORDS
5-lipoxygenase; p38 MAPK; microglia; spinal cord; peripheral nerve injury

ABSTRACT
Leukotrienes (LTs) belong to a large family of lipid mediators, termed eicosanoids, which are derived from arachidonic acids and released from the cell membrane by phospholipases. LTs are involved in the pathogenesis of inflammatory diseases, such as asthma, rheumatoid arthritis, and peripheral inflammatory pain. In the present study, we examined whether LTs were implicated in pathomechanism of neuropathic pain following peripheral nerve injury. Using the spared nerve injury (SNI) model in rats, we investigated the expression of LT synthases (5-lipoxygenase; 5-LO, Five lipoxygenase activating protein; FLAP, LTA4 hydrolase; LTA4h and LTC4 synthase; LTC4s) and receptors (BLT1, 2 and CysLT1, 2) mRNAs in the rat spinal cord. Semi-quantitative RT-PCR revealed that 5-LO, FLAP, LTC4s, BLT1, and CysLT1 mRNAs increased following SNI, but not CysLT2 mRNAs. Using double labeling analysis of *in situ* hybridization with immunohistochemistry, we observed that 5-LO, FLAP, and CysLT1 mRNAs were expressed in spinal microglia. LTA4h and LTC4s mRNAs were expressed in both spinal neurons and microglia. BLT1 mRNA was expressed in spinal neurons. The p38 mitogen-activated protein kinase inhibitor, but not MEK inhibitor, reduced the increase in 5-LO in spinal microglia. Continuous intrathecal administration of the 5-LO inhibitor or BLT1 and CysLT1 receptor antagonists suppressed mechanical allodynia induced by SNI. Our findings suggest that the increase of LT synthesis in spinal microglia produced via p38 MAPK plays a role in the generation of neuropathic pain.

INTRODUCTION

Peripheral nerve injury induces a number of alterations in gene expression, protein synthesis, and intracellular signaling in nociceptive pathways (Scholz and Woolf, 2002; Woolf and Salter, 2000; Zhuang et al., 2006). Recent evidence suggests that glial cells in the spinal cord undergo dynamic changes in their gene expression and produce a number of important mediators, triggering the activation of an excitatory circle among glial cells. And importantly, activated glial cells produce and release proinflammatory cytokines, such as IL-1β, TNF-α, and neurotrophins (DeLeo and Yezierski, 2001; Milligan and Watkins, 2009; Scholz and Woolf, 2007; Watkins and Maier, 2003; Watkins et al., 2001a,b), resulting in an enhanced excitability of nociceptive dorsal horn neurons.

One of most interesting subjects in pain research is the discovery of lipid mediators and their receptors in nociceptive pathways, and their crucial roles in pathological pain conditions (Ma and Quirion, 2008; Tsuda et al., 2007; Yao et al., 2009). The metabolism of arachidonic acid via cyclooxygenase and lipoxygenase pathways produces prostaglandins and several lipoxygenase metabolites, respectively. It is widely accepted that prostaglandins generated by cyclooxygenase act locally to sensitize peripheral nociceptors to external stimuli, resulting in the development of hyperalgesia or allodynia (Moriyama et al., 2005). Prostaglandins are also suggested to have a role in the spinal cord in modulating nociceptive pathways (O'Rielly and Loomis, 2006; Zhao et al., 2000). Another lipid mediator which has collected much attention as a possible pain mediator is lysophosphatidic acid (LPA). LPA may be released in the spinal cord after nerve injury and affect the excitability of dorsal horn neurons, which may be involved in the hyperalgesia/allodynia after peripheral nerve injury (Inoue et al., 2004; Ueda, 2008).

Leukotrienes (LTs) are a group of lipid mediators derived from arachidonic acid and comprise several products of the 5-lipoxygenase (5-LO) pathway. Arachidonic acid is converted to leukotriene A4 (LTA4) and LTA4 is converted to LTB4, or LTC4, LTD4, and LTE4 enzymatically, that are known as bioactive LTs. LTC4, LTD4, and LTE4 are collectively termed as the cysteinyl leukotrienes (CysLTs). LTs act by binding to specific receptors that are located on the outer plasma membrane of structural and inflammatory cells (Henderson, 1994). Four G-protein coupled LT receptors have been cloned (Heise et al., 2000; Lynch et al., 1999; Yokomizo et al., 1997, 2000). Leukotriene B4 receptor 1 (BLT1) is a receptor which recognizes LTB4 with high affinity, and BLT2 binds LTB4 and many other LTs with low affinity.

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The cysteinyl leukotriene receptor 1 and 2 (CysLT1 and 2) are known to recognize CysLTs with different affinities (Peters-Golden and Henderson, 2007). LTs have a variety of biological actions and have been recognized as important factors in numerous disease processes including allergic diseases such as asthma and atopic dermatitis, local and systemic inflammatory diseases such as rheumatoid arthritis and psoriasis, cancer, and cardiovascular diseases (Henderson, 1994; Peters-Golden and Henderson, 2007).

Recently, it was reported that LTs have a key role in the pain mechanisms of peripheral inflammation and some papers indicated the involvement of spinal lipoxygenase metabolites in hyperalgesic responses (Bisgaard and Kristense, 1985; Jain et al., 2001; Martin et al., 1988). In this study, the detailed expression patterns of enzymes for LTs synthesis and the receptors in the rat spinal cord were examined. Moreover, we explored in detail the alteration of expression of these molecules after peripheral nerve injury and their roles in neuropathic pain. We hypothesize that the expression and the dynamic changes in LTs and their receptors have important roles in pathological pain.

