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研究課題名（和文）神経因性疼痛モデルにおけるグリシン及び GABA トランスポーター阻害薬の鎮痛効果
研究課題名（英文）Antinociceptive effects of glycine- and GABA- transporter inhibitors in neuropathic pain models

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研究成果の概要（和文）：侵害受容伝達で重要な脊髄において抑制性神経のグリシン作動性神経伝達を調節するグリシントランスポーター-2 (GlyT-2)の阻害薬 ALX1393 をラット急性痛モデルで髄腔内投与すると運動機能には影響を与えない用量で抗侵害受容作用を示した。GlyT-2 阻害薬の新たな急性痛治療薬として可能性が示唆された。同様に脊髄で抑制性神経伝達物質として働くタウリンは神経障害性疼痛モデルで抗侵害受容作用を示した。本研究から疼痛治療におけるグリシン作動性神経への介入の重要性が示唆された。

研究成果の概要（英文）：Inhibitory neurotransmitter transporters are promising targets for treatments of acute, inflammatory, and neuropathic pains, and glycinergic neurons may be key component of modulating nociceptive transmittion.

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キーワード：(1)神経障害性疼痛 (2)グリシントランスポーター阻害剤 (3)GABA トランスポーター阻害剤 (4)疼痛行動実験 (5)坐骨神経結紮モデル (6)　抗侵害作用(7)運動協調機能

１．研究開始当初の背景

神経因性疼痛には有効性の高い薬物が無く、慢性疼痛の成因の大部分を占める。このため臨床でその治療に難渋している。神経因性疼痛に対する研究は近年精力的に行われており複雑な病態と機序が少しずつ明らかにされている。中枢神経での主要な抑制性神経伝達物質の GABA とグリシンは疼痛シグナル伝達の生理的調節因子で主に脊髄の介在ニューロンとして抑制性に働いており、神経因性疼痛の病態にも関与すると考えられる。グリシン受容体アンタゴニストのストリキニーネを髄腔内投与すると痛覚過敏やアロディニアが起こることが動物実験で確かめられている。また、$GABA_A$受容体アンタゴニストが痛覚過敏やアロディニアを起こしアゴニストを投与することで抑制される。筆者らはシナプス前膜に存在し、放出された神経伝達物質を再取り込みすることでシナプス伝達を終了させる働きをする膜蛋白質でシナプス間隙での GABA 及びグリシンの動態に大きな役割を持つトランスポーターに注目しそれらの阻害薬が抑制性ニューロンの働きを増強させ、神経因性疼痛を抑制する可能性があると考える。GLYT には 2 つのサブタイプが存在する。GLYT-1 はグルタミン酸ニューロンに近接する星状細胞の突起に存在し NMDA 型受容体の co-agonist であるグリシンの濃度を調節する役割をしており、GLYT-1 機能の抑制は興奮性グルタミン酸ニューロンの働きを増強させると考えられる。一方 GLYT-2 は脊髄のグリシンニューロン・シナプス前膜に存在し、その機能を抑制することは脊髄の主要な抑制性ニューロンの働きを増強させると考えられる。

痛みの伝達物質や抑制物質のシナプス伝達に関する研究は受容体に対するものがほとんどである。シナプスでの神経伝達物質の濃度に大きな影響を持つトランスポーターについてはあまり知られていない。中枢神経の下行性抑制系を構成するノルアドレナリンおよびセロトニンのトランスポーターの阻害薬が痛覚過敏に部分的に効果を示すことは知られているが脊髄の介在ニューロンであるグリシンおよび GABA のトランスポーター阻害薬の作用を調べた研究はない。

２．研究の目的

慢性疼痛の大部分の原因である神経因性疼痛には現在有効な薬物が無い。中枢神経での主要な抑制性神経伝達物質の GABA とグリシンは疼痛シグナル伝達の生理的調節因子として抑制性に働いていることが知られており神経因性疼痛の病態にも関与すると考えられる。シナプス前膜に存在し、シナプス間隙での GABA 及びグリシンの動態に大きな役割を持つ各々のトランスポーターの阻害薬が抑制性ニューロンの働きを増強させ、神経因性疼痛を抑制する可能性があると考える。ラットの神経因性疼痛モデルを用いて GAT 及び GLYT 阻害薬の鎮痛作用を急性疼痛に対する作用と併せて疼痛行動実験で検討する。

筆者は GLYT-1 と GLYT-2 に特異的な阻害薬を用いて動物疼痛行動実験で鎮痛作用を見出したいと考えている。一方、中枢神経系におけるもうひとつの抑制性ニューロンの GABA 作動性ニューロンも GAT-1-4 の 4 つのサブタイプにより調節されている。同様にサブタイプに特異的な阻害薬を用いて侵害受容神経伝達における GAT の役割を探る。尚、GLYT, GAT とも筆者が過去に研究してきたモノアミントランスポーターと同様に Na/Ca-dependent トランスポーターで薬理学的性質に多くの類似点がある。

これまで神経因性疼痛に対して GAT 及び GLYT サブタイプに特異的な阻害薬の作用を調べた報告はない。GLYT-2 や GAT-1, GAT-3 の阻害薬がラット神経因性疼痛モデルでの熱刺激および機械刺激に対する痛覚過敏を抑制する可能性がある。併せて急性疼痛に対する効果についても検討する。急性疼痛についての研究は GAT-1 を overexpression させたマウスが痛覚過敏状態になることが報告されている(1)。また、運動機能への副作用の有無についても調べるためロータロッドテストを行い、臨床応用可能な神経因性疼痛の新しい治療薬の可能性を探る。

(引用文献)

(1) Hu JH, Yang N, Ma YH et al.：
Hyperalgesic effects of gamma-aminobutyric acid transporter I in mice.
J Neurosci Res 73:565-72

３．研究の方法

(1)グリシントランスポーター(GLYT)阻害薬の作用に関する実験

①くも膜下カテーテル留置

薬物を髄腔内投与するため Yaksh らの方法により、ペントバルビタール麻酔下に大槽からポリエチレンカテーテル(PE-10)を尾側に向かって挿入した。

②急性疼痛に対する実験

無処置のラットを用いる。

(i) Tail-flick test(脊髄反射への影響): UGO BASILE 社製 7360 を用いた。尻尾に radiant heat を当て逃避反応が起こるまでの潜時を測定した。

cut-off: 15 秒 %MPE = [(ALX1393-treated latency) - (vehicle-treated latency)] / [15 (cut-off) - (vehicle-treated latency)] × 100

(ii) Hot-plate test(上位中枢への影響): Columbus Instruments 社製 Hotplate Analgesia Meter を用いた。熱板にラットを乗せ逃避反応が起こるまでの潜時を測定した。

cut-off: 30 秒 %MPE = [(ALX1393-treated latency) - (vehicle-treated latency)] / [30 (cut-off) - (vehicle-treated latency)] × 100
(iii) Paw pressure test: 後肢第3/4趾間に32 or 48g/sec で加圧した。啼くまでの閾値(g)を測定した。cut-off: 750g
(iv) ホルマリンテスト:5%ホルマリン 10μL をラットの後肢の足背に皮下注入し疼痛行動(licking, flinching)の回数をカウントし、Phase 1 と Phase 2 での影響を観察する。
それぞれの test について GLYT 阻害薬 Sarcosine 及び ALX 1393 [0 (control), 4, 20, 40 μg/kg, i.t.]の影響を調べた。
③運動機能に対する実験
ロータロッドテスト(Rotarod test)は薬物が運動機能、運動協調機能、運動学習機能に影響を与えるか否か調べる検査で薬物による疼痛行動の変化が鎮痛機序に及ぼす作用に由来するものなのか運動機能に対する作用によるものなのかを評価・鑑別する際には必要不可欠である。一方でロータロッドテストは鎮痛効果を有する物質の運動機能に関する副作用の有無をスクリーニングし、運動失調などの副作用の情報が得られる。UGO BASILE 社製 Model 47700 を用いて ALX 1393 [0 (control), 20, 40, 60 μg/kg, i.t.]の影響を調べた。
④神経因性疼痛モデルの作成
(i) Chronic constriction injury (CCI)モデル：Bennett らの方法により、雄 Sprague- Dawley rat をペントバルビタール麻酔下に大腿骨上の皮膚を切開し片側の坐骨神経を 4.0-silk 糸で 4 箇所緩く結紮して作成した。コントロール群には麻酔下に坐骨神経同部を剥離・露出させる sham operation を施した。
(ii) I 型糖尿病性ニューロパチー(DM)モデル：ストレプトゾトシン(STZ)75mg/kg を腹腔内投与し 1 週間後に血糖値が 250mg/dL 以上のラットを糖尿病とした。STZ 注入後 4-6 週のラットを実験に使用した。
⑤熱刺激および機械刺激過敏性に対する実験
(i) Plantar test (thermal hyperalgesia を調べる): UGO BASILE 社製 7370 を用いた。神経因性疼痛に随伴する熱刺激に対する痛覚過敏を評価するための検査で患肢足底に radiant heat を当て逃避反応が起こるまでの時間(潜時)を測定した。
(ii) Electronic von Frey test (mechanical allodynia を調べる): IITC Life Science 社製 model 2391C を用いた。患肢足底に力がかかるように filaments を当て、逃避反応が起こる閾値(g)を測定した。
GLYT 阻害薬 Sarcosine 及び ALX 1393 [0 (control), 4, 20, 40 μg i.t.]の影響を調べた。
(iii) Paw pressure test: 後肢第3/4趾間に32 or 48g/sec で加圧した。啼くまでの閾値(g)を測定した。cut-off: 750g

(2)GABA トランスポーター(GAT)阻害薬の作用に関する実験
①神経因性疼痛モデルの作成
②くも膜下カテーテル留置
③熱刺激および機械刺激過敏性に対する実験
(i) Plantar test
(ii) Electronic von Frey test

GAT 阻害薬 Nipecotic acid [0 (control), 1, 10 mg/kg, i.p.; 0 (control), 1, 5 mM, i.t.]及び NO-711 [0 (control), 1, 5 mM, i.t.] の影響を調べた。薬物の作用部位が脊髄であるか否か(末梢あるいは脳)を調べるためそれぞれの薬物について腹腔内投与あるいは髄腔内投与を行い効果の違いを観察した。