MATERIALS AND METHODS

Animal Procedures

Male Sprague Dawley rats weighing 200–250 g were anesthetized with sodium pentobarbital (50 mg kg ^{1}, i.p.) and received spared nerve injury (SNI) (Decosterd and Woolf, 2000). At several time points (1, 3, 7, and 14 days) after the SNI, groups of rats were processed for histological analysis (n = 4 at each time point). Every effort was made to minimize animal suffering and reduce the number of animals used. All animal experimental procedures were approved by the Hyogo College of Medicine Committee on Animal Research and were performed in accordance with the National Institutes of Health guidelines on animal care.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and *In Situ* Hybridization Histochemistry (ISHH)

The rats were killed by decapitation under deep ether anesthesia. Spinal cords (L4-L5) were removed and rapidly frozen with powdered dry ice and stored at −80°C until use. Extraction of total RNA was done using a single step extraction method with ISOGEN (Nippon Gene, Tokyo, Japan) as described in a previous paper (Yamanaka et al., 2005). PCR primers for LT synthase, receptor, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA were designed as follows:

5-LO (accession number J03960) primers, sense 5′-CGGGCACCGACGACTACATT-3′ and antisense 5′-GGG GCAGATCCTTGTGGCAT-3′;

FLAP (accession number X52196) primers, sense 5′-GGCCATCGTCACCCTCATCA-3′ and antisense 5′-GAT GAGAAGCAGCGGGGAGA-3′;

LTA4h (accession number S87522) primers, sense 5′-GGGGACCCTATCTTTGGGGA-3′ and antisense 5′-CAG GAGCATAGAGCCAGGCA-3′;

LTC4s (accession number AB048790) primers, sense 5′-GGTGATCTCTGCGCGAAGAA-3′ and antisense 5′-TCAGGCCATCGGCAGGAGCA-3′;

BLT1 (accession number AB025230) primers, sense 5′-GGTGGCATGTCCCTGTCTT-3′ and antisense 5′-GACCGGTATGGCCAGCAGAA-3′;

BLT2 (accession number AB052660) primers, sense 5′-GGGGTGATCGCGTGTGTCAA-3′ and antisense 5′-CTCCCCAGAGCCTTCGAAGA-3′;

CysLT1 (accession number AB052685) primers, sense 5′-TGCTGGTGCTGAGTATGTCG-3′ and antisense 5′-GCATGTCAGATCCACAACGG-3′;

CysLT2 (accession number AB052661) primers, sense 5′-CTGGGGAGCCTTGGGAAATG-3′ and antisense 5′-GTGGACCTGATTCTGGAAATC-3′;

GAPDH (accession number M17701) primers, sense 5′-TGCTGGTGCTGAGTATGTCG-3′ and antisense 5′-GCATGTCAGATCCACAACGG-3′.

The resulting PCR products were used to generate the cRNA probes for ISHH. The rats were killed by decapitation under deep ether anesthesia. The bilateral L4-L5 spinal cord were dissected out, rapidly frozen in powdered dry ice, and cut on a cryostat at a 10 μm thickness. The protocol for ISHH was based on a published method (Chen et al., 1995). Using the enzyme-digested clones, ^{35}S UTP-labeled antisense and sense cRNA probes were synthesized. The ^{35}S-labeled probes in hybridization buffer were placed on the section, and then incubated at 55°C overnight. Sections were then washed and treated with 1 μg mL ^{1} RNase A. Subsequently, sections were dehydrated and air-dried. After the hybridization reaction, the slides were coated with NTB emulsion (Kodak, Rochester, NY) and exposed for 3–4 weeks. Once developed in D-19 (Kodak), the sections were stained with hematoxylin-eosin and coverslipped.

Immunohistochemistry

Rats that received the SNI were used for immunohistochemistry. The treatment of rats and methods of immunohistochemistry were described before (Yamanaka et al., 2004). The following antibodies were used: rabbit anti-ionized calcium-binding adapter molecule1 (Iba1) polyclonal antiserum (1:100; Wako Chemicals, Tokyo, Japan), goat anti-Iba1 polyclonal antiserum (1:1,000; Abcam, Cambridge, MA), rabbit anti-phospho-p38 MAPK (p-p38) polyclonal antiserum (1:500; Cell Signaling Technology, Beverly, MA), mouse anti-neuronal specific nuclear protein (NeuN) monoclonal antiserum (1:1,000; Chemicon, Temecula, CA), and rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antiserum (1:1,000; DakoCytomation, Glostrup, Denmark). Double-immunofluorescent staining was performed with anti-

rabbit Alexa Fluor 594 IgG (1:1,000; Invitrogen, San-Diego, CA) and anti-goat Alexa Fluor 488 IgG (1:1,000) after incubation with respective primary antibodies.

Double-Labeling Analysis of *In Situ* Hybridization with Immunohistochemistry

To examine the distribution of mRNAs for LT synthase and receptors in neurons versus glial cells, we used a combined ISHH with immunohistochemistry (IHC) (Kobayashi et al., 2006). The frozen spinal cord sections were processed for IHC using the ABC method. The following antibodies were used: rabbit anti-Iba1 polyclonal antiserum, mouse anti-NeuN monoclonal antiserum, and rabbit anti-GFAP polyclonal antiserum. After immunohistochemistry, these sections were immediately processed for ISHH. These sections were fixed again in 4% formaldehyde in PB for 5 min, treated with 10 μg mL^{-1} protease K in 50 mM Tris-5 mM EDTA (pH 8.0) for 5 min, postfixed in the same fixative, acetylated with acetic anhydride in 0.1 M triethanolamine, rinsed in PB, and dehydrated through an ascending ethanol series. The following processes are the same as those mentioned in ISHH.