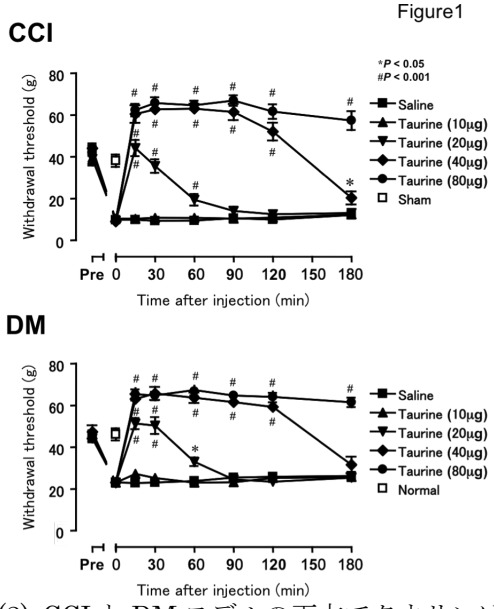
(3)タウリンの神経因性疼痛に対する作用に関する実験
グリシンと同様に中枢神経系で抑制性神経伝達物質として働くアミノ酸であるタウリン(10, 20, 40, 80 μg/10μL, i.t.)の鎮痛効果を併せて調べた。
①神経因性疼痛モデルの作成
(i) Chronic constriction injury (CCI)モデル
(ii) I 型糖尿病性ニューロパチー(DM)モデル
②くも膜下カテーテル留置
③熱刺激および機械刺激過敏性に対する実験
(i) Plantar test
(ii) Electronic von Frey test
(iii) Paw pressure test
④運動機能に対する実験
⑤作用機序に関する実験
タウリンの抗侵害受容作用にどの内因性鎮痛機構が関与しているか調べるためグリシン受容体阻害薬 strychnine (1mg/kg)、$GABA_A$ 受容体阻害薬 bicuculline (2 mg/kg)、$\alpha_2$受容体阻害薬 yohimbine (3 mg/kg)、$5\text{-}HT_3$ 受容体阻害薬 ondansetron (0.1 mg/kg)、オピオイド受容体阻害薬 naloxone (2 mg/kg)をタウリン注入 10 分前に腹腔内投与しタウリン作用への影響を調べた。

４．研究成果
《抑制性神経伝達物質トランスポーター阻害薬に関する研究》
(1) ALX1393 は侵害性熱刺激に対して用量依存性に抗侵害受容作用を示した。その作用はストリキニーネの同時投与により完全に消失した。最大効果は注入後 15-30 分で見られ 60 分間持続した。(2) 侵害性機械刺激に対して高用量で抗侵害受容作用を示した。その作用はストリキニーネの同時投与により完全に消失した。最大

効果は注入後 15 分で見られ 60 分後に消失した。(3) ホルマリンテストで第 1 相および 2 相反応を共に抑制したが第 2 相反応をより強く抑制した。(4) 運動機能を抑制しない用量で抗侵害受容作用が現れた。(5) 一方、GlyT-1 阻害薬 Sarcosine は全てのテストで抗侵害受容作用を示さなかった。(6) Nipecotic acid 及び NO-711 は用量依存性に抗侵害受容作用を示した。
GlyT-2 阻害薬 ALX1393 は侵害性熱刺激・機械刺激・化学刺激に対して疼痛行動を抑制し、作用発現時間と持続時間およびストリキニーネの効果から抗侵害受容作用はグリシン作動性神経伝達の増強によるものと考えられた。抗侵害受容作用が現れる用量で運動機能に影響しないことから ALX1393 を臨床に応用できる可能性があるが治療域が狭いことも示唆された。以上の結果から脊髄において抑制性ニューロンの機能を増強させると強い抗侵害受容作用が発現することがわかった(Anesth Analg 2010;110:615-21)。

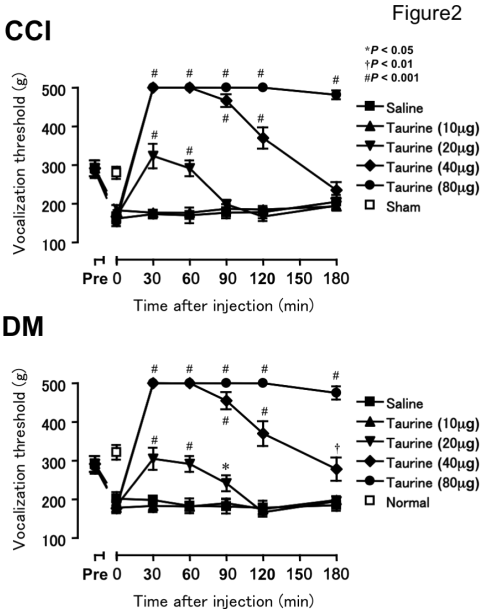
《抑制性神経伝達物質タウリンに関する研究》
次に著者らは中枢神経系で最も多く存在するタウリンが抑制性神経伝達物質として生理学的役割を持つことに注目した。タウリンは急性疼痛モデルと炎症性疼痛モデルにおいて抗侵害受容作用があることが報告されている。しかし神経障害性疼痛に対する作用についてはほとんど理解されていない。そこで CCI と DM の 2 種類の神経障害性疼痛モデルでのタウリンの髄腔内投与による作用を調べた。その結果、(1) CCI と DM モデルの両方でタウリンは用量依存性に機械刺激によるアロディニアを抑制した(Fig.1)。

Figure1



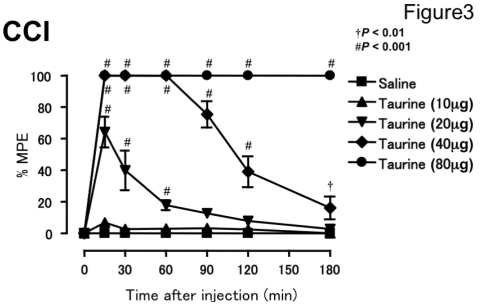

(2) CCI と DM モデルの両方でタウリンは用量依存性に機械刺激による痛覚過敏を抑制した(Fig.2)。

Figure2



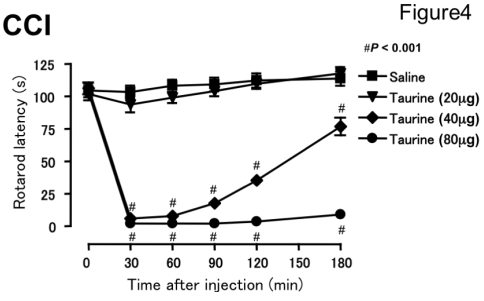

(3)タウリンの効果は二つのモデルで類似していた。
(4)CCI モデルでタウリンは用量依存性に熱刺激による痛覚過敏を抑制した(Fig.3)。

Figure3




(5) CCI モデルでタウリンは用量依存性に運動機能を抑制した(Fig.4)。

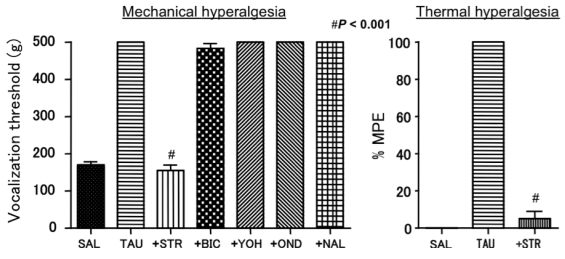
Figure4




(6) CCI モデルでタウリンの機械刺激および熱刺激に対する痛覚過敏はグリシン受容体阻害薬 strychnine の前処置により完全に消失した。一方、$GABA_A$ 受容体阻害薬、$\alpha_2$ 受容体阻害薬、$5\text{-}HT_3$ 受容体阻害薬、オピオイド受容体阻害薬はタウリンの抗侵害受容作用に影響しなかった(Fig.5)。

CCI

Figure5




に GlyT-2 阻害薬では高用量で運動機能抑制がみられるものの作用が強いことである。また関連して行った実験でタウリンが主にグリシン受容体を介して強力な抗侵害受容作用を示した。これらの結果を併せて、本研究によりこれまで鎮痛薬のターゲットとしてあまり注目されていないグリシン作動性神経の調節がこれから慢性疼痛治療の新たなターゲットとなる可能性が示唆された。

５．主な発表論文等

〔雑誌論文〕（計１件）

①Haranishi Y, Hara K, Terada T, Nakamura S, Sata T.:
The antinociceptive effect of intrathecal administration of glycine transporter-2 inhibitor ALX1393 in a rat acute pain model.
Anesth Analg 2010;110:615-21.［査読有］

Pain Mechanisms
*Section Editors:* **Tony L. Yaksh/Quinn H. Hogan**

# The Antinociceptive Effect of Intrathecal Administration of Glycine Transporter-2 Inhibitor ALX1393 in a Rat Acute Pain Model

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**BACKGROUND:** Glycinergic neurons in the spinal dorsal horn have been implicated in the inhibition of spinal pain processing in peripheral inflammation and chronic pain states. Neuronal isoform glycine transporter-2 (GlyT2) reuptakes presynaptically released glycine and regulates the glycinergic neurotransmission. In this study, we examined whether a selective GlyT2 inhibitor, ALX1393, elicits an antinociceptive effect in a rat acute pain model.
**METHODS:** Male Sprague-Dawley rats were implanted with a catheter intrathecally. The effects of intrathecal administration of ALX1393 (4, 20, or 40 μg) on thermal, mechanical, and chemical nociception were evaluated by tail flick, hot plate, paw pressure, and formalin tests. Furthermore, to explore whether ALX1393 affects motor function, a rotarod test was performed.
**RESULTS:** ALX1393 exhibited antinociceptive effects on the thermal and mechanical stimulations in a dose-dependent manner. The maximal effect of ALX1393 was observed at 15 min after administration, and a significant effect lasted for about 60 min. These antinociceptive effects were reversed completely by strychnine injected immediately after the administration of ALX1393. In the formalin test, ALX1393 inhibited pain behaviors in a dose-dependent manner, both in the early and late phases, although the influence was greater in the late phase. In contrast to antinociceptive action, ALX1393 did not affect motor function up to 40 μg.
**CONCLUSIONS:** This study demonstrates the antinociceptive action of ALX1393 on acute pain. These findings suggest that the inhibitory neurotransmitter transporters are promising targets for the treatment of acute pain and that the selective inhibitor of GlyT2 could be a novel therapeutic drug.
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Glycine is a major inhibitory neurotransmitter in the central nervous system (CNS). Glycinergic neurons in the spinal dorsal horn have been implicated as having a crucial role in the inhibition of spinal pain processing in peripheral inflammation and the chronic pain state.[1] Previous studies have shown that intrathecal administration of the glycine receptor antagonist, strychnine, can elicit nociceptive responses,[2–4] whereas intrathecal glycine was found to prevent mechanical hyperalgesia in a rat neuropathic pain model.[5] The synaptic function of glycine released presynaptically is terminated by uptake via $Na^+/Cl^-$-dependent glycine transporters (GlyTs). The concentrations of glycine at the glycinergic synaptic cleft can be controlled by GlyTs activities. Two GlyT subtypes have been identified (GlyT1 and GlyT2).[6] GlyT1 is expressed widely in the CNS and is localized mainly in glial cells surrounding both inhibitory and excitatory synapses. GlyT1 is also found on the terminals of some excitatory neurons expressing *N*-methyl-D-aspartate (NMDA) receptors, where glycine acts as a coagonist of glutamate to facilitate excitatory neurotransmission mediated by NMDA receptors. Thus, the inhibition of GlyT1 can enhance the activities of excitatory neurons and may counteract the enhanced glycinergic neurotransmission. In contrast, GlyT2 is localized mainly at the presynaptic terminals of glycinergic neurons in the spinal cord, brainstem, and cerebellum. The overall distribution of GlyT2 parallels that of glycine receptors. GlyT2 is thought to be the main isoform mediating the clearance of glycine presynaptically released at the inhibitory synaptic cleft.[7,8] Therefore, one can postulate that the GlyT2 inhibitor facilitates the glycinergic neurotransmission and has the ability to suppress spinal nociceptive processing.