Drug Treatments

Two or six days after, or at the same time as SNI surgery, the L5 vertebra was laminectomized under adequate anesthesia with sodium pentobarbital, and a soft tube (Silascon, Kaneka Medix Company, Osaka, Japan; outer diameter, 0.64 mm) filled with 5 μL of saline was inserted into the subarachnoid space for an ~0.5-cm length (tube were pointed caudally). Mini-osmotic pumps (model 2001; 7d pump, 1 μL h^{-1}; Alzet, Corporation, Palo Alto, CA) filled with saline or 5-LO inhibitor AA-861 (2-(12-Hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone) (BIOMOL, PA, USA) or CysLT1 receptor antagonist pranlukast (*N*-[4-oxo-2-(1H-tetrazol-5-yl)-4H-1-benzopyran-8-yl]-4-(4-phenylbutoxy)-benzamide (Cayman Chemical, Ann Arbor, MI) or BLT1 receptor antagonist U-75302 (6-[6-(3-Hydroxy-1E,5Z-undecadienyl)-2-pyridinyl]-1,5-hexanediol) (Sigma, Poole, UK) were connected to the tube. Then, the pump was laid under the skin and the incision was closed. The concentrations of AA-861 and pranlukast were 0.24 nmol day^{-1} and 2.4 nmol day^{-1} diluted in 0.02–0.2% ethanol or 50% dimethyl sulfoxide (DMSO), respectively (n = 6–8, for behavior test). The concentrations of U-75302 were 2.4 nmol day^{-1} and 24 nmol day^{-1} diluted in 10% DMSO/saline (n = 6–8, for behavior test).

The intrathecal delivery of the p38 MAPK inhibitor, SB203580 hydrochloride (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1Himidazole, HCl) (Calbiochem, La Jolla, CA) and MAPK kinase (MEK) 1/2 inhibitor, U0126 (1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene) (Calbiochem, La Jolla, CA) were performed at the same time as the SNI. To obtain a sustained drug infusion, an Alzet osmotic pump (model 1003D; 3d pump, 1 μL h^{-1}; Durect, Cupertino, CA) was filled with SB203580 in saline or U0126 in 50% DMSO, and the associated catheter was implanted intrathecally before SNI. The concentrations of SB203580 were 36 nmol day^{-1} and 196 nmol day^{-1} (n = 4, for histochemical analysis). The concentration of U0126 was 29.8 nmol day^{-1} (n = 4, for histochemical analysis).

Behavioral Tests

All SNI rats were tested for mechanical allodynia on the plantar surface of the hindpaw 1 day before surgery and 1, 3, 5, 7, 9, 11 and 14 days after surgery. Mechanical allodynia was assessed with a dynamic plantar anesthesiometer (Ugo Basile, Comerio, Italy) (Kalmar et al., 2003; Lever et al., 2003). The detailed method of mechanical sensitivity measurement in rat hindpaw was described previously (Kobayashi et al., 2008).

Statistics

Data are expressed as mean ± SEM. Differences in changes of values over time of each group were tested using one-way ANOVA, followed by individual *post hoc* comparisons (Fisher's exact test) or pairwise comparisons (t test) to assess differences of values between naive versus each time point of the SNI groups. A difference was accepted as significant if $P < 0.05$.

RESULTS

The gene expression of the LT synthetic enzymes from the substrate arachidonic acid and the receptors of LTs were examined in the rat spinal dorsal horn using RT-PCR methods. At first, the mRNA level of LT synthases was determined after peripheral nerve injury (SNI) (Fig. 1A,C). The L4-5 spinal cord was taken at 0 (naive), 3, 7, and 14 days after surgery. Three of 4 LT synthases examined, 5-LO, FLAP, and LTC4s, increased their mRNA content after SNI surgery. Significant increases were observed 3 days after nerve injury and continued at least for 14 days except 5-LO. LTA4h mRNA did not change after SNI. The expression of LT receptors was also examined using RT-PCR (Fig. 1B,C). The mRNA of BLT1 increased gradually after SNI and was significantly increased at 7 days after injury. In contrast, the level of CysLT1 rapidly increased at 3 days after injury and remained statistically significant at least until 14 days. CysLT2 showed no change in mRNA after SNI. We could not detect BLT2 mRNA in the rat spinal cord by RT-PCR method.

The presence of the mRNA of synthetic enzymes and the receptors of LTs in the spinal cord led us to behavioral experiments to study whether the LTs have a role in abnormal pain. First, the effect of an intrathecal injection of the 5-LO inhibitor was examined using the