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Recently, Tanabe et al.[9] reported the antinociceptive and antiallodynic effects of selective GlyT1 inhibitors in mice inflammatory and neuropathic pain models. Other investigations have demonstrated that selective inhibitors of GlyT2, as well as GlyT1, are effective in regulating nociceptive responses in mouse and rat neuropathic pain models.[10,11] Exogenous glycine injected intrathecally can partially activate the NMDA receptor, and a GlyT1 inhibitor also mediates both excitatory and inhibitory actions, as observed in previous studies.[10,11] Therefore, a GlyT2 inhibitor is expected to produce a more potent antinociceptive effect than glycine or the GlyT1 inhibitor, with little adverse action. GlyT2 may be a promising target of an ideal therapeutic drug for acute and chronic pain states.

In this study, we first examined whether the intrathecal administration of ALX1393, a selective GlyT2 inhibitor,[12,13] has an antinociceptive effect on thermal, mechanical, and chemical stimulations in a rat acute pain model. To explore the clinical availability, the effect of ALX1393 on motor function was also examined.

## METHODS

### Animals and Drug Preparation

This study was approved by the Ethics Committee of Animal Care and Experimentation at the University of Occupational and Environmental Health, Japan. One hundred ninety male Sprague-Dawley rats (Kyudo, Fukuoka, Japan) weighing 180–230 g were used in this study. Rats were housed with free access to food and water and maintained on a 12:12 h light-dark cycle at constant room temperature 22°C ± 2°C and humidity 50% ± 5%. All experiments were performed at the same time (between 10:00 and 17:00) during the light period. Rats were assigned randomly to treatment groups, with the experimenter blinded to the treatments. All experimental groups consisted of 6–10 rats, unless otherwise noted.

ALX1393 (O-[(2-benzyloxyphenyl-3-flurophenyl) methyl]-L-serine), dimethyl sulfoxide (DMSO), pentobarbital sodium, and strychnine hydrochloride were purchased from Sigma (St. Louis, MO). Polyethylene catheters (PE-10) were obtained from Becton, Dickinson and Company (San Jose, CA). ALX1393 was first dissolved in DMSO and then diluted in 0.9% physiological saline. The highest final concentration of DMSO was 50% for 40 and 60 μg of ALX1393 and 25% for 4 and 20 μg. Strychnine was dissolved in saline.

### Intrathecal Catheter Implantation

For multiple intrathecal administration of drugs, lumbar catheters were implanted in all rats according to the procedure by Yaksh and Rudy.[14] Under anesthesia using pentobarbital sodium (75 mg/kg, IP, supplemented as necessary), a stretched PE-10 polyethylene catheter (8.5 cm) was inserted into the intrathecal space and advanced caudally to the rostral edge of the lumbar enlargement through an incision in the atlantooccipital membrane. A 7-day interval was allowed to elapse before including an animal in the study. Rats with any neurological dysfunction, such as hindlimb paralysis or urine incontinence, were excluded from the study. Proper location of the catheter was confirmed by hindlimb paralysis after the injection of 10 μL of 2% lidocaine 2 days before the study. For assays, 10 μL of ALX1393 (4, 20, 40, or 60 μg) or DMSO (25% or 50%) was administered intrathecally, followed by 10 μL of saline to flush the catheter.

### Tail Flick Test

A radiant heat source was focused on the middle part of the rat's tail. The time interval from the onset of the stimulus until the tail flick response was measured using a tail flick unit (7360, Ugo Basile, Comerio, Italy). The intensity of the radiant heat was adjusted to give a tail flick latency of 4–5 s before the administration of ALX1393 or vehicle (DMSO 25% or 50%). In the absence of a response, the stimulus was terminated after 15 s (cutoff) to prevent tissue damage. The effects of ALX1393 (4, 20, and 40 μg) were assessed at 15 min after administration, and a time course for the action of ALX1393 (40 μg) was recorded for 180 min.

The measured reaction latencies (s) were converted to the percentage of the maximum possible effect (%MPE) according to the formula: %MPE = ([ALX1393-treated latency] − [vehicle-treated latency])/(15[cutoff] − [vehicle-treated latency]) × 100.

### Hot Plate Test

The hot plate test was performed using a hotplate analgesia meter (model 0134-003M, Columbus Instruments, Columbus, OH). Rats were placed on a metal plate enclosed by Plexiglass walls maintained at 52.5°C ± 0.1°C. The behavioral end point was the time (s) at which the rats exhibited licking or shaking of the hindpaw or jumping. Rats were removed from the hot plate if they did not respond within 30 s (cutoff) to prevent tissue damage. The effects of ALX1393 (4, 20, and 40 μg) and vehicle (DMSO 25% or 50%) were assessed at 15 min after administration, and the time course of the action of ALX1393 (40 μg) was recorded for 180 min. The measured reaction latencies (s) were converted to the %MPE according to the formula: %MPE = ([ALX1393-treated latency] − [vehicle-treated latency])/(30[cutoff] − [vehicle-treated latency]) × 100.

### Paw Pressure Test

The response to noxious mechanical stimulation was determined by measuring the vocalization threshold to paw pressure as described by Randall and Selitto[15] using an analgesy-meter (Ugo Basile). Increasing pressure (32 or 48 g/s) was applied through a plastic tip onto the dorsal surface between the third and fourth metatarsus of the hindpaw until the rat squeaked. Vocalization thresholds were expressed in grams, and the cutoff was 750 g. Threshold measurements were repeated 3 times and the average was taken. The effects of ALX1393 (4, 20, and 40 μg) and vehicle (50% DMSO) were assessed at 15 min after administration, and the time course of action for ALX1393 (40 μg) was observed for 120 min.