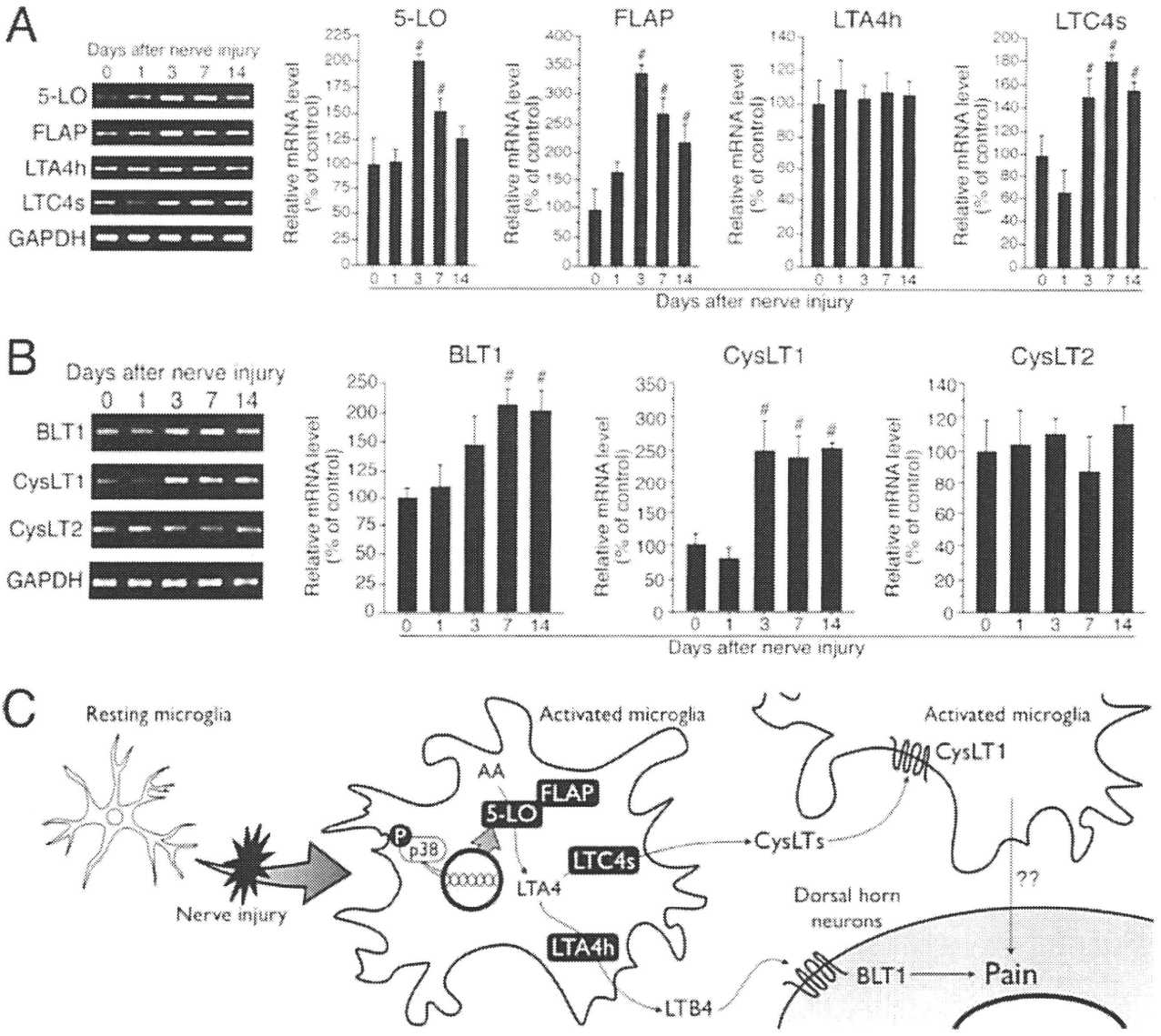

Fig. 1. Peripheral nerve injury (SNI) induces mRNAs for a series of LT synthase and receptors in the ipsilateral spinal cord. The levels of (**A**) LT synthase and (**B**) receptor mRNAs were determined using the RT-PCR technique. Gel panels show PCR products from the L4-L5 spinal cord taken from 0 (naive), 1, 3, 7, and 14 days after surgery. Right graphs show quantification of the relative mRNA levels of LT synthases and the receptors. LT synthase and receptor mRNA levels were normalized against GAPDH ($n = 4$, mean ± SEM, #; $P < 0.05$ compared with naive). (**C**) Proposed model for a neuropathic pain mechanism of LT synthases and the receptors in the dorsal horn. Injury to peripheral nerve activates microglia in the spinal dorsal horn. p38 MAPK is phosphorylated and up-regulates 5-LO expression. Peripheral nerve injury also induces FLAP, LTA4h, and LTC4s expression in activated microglia. CysLT1 and BLT1 receptors increased in microglia and neurons, respectively. Activation of CysLT1 and BLT1 receptors may contribute to neuropathic pain.

SNI model (Fig. 2A,B). The osmotic pump was set and the administration of inhibitor started at 2 days after SNI surgery. The nerve injury induced a decrease in the withdrawal threshold indicating mechanical hypersensitivity and the administration of 5-LO inhibitor (2.4 nmol days^{-1}) attenuated the mechanical hypersensitivity significantly compared with the vehicle-treated group. Low dose of the 5-LO inhibitor did not produce a significant effect. On the contralateral side to the nerve injury, the 5-LO inhibitor did not produce any effect on the withdrawal threshold. Next, we examined the effect of intrathecal injection of the BLT1 receptor antagonist on the pain behaviors (Fig. 2C,D). Because constitutive expression of BLT1 expression was observed in the spinal cord, the BLT1 antagonist was administered from the time of SNI surgery for 1 week. Only a high dose of BLT1 antagonist (24 nmol days^{-1}) attenuated the mechanical hypersensitivity significantly compared with vehicle control. This treatment did not have any effect on the contralateral side.

The effect of intrathecal injection of a CysLT1 receptor antagonist was also examined (Fig. 2E,F). The intrathecal administration of the CysLT1 antagonist attenuated the decrease in withdrawal threshold after SNI. There was a significant effect of the antagonist from 3 to 7 days after injury, with a similar time course to the 5-LO inhib-