### Formalin Test

Rats were first placed in a plastic observation chamber (25 × 25 × 30 cm) for at least 15 min to acclimate to the environment. The rats were subcutaneously injected into the plantar surface of the hindpaw with 50 μL of 5% formalin solution using a 27-gauge needle. The formalin injection produced the characteristic pain response: biphasic flinching/shaking of the injected paw. Such pain behaviors were quantified by periodically counting the number of spontaneous flinching/shaking responses. They were counted for 1-min periods at 1 to 2 min, 5 to 6 min, and 10-min intervals from 10 to 60 min after the formalin injection. Because the observed pain behavior was biphasic, the evaluation of the flinching/shaking response was divided into 2 phases, Phase I (0–10 min) and Phase II (10–60 min), after formalin injection. To investigate the effect of ALX1393, the vehicle (50% DMSO) or ALX1393 (4, 20, or 40 μg) was administered intrathecally 15 min before formalin injection.

### Rotarod Test

The influence on motor performance was assessed using an accelerating rotarod (model 47700, Ugo Basile) in which the rats were placed on a rotating drum, with the speed increasing from 4 to 40 rpm over 5 min, and forced to make forward walking movements to avoid falling. The latencies (s) to fall were measured. Training sessions were performed 1 and 2 days before the experiments with 3 trials on each day. On the experimental day, a baseline response was obtained, and the rats were subsequently administered ALX1393 (20, 40, or 60 μg) or vehicle (50% DMSO). The time course of motor performance was assessed every 30 min for 120 min after injection.

### Effects of Strychnine on the Antinociceptive Actions of ALX1393

To confirm whether ALX1393 actions are mediated by glycinergic neurotransmission, an antagonist of the glycine receptor, strychnine (10 μg), was administered intrathecally immediately after ALX1393 injection in the thermal and mechanical tests.

Data are represented as the mean ± SEM. Data were analyzed by one-way analysis of variance, followed by the Dunnett test for multiple comparison and the unpaired *t*-test. Statistical analyses and calculations of area under the curve (number of flinching/shaking × minutes) in the formalin test were performed using StatView-J 5.0 (Abacus Concepts, Berkeley, CA) and GraphPad Prism 4.03 software (GraphPad, San Diego, CA). Differences were considered significant at $P < 0.05$.

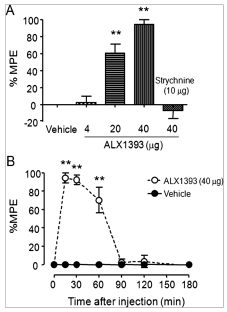


**Figure 1.** A, Effect of intrathecal administration of ALX1393 on thermal nociception in the rat tail flick test. Intensity of the radiant heat was adjusted to give a tail flick latency of 4–5 s before administration of ALX1393 or vehicle. ALX1393 increased the tail flick latency in a dose-dependent manner at 15 min after administration. The antinociceptive effect of ALX1393 (40 μg) was reversed completely by intrathecally administered strychnine (10 μg). Data are expressed as the percentage of the maximum possible effect (%MPE) of means ± SEM. B, Time course of the antinociceptive effect of ALX1393. The maximal effect of ALX1393 (40 μg) was observed at 15 min postinjection, and the antinociceptive effects lasted for 60 min. Each group consists of 8 rats. $^{**}P < 0.01$ compared with the vehicle.

## RESULTS

### Antinociceptive Effects of ALX1393 on Thermal Stimulation

To determine whether ALX1393 modulates thermal pain, tail flick and hot plate tests were performed. ALX1393 prolonged the tail flick latencies in a dose-dependent manner at 15 min after administration (Fig. 1A). Significant effects were observed at 20 μg ALX1393 (60.3% ± 10.6%, $P < 0.001$). The antinociceptive effects of 40 μg ALX1393 lasted for 60 min (Fig. 1B). The effect of ALX1393 was reversed completely by intrathecally administered strychnine (−7.2% ± 9.3%; Fig. 1A).

Baseline measurements of the latency in the hot plate test were 9.0 ± 0.6 s. ALX1393 also displayed antinociceptive responses in a dose-dependent manner at 15 min after administration (Fig. 2A). Significant effects were observed at 20 μg ALX1393 (29.6% ± 11.6%, $P = 0.011$). The antinociceptive effects of 40 μg ALX1393 lasted for 60 min (Fig. 2B). The effect of ALX1393 was abolished by intrathecally administered strychnine (3.2% ± 5.5%; Fig. 2A). In preliminary experiments, the effects of strychnine (10 μg) alone on

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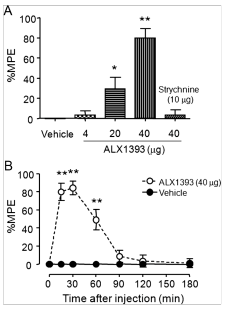


**Figure 2.** A, Effect of intrathecally administering ALX1393 on thermal nociception in the rat hot plate test. ALX1393 increased the latency in a dose-dependent manner at 15 min after administration. The antinociceptive effect of ALX1393 (40 μg) was abolished completely by intrathecally administered strychnine (10 μg). Data are expressed as the percentage of the maximum possible effect (%MPE) of means ± SEM. B, Time course of the antinociceptive effect of ALX1393. The maximal effect of ALX1393 (40 μg) was observed at 15 min postinjection, and the antinociceptive effects lasted for 60 min. Each group consists of 8 rats. *$P < 0.05$; **$P < 0.01$ compared with the vehicle.