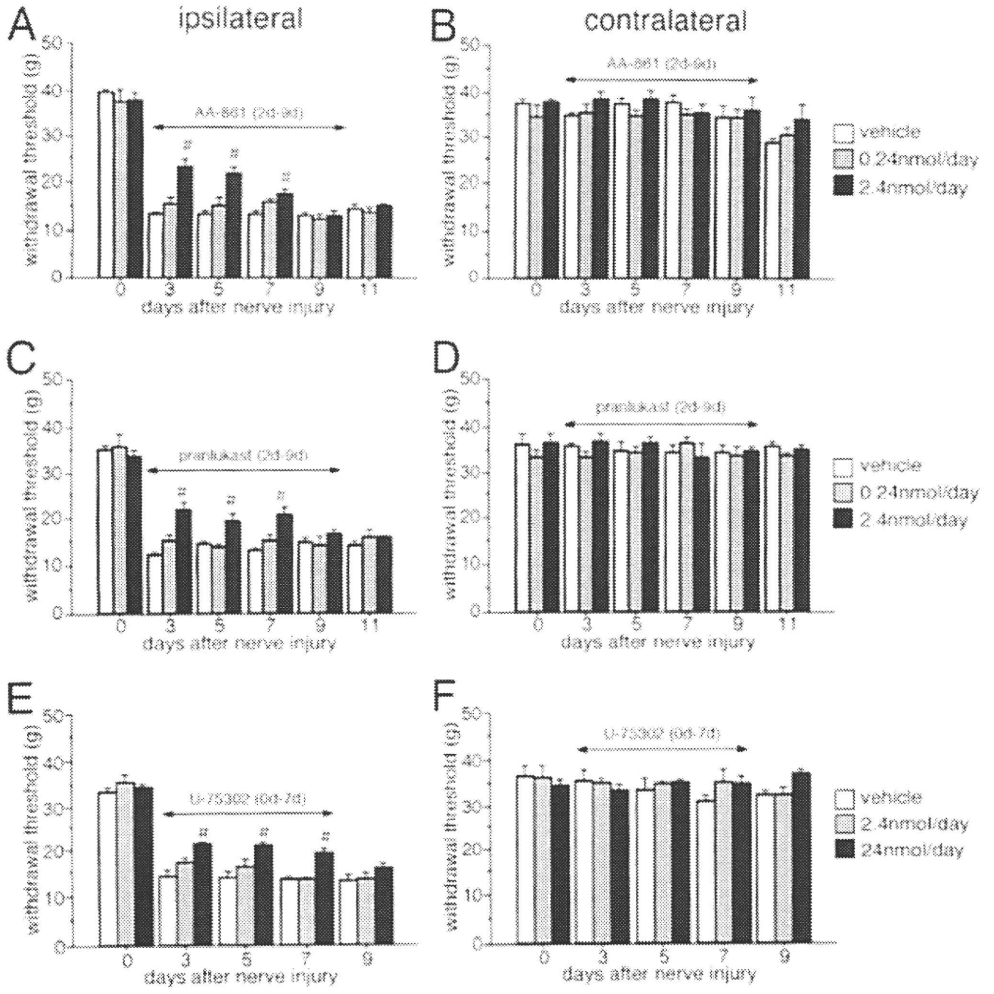

Fig. 2. Intrathecal administration of the 5-LO inhibitor, CysLT1, and BLT1 antagonists suppressed the mechanical allodynia induced by SNI. Continuous infusion of (**A**) 5-LO inhibitor (AA-861), (**C**) CysLT1 (Pranlukast), and (**E**) BLT1 (U-75302) antagonists inhibited the mechanical allodynia on the ipsilateral side to the SNI surgery. (**A–D**) The osmotic pump was set 2 days after surgery and drug administration continued for 7 days. (**E, F**) The osmotic pump was set at the same time as SNI surgery and drug administration continued for 7 days. (B, D, F) The treatment with these drugs did not change the mechanical sensitivity on the contralateral side. In all graphs, values are mean ± SEM (n = 6–8 in each group, # $P < 0.05$ compared with vehicle).

itor. The low dose of the antagonist had no effect. On the contralateral side to the nerve injury, the CysLT1 antagonist had no effect on base line mechanical sensitivity.

To determine the effect of antagonists of LT receptors on established pain behaviors, the osmotic pump with antagonist of BLT1 or CysLT1 was implanted at 6th day after SNI surgery and pain behaviors were measured from 7 to 14 days (Fig. 3A–D). Both antagonists did not attenuate the decrease of withdrawal threshold after SNI surgery (Fig. 3A,C). These treatments did not affect the threshold on the contralateral side (Fig. 3B,D).

These findings suggest a novel idea that LT synthesis increases in the spinal cord after peripheral nerve injury and the inhibitors of these enzymes affect pain behaviors in the rat, especially during the development of neuropathic pain. To determine the mechanisms of LTs dynamics in the spinal cord, we further examined the detailed expression and the modulation by mitogen-activated protein kinase (MAPK) signaling. Initially, we examined the mRNA of 5-LO in the spinal cord. In the spinal cord of naive rats, we detected very few aggregations of silver grains, suggesting very low mRNA levels (Fig. 4A). Three days after SNI surgery, aggregations of signals were observed in the dorsal horn on the ipsilateral side to the nerve injury (Fig. 4B), especially in cells with small nuclei deeply stained by hematoxylin (Fig. 4C). There was no induction of 5-LO mRNA in the contralateral dorsal horn (Fig. 4B). The changes in FLAP mRNA in the spinal cord were more dramatic. The increase of FLAP mRNA was apparent in ipsilateral dorsal horn (Fig. 4D–F). The aggregation of grains occurred in cells with small nuclei stained by hematoxylin (Fig. 4F). Next, the detailed expression of LTA4h and LTC4s mRNA in the spinal cord was examined using ISHH. We found that both mRNAs were expressed throughout the gray matter in the spinal cord. Although the PCR results showed no increase in LTA4h mRNA after nerve injury, ISHH sections appeared to have an increase in

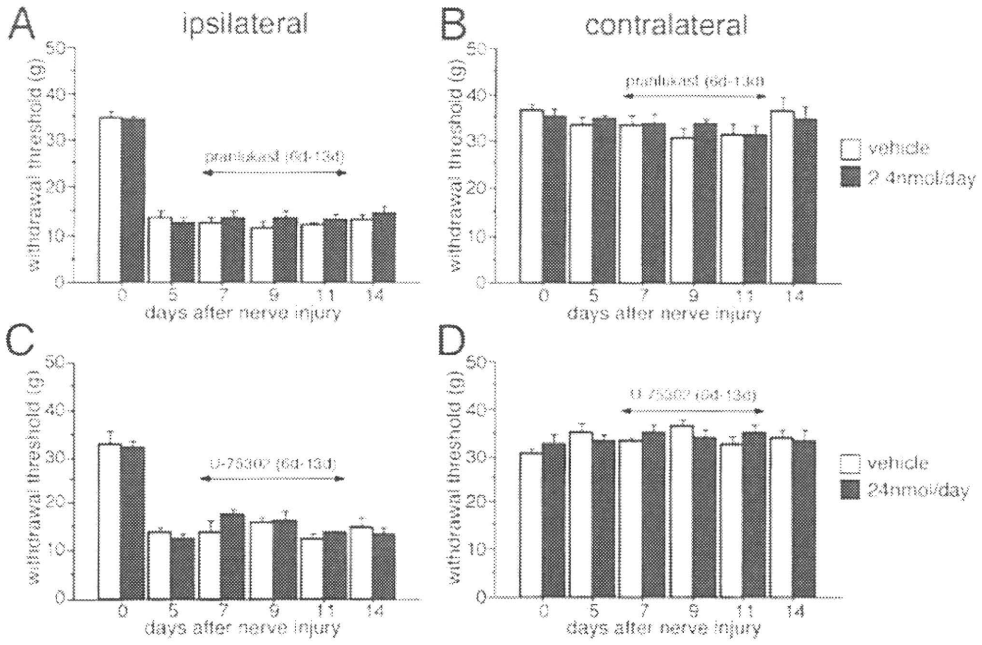

Fig. 3. The delayed administration of the LT receptor antagonists did not reverse the mechanical allodynia induced by SNI. Post-treatment with pranlukast (**A**, **B**) and U-75302 (**C**, **D**) had no effect on mechanical allodynia on the ipsilateral side and mechanical sensitivity on the contralateral side. The osmotic pump was implanted 6 days after SNI and worked for 7 days. In all graphs, values are mean ± SEM (n = 6–7 in each group, # $P < 0.05$ compared with vehicle).

the signal in dorsal horn (Fig. 4H). Brightfield photographs showed constitutive signals in cells with large nuclei lightly stained by hematoxylin and increased signals after nerve injury in cells with small nuclei deeply stained by hematoxylin (Fig. 4I). LTC4s mRNA was expressed constitutively in throughout the grey matter and increased after nerve injury in dorsal horn (Fig. 4J,K). This increase in LTC4s mRNA mainly occurred in cells with small nuclei (Fig. 4L).

To elucidate what cells expressed mRNAs of LT synthetic enzyme and receptor before and after peripheral nerve injury, we performed double labeling with IHC and radioisotope-labeled ISHH. The double-labeling experiments revealed that the expression of 5-LO mRNA in the spinal cord 3 days after nerve injury occurred in cells labeled for Iba1, not NeuN or GFAP (Fig. 5A–C). This data suggest 5-LO mRNA was expressed in microglia in the spinal cord after nerve injury. The double labeling with FLAP mRNA and cell markers also indicated that microglia expressed FLAP mRNA (Fig. 5D–F). In contrast, mRNA expression of LTA4h and LTC4s were observed in cells labeled for both NeuN and Iba1 (Fig. 5G–L), suggesting the expression in neurons and microglia. These four mRNAs were not expressed in cells labeled for GFAP, suggesting no expression in astrocytes.

Next, we performed a detailed morphological analysis of LT receptors in the spinal cord after peripheral nerve injury using ISHH. As predicted from the RT-PCR data, the expression level of BLT1 mRNA was low, even after spinal nerve injury, and therefore the increase in BLT1 mRNA in the spinal cord was hard to see in darkfield photographs (Fig. 6A,B). The brightfield photograph showed the aggregation of silver grains on cells with large nuclei that were lightly stained by hematoxylin in the gray matter after SNI (Fig. 6C). On the contralateral side to the nerve injury, there were fewer aggregations of silver grains (data not shown). The expression in CysLT1 mRNA in the spinal cord increased in the dorsal horn after SNI (Fig. 6D,E). The labeled cells for CysLT1 mRNA contained small nuclei deeply stained by hematoxylin (Fig. 6F). In contrast, the CysLT2 mRNA was expressed in the white matter of the spinal cord (Fig. 6G–I), and this expression was not changed after SNI compared with the controls. To identify the cell type expressing LT receptor mRNAs, double labeling was performed with cell markers (Fig. 6J–O). We found the BLT1 mRNA was co-labeled with NeuN, but not with GFAP or Iba1 (Fig. 6J–L). This finding suggests the BLT1 mRNA was expressed in neurons in the spinal cord and increased after SNI. The expression of CysLT1 mRNA was also examined using double labeling and we found that CysLT1 mRNA was colocalized with Iba1, not with NeuN or GFAP (Fig. 6M–O), suggesting this mRNA was expressed in microglia in the spinal cord.

LT synthetic enzymes, such as 5-LO and FLAP, and LT receptor, CysLT1 were found to be increased in microglia in the spinal cord after peripheral nerve injury. We found that only BLT1 was increased in neurons in spinal gray matter after nerve injury. 5-LO and FLAP are first step enzymes of the LT metabolic pathway (Fig. 1C), and the changes in the signal transduction cascade in microglia is known to be important step for activation of microglia. We examined whether p38 inhibitor (SB203580) application could suppress or inhibit