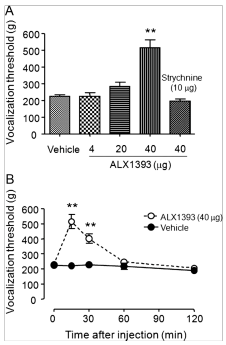


**Figure 3.** A, Effect of intrathecally administering ALX1393 on mechanical nociception in the rat Randall and Selitto test. ALX1393 (40 μg) significantly increased the vocalization threshold to paw pressure at 15 min after administration. The antinociceptive effect of ALX1393 was reversed completely by intrathecally administered strychnine (10 μg). Data are expressed as the threshold in grams (means ± SEM). B, Time course of the antinociceptive effect of ALX1393. The maximal effect of ALX1393 (40 μg) was observed at 15 min postinjection, and the antinociceptive effects lasted for 30 min. Each group consists of 10 rats. **$P < 0.01$ compared with the vehicle.

the tail flick and hot plate latencies were −14.4% ± 2.3% and −11.5% ± 2.6%, respectively ($n = 5$).

### Antinociceptive Effects of ALX1393 on Mechanical Stimulation

To determine whether ALX1393 reduces pressure-evoked pain, rats were subjected to the Randall-Selitto test. The baseline of vocalization threshold to paw pressure was 224 ± 11 g. ALX1393 (40 μg) increased the vocalization threshold significantly (514 ± 48 g, $P < 0.001$; 20 μg: 283 ± 25 g, $P = 0.158$; Fig. 3A), and the effects lasted for 30 min after administration (Fig. 3B). The effect of ALX1393 was reversed completely by intrathecally administered strychnine (198 ± 11 g; Fig. 3A).

### Antinociceptive Effects of ALX1393 on Chemical Stimulation

To further examine whether ALX1393 causes an antinociceptive effect on chemical stimulation, the formalin test was performed. ALX1393 suppressed the flinching/shaking behavior in both phases dose dependently (Fig. 4A). Calculation of the area under the curve revealed that only 40 μg ALX1393 significantly decreased the flinching/shaking behavior in Phase I ($P = 0.002$), whereas significant decreases were observed at 20 μg in Phase II ($P = 0.003$), indicating that ALX1393 predominantly inhibits the Phase II response (Fig. 4B).

### Effects of ALX1393 on Motor Function

The effect of ALX1393 on motor activity was determined using the rotarod test. Baseline latency was 124 ± 3 s. ALX1393 produced no significant change in the rotarod latency up to 40 μg (Fig. 5). At the highest dose (60 μg), at which one-third of the rats tested (3 of 9) died soon thereafter because of respiratory suppression, the rats displayed disturbed motor function but tended to recover to the vehicle value.

## DISCUSSION

This study first demonstrates the antinociceptive effects of an intrathecally administered GlyT2 inhibitor on acute pain in rats. The inhibitory neurons that include glycine and/or γ-aminobutyric acid in the spinal cord dorsal horn have important roles in the inhibition of spinal nociceptive processing. The primary nociceptive afferents from the periphery make



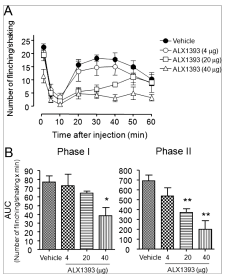


**Figure 4.** Antinociceptive effect of intrathecal administration of ALX1393 on formalin-induced paw flinching/shaking behavior. An injection of formalin into the hindpaw of rats produced a biphasic pain response. ALX1393 or vehicle was applied 15 min before formalin injection. Flinching/shaking was counted for 1-min periods at 1 to 2 min, 5 to 6 min, and 10-min intervals from 10 to 60 min after the formalin injection. Data are expressed by (*A*) the time course curves and (*B*) the area under the curve (AUC, number of flinching/shaking × minutes; means ± SEM). Each group consists of 8 rats. *$P < 0.05$; **$P < 0.01$ compared with the vehicle.

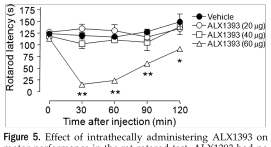


**Figure 5.** Effect of intrathecally administering ALX1393 on motor performance in the rat rotarod test. ALX1393 had no influence on motor function up to 40 μg, at which point it represented the antinociceptive effect. The highest dose of ALX1393 (60 μg) significantly interfered with motor function. Data are expressed as latencies of the means ± SEM. Each group consists of 6 rats. *$P < 0.05$; **$P < 0.01$ compared with the vehicle.

synaptic connections with spinal projection neurons. The activation of these projection neurons not only depends on the primary afferent input but also is under the control of a local network of excitatory and inhibitory interneurons, as well as descending pain-modulating tracts.[1] Because the glycinergic neurons are localized between axons from primary mechanoreceptive afferents and central projection neurons, the glycinergic neurotransmission is considered to contribute to the processing of mechanical input.[16] In fact, spinal glycinergic interneurons have been shown to be directly activated by input from mechanoreceptors using an *in vivo* patch-clamp technique.[17] In addition, local glycinergic interneurons are probably activated by descending antinociceptive pathways that can be stimulated in response to mechanical and thermal nociception.[18] Furthermore, inhibitory input to the dorsal horn neurons also originates from the rostral ventromedial medulla and contact nociceptive spinothalamic projection neurons,[19] which suggests that glycinergic neurons are also associated with the supraspinal control of the spinal nociceptive processing.

ALX1393 increased the thresholds for thermal stimulation in both the tail flick and the hot plate tests. The tail flick response is recognized as a spinally integrated nociceptive reflex, whereas the hot plate response is considered to be a complex response integrated supraspinally on acute nociceptive processing. Consequently, the prolonged reaction latency observed in the ALX1393-treated rats seems to have been derived from the activation of glycinergic interneurons and descending glycinergic neurons at the spinal dorsal horn.

The restricted distribution of GlyT2 in glycinergic neurons suggests its pivotal role in the regulation of glycinergic neurotransmission. Whitehead et al.[20] demonstrated an increase in extracellular glycine via microdialysis perfusion with a GlyT2 inhibitor in the rat dorsal spinal cord. Bradaïa et al.,[21] using whole-cell voltage clamp recording, concluded that the GlyT2 inhibitor increased the level of glycine in rat spinal cord, and in turn prolonged the duration of the glycinergic synaptic current. To confirm that the antinociceptive effect of ALX1393 was attributable to the inhibition of GlyT2 and the subsequent activation of postsynaptic glycine receptors, the effect of strychnine, an inhibitor of the glycine receptor, was tested in response to thermal and mechanical nociception. The antinociceptive effect of ALX1393 was reversed completely by strychnine administered concurrently with ALX1393, indicating that the ALX1393 action is induced by the enhancement of the glycinergic neurotransmission.

ALX1393 exhibited analgesic action on mechanical nociception. The vocalization threshold to paw pressure increased significantly with a high dose of ALX1393. Although paw withdrawal behavior is regarded as a spinally integrated pain response, the vocalization is a more integrated pain response that involves a supraspinal mechanism.

The formalin test is used widely for chemically induced acute and persistent pain assessment.[22] Typically, a biphasic response is observed after the intraplantar injection of formalin in the hindpaw of rats. The early phase is the response to direct chemical stimulation of nociceptors through Aδ and C-fibers, and the late phase can be attributed to peripheral inflammatory processes and subsequent sensitization

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of nociceptive spinal neurons.[23] Our results from the Phase I response indicate that the inhibition of GlyT2 can suppress the nociception by direct chemical stimulation. In Phase II, ALX1393 also inhibited the nociceptive response to persistent pain induced by formalin in a dose-dependent manner. The inhibitory potency in Phase II seemed to be greater than that in Phase I. It has been shown that formalin releases prostaglandins (PGs), excitatory amino acids, nitric oxide, and neuropeptides,[24] which in turn induce inflammatory processes. Ahmadi et al.[25] reported that $PGE_2$ selectively interfered with strychnine-sensitive glycinergic neurotransmission in the superficial layers of the dorsal horn, where nociceptive afferents mainly terminate. The $PGE_2$-mediated disinhibition of the glycinergic neurotransmission on the postsynaptic lesion is at least in part thought to underlie central inflammatory hyperalgesia.[1] However, it is likely that the enhanced glycinergic neurotransmission in the spinal cord by ALX1393 could overcome the disinhibitory sequel derived from formalin injection and strengthen the glycinergic inhibitory system. From this result, the glycinergic neurons might participate in the regulation of the Phase II response to a larger extent than that of Phase I.

In this study, the maximal responses to ALX1393 were observed at 15 min after administration, and significant effects lasted for approximately 60 min. Given the rapid onset and short duration of action, as well as the effects of strychnine, the analgesic action of ALX1393 seems to be derived only from its primary pharmacological action and is not involved in other mechanisms.

To exclude the possibility that the GlyT2 inhibition affects motor function and to explore the clinical availability of the GlyT2 inhibitor, we performed the rotarod test. ALX1393 displayed no influence on motor function up to 40 μg, at which point it elicits a marked antinociceptive effect. Thus, the dose of ALX1393 having analgesic action would be clinically relevant. However, at the highest dose of ALX1393 (60 μg), the motor function was disturbed, and some rats died probably because of respiratory suppression. It is conceivable that the spinally applied ALX1393 partly diffused to the brainstem, resulting in respiratory suppression. Properties of ALX1393 action in this study seem to be different from those in the previous studies with neuropathic pain models. Morita et al.[10] showed in mice that the antiallodynic action of ALX1393 lasted for 72 h, and motor function was preserved even at the highest dose. In contrast, Hermanns et al.[11] found that the antiallodynic effect occurred only at the concentration at which motor performance was disturbed in rats. The reason for the discrepancy between their findings and ours remains to be elucidated; however, differences in experimental models, species, and solvents of ALX1393 might be involved.

A recent generation of GlyT2 knockout mice revealed a decreased glycine release at the glycinergic nerve terminals.[26] Because this study did not investigate the effect of prolonged administration of ALX1393 on acute pain, further experiments are needed to understand a chronic effect of ALX1393. ALX1393 can penetrate the blood–brain barrier; a comparison of its systemic action with intrathecal action observed in this study would be worth investigating for a better understanding of how the GlyT2 inhibitor elicits antinociception in the CNS and which administration route is desirable.

Our previous investigations suggested that neurotransmitter transporters on the presynaptic plasma membrane are one of the targets for anesthetics, and that their inhibition may at least in part mediate the anesthetic action.[27,28] Many studies of inhibitory neurons have focused on postsynaptic receptors. We propose that presynaptic neurotransmitter transporters may provide another clue to the solution of acute and persistent pain.

In conclusion, this study demonstrates the antinociceptive effects of intrathecal ALX1393 against thermal, mechanical, and chemical nociception, which induce both spinally and supraspinally integrated pain responses. The findings from this study suggest that the selective inhibitor of GlyT2 can be a candidate for a novel remedy to ameliorate acute pain; however, further research is needed for a clinical application of the drug.

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