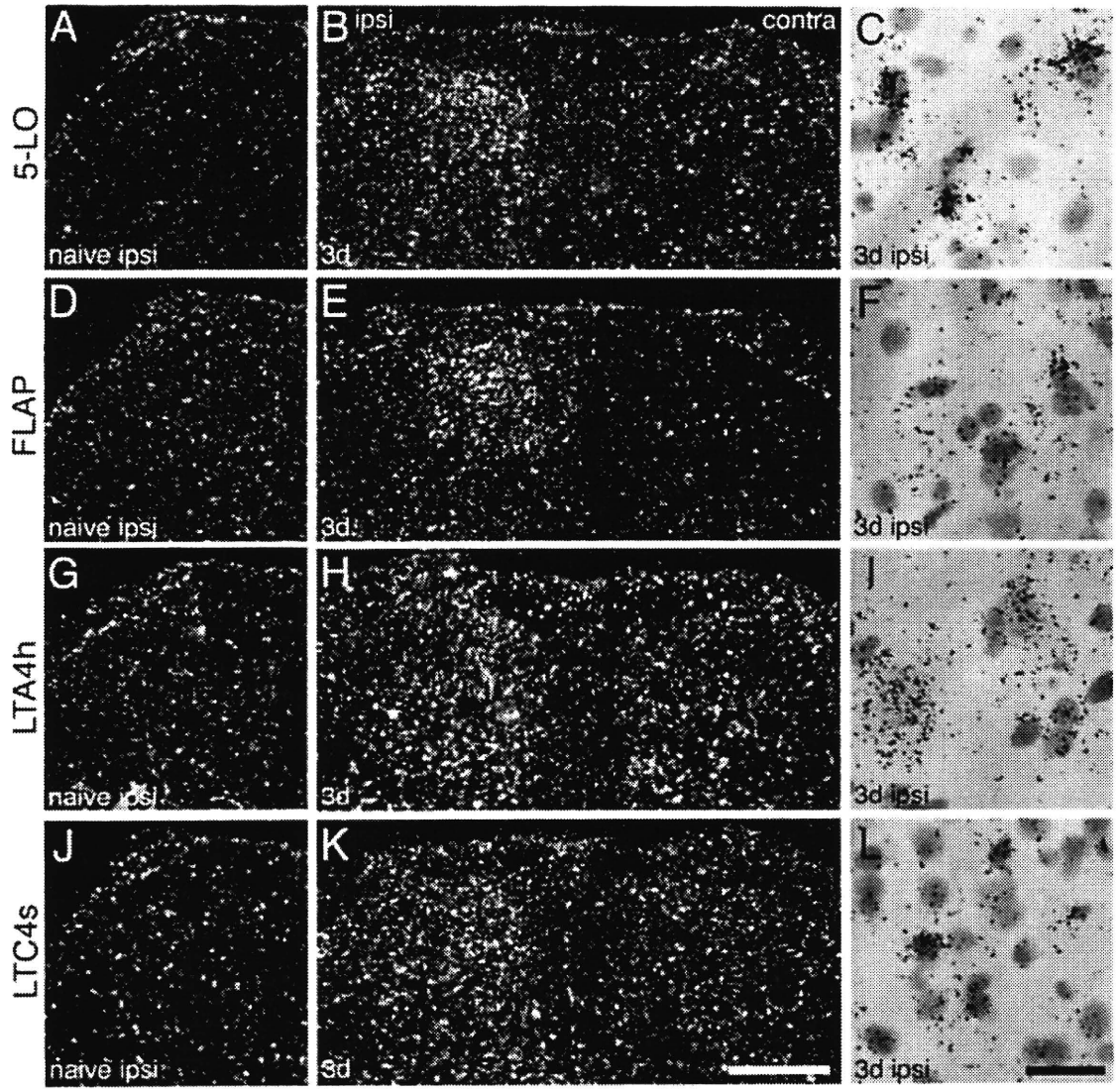

Fig. 4. LT synthase mRNAs induced by SNI were expressed in the ipsilateral dorsal horn. ISHH revealed the mRNA expression of 5-LO (A–C), FLAP (D–F), LTA4h (G–I), and LTC4s (J–L) in naive rats (A, D, G, J) and 3 days after nerve injury (B, E, H, K). (C, F, I, L) Brightfield photographs of lamina I–II in the ipsilateral spinal cord 3 days after nerve injury showed the mRNA expression for LT synthases. Scale bars: darkfield images; 500 μm, brightfield images; 25 μm. 3 d; 3 days after surgery, ipsi; ipsilateral, contra; contralateral. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the upregulation of LT synthesis in microglia after SNI. At first, we confirmed the upregulation of phosphorylated p38 in microglia in the spinal cord after nerve injury, and found that phospho-p38-labeled cells were also labeled for Iba1 extensively after SNI (Fig. 7A). We tested the effect of several doses of the p38 inhibitor on mechanical allodynia following SNI surgery (Fig. 7B). All doses of the p38 inhibitor significantly suppressed mechanical allodynia. On the contralateral side to the nerve injury, the p38 inhibitor did not produce any effect on the withdrawal threshold (data not shown).

RT-PCR analysis of spinal tissues 3 days after SNI revealed that the pretreatment with the p38 MAPK inhibitor (192 nmol days^{-1}) suppressed the increase in 5-LO mRNA (Fig. 7C). Lower dose of the p38 inhibitor did not significantly attenuate the increase in 5-LO mRNA after nerve injury. MEK inhibitor, U0126, could not suppress mRNAs for synthase and receptor induced by SNI. Other enzymes, such as FLAP and LTC4s and the LT receptor CysLT1, were not suppressed by the pretreatment with the p38 inhibitor (Fig. 7C). The content of Iba1 mRNA observed after SNI was not changed by the pretreatment. Finally, we confirmed the effects of the p38 inhibitor on 5-LO and Iba1 using morphological methods (see Fig. 8). The signal of 5-LO mRNA was clearly decreased by the application of p38 inhibitor, but there was no effect on Iba1 immunoreactivity.

DISCUSSION

In the present study, we examined the distribution of mRNAs of LT synthase and receptor in the rat spinal

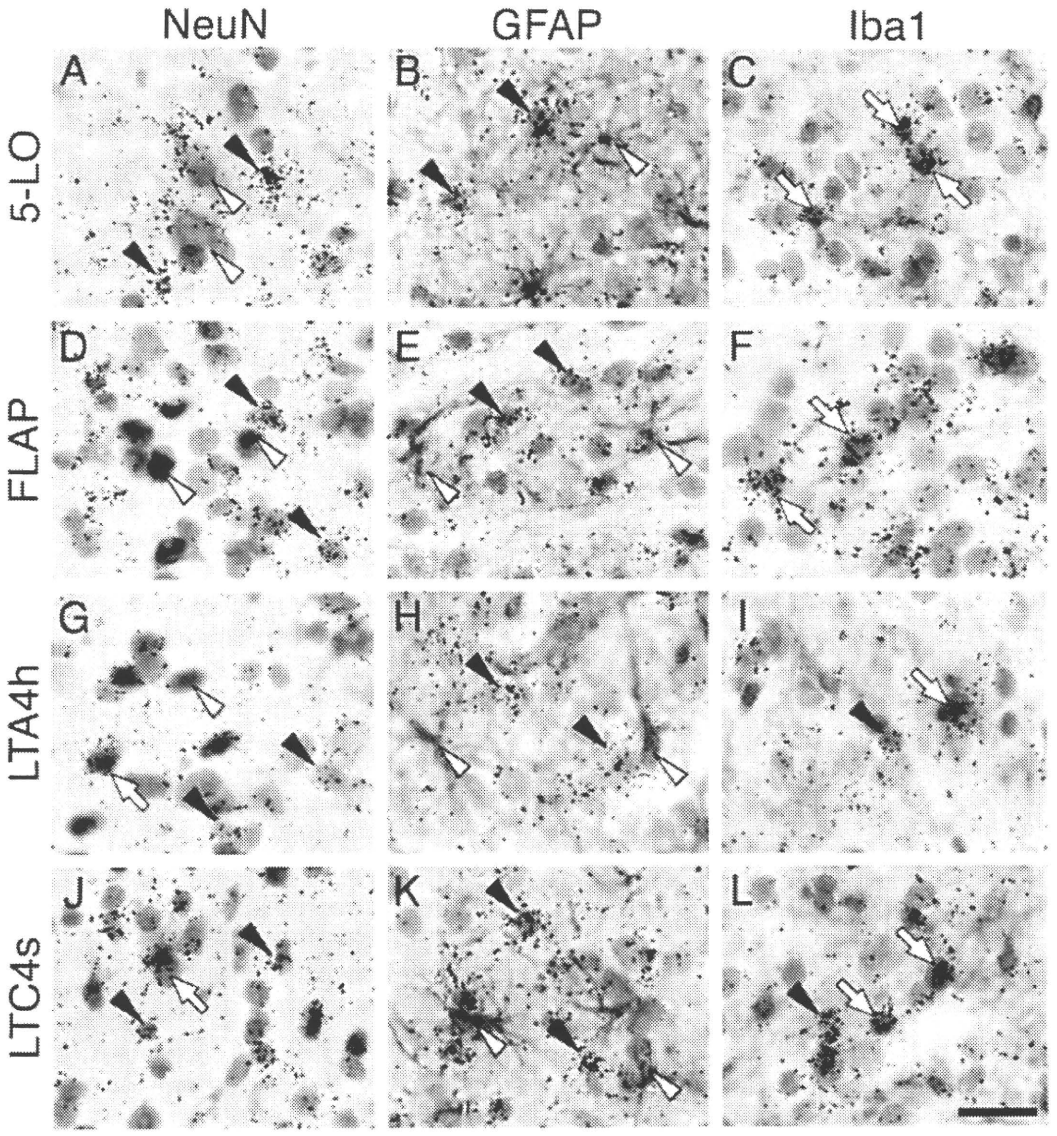

Fig. 5. Induction of LT synthase mRNAs occurred in the spinal microglia 3 days after SNI. Photographs show combined ISHH for LT synthase; (A–C) 5-LO, (D–F) FLAP, (G–I) LTA4h, and (J–L) LTC4s mRNAs with immunostaining of NeuN (A, D, G, J), GFAP (B, E, H, K) and Iba1 (C, F, I, L). Scale bar; 25 μm. Open arrows indicate double-labeled cells. Arrowheads indicate single-labeled cells by ISHH (aggregation of grains), and open arrowheads indicate single immunostained cells (brown staining). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cord using RT-PCR and ISHH. One of most important findings was that 5-LO and FLAP mRNAs increased in spinal microglia and after SNI surgery (Fig. 1C). The 5-LO is the most important enzyme in the process of synthesis of LTs and FLAP enhances the ability of 5-LO to interact with its substrate. We found 5-LO and FLAP mRNAs in spinal microglia, suggesting that LTs are synthesized and playing a role in the microglia in the spinal cord. The following enzymes, LTA4h and LTC4s mRNAs, were also increased in the spinal cord by ISHH. However, RT-PCR confirmed the significant increase in mRNA content only of the LTC4s, but no increase of LTA4h after nerve injury. This discrepancy may be derived from the ample constitutive expression of mRNA in numerous cells, including ventral horn neurons, which results in the saturation of RT-PCR signals in all conditions. In any case, we believe that LTB4 and CysLTs are synthesized in spinal microglia a few days after peripheral nerve injury.

Another important finding in the present article is that the receptors of LTs, BLT1, and CysLT1, are expressed in the gray matter in the spinal cord. ISHH and double-labeling studies revealed that BLT1 is localized in neurons and CysLT1 in microglia. We also found that these receptors were upregulated by peripheral nerve injury in each cell type. These findings are interesting, because LTs synthesized in the spinal cord after SNI may have an affect on different cells, neurons, and microglia. Increasing evidence in pain research indicates that glial cells in the spinal cord have substantial roles in the development