believes that palliative care units are places where patients simply wait for death, these misperceptions are significantly decreased after individuals actually use a specialized palliative care service.[3,4] Thus, the lack of knowledge and general misperceptions regarding palliative care are considerable barriers to palliative care and appropriate pain control, and further education of the general public would be of great value.

This research has revealed prevalence and relationships among general knowledge and perceptions for barriers but has not clarified intentions, acceptance, and knowledge of the availability if the people use the services in a local region. To develop effective strategies to promote the enhanced utilization of palliative care services, we must explore the demographics (such as gender, age, or residential status) and barriers related to not only general public awareness of palliative care, but also intention for use, knowledge of the availability, and actual rate of service utilization. As the previous study revealed,[3,4] it is also expected that opinions of palliative care, which are supposed to be formed from personal experiences, affected not only general awareness but intention or readiness. In particular, sources to form opinions of cancer palliative care are supposed to be cancer-related experiences as the patient themselves or as the patient's family members.[5]

This article, therefore, has the following aims: (1) to clarify the distribution of public awareness, knowledge of availability, and readiness for palliative care services, (2) to clarify the differences in awareness, knowledge, and readiness among demographic variables and between healthy individuals and those who have cancer-related experiences (either personally or via family), (3) to clarify the differences of typical opinions of palliative care in awareness, knowledge, and readiness, in relation to cancer-related experiences and other demographics.

Methods

Subjects

This study was a part of OPTIM, and the overall protocol has been provided in detail elsewhere.[1] Our investigation was a survey of the general population, including patients with cancer and their families in four regions from the OPTIM study. These consisted of a large urban area (Chiba), an urban area (Shizuoka), and two rural areas (Nagasaki and Yamagata). The first three areas are places in which palliative care services are available and the last one (Yamagata) is, in comparison, a location in which services are practically unavailable.

A cross-sectional anonymous questionnaire survey was conducted in a sample of the general population selected by stratified two-stage random sampling in each area (2,000 subjects in each of four regional areas). As a result, this sample included cancer patients (outpatients receiving or having received cancer treatment). We mailed a total of 8,000 questionnaires to these potential participants in June 2007 and on a later date sent a reminder postcard. On the questionnaire, we explained the aim of the study and regarded completion and return of the questionnaire as consent for participation in this study. The institutional review boards of Tokyo University confirmed the ethical and scientific validity.

Questionnaire

We developed our own questionnaire on the basis of the aims of OPTIM and through literature reviews, existing surveys, and consensus among the authors as follows. On the cover page of the questionnaire, palliative care was defined as: attempts to make patients with cancer and their family less anxious or to experience less pain, to immediately start consultations about anxiousness and pain regardless of the state of cancer development, and in addition to treatment, to facilitate the teamwork of doctors and nurses in the practice of treating patients who are suffering from the physical and/or emotional effects of cancer.

The questionnaire included three parts. First, it included questions covering the demographic information of the subjects (age, gender, length of living in each area) and whether subjects are undergoing (or had undergone) cancer treatment or had family members who had experiences of undergoing cancer treatment. Second, it included an item originally designed to determine the extent of public awareness, knowledge of availability, and readiness and actual utilization of palliative care service. We asked the participants to choose only one option from six sequential options regarding palliative care and such services: (1) no knowledge (I have no knowledge regarding palliative care; I); (2) lack of knowledge of availability (I have heard of palliative care, but I do not know if there are any available facilities in my municipality; II); (3) no interest (I know about palliative care and its availability in my residential area, but I have no interest in the service; III); (4) no intention (I know about palliative care and its availability in my residential area, and have an interest, but I have no intention of using the service as a patient or for a family member; IV); (5) preparation (I am preparing to use palliative care services; V); (6) under utilization (I currently use palliative care services; VI; Fig. 1). We converted the subjects' responses for these responses (I to VI) into a numeric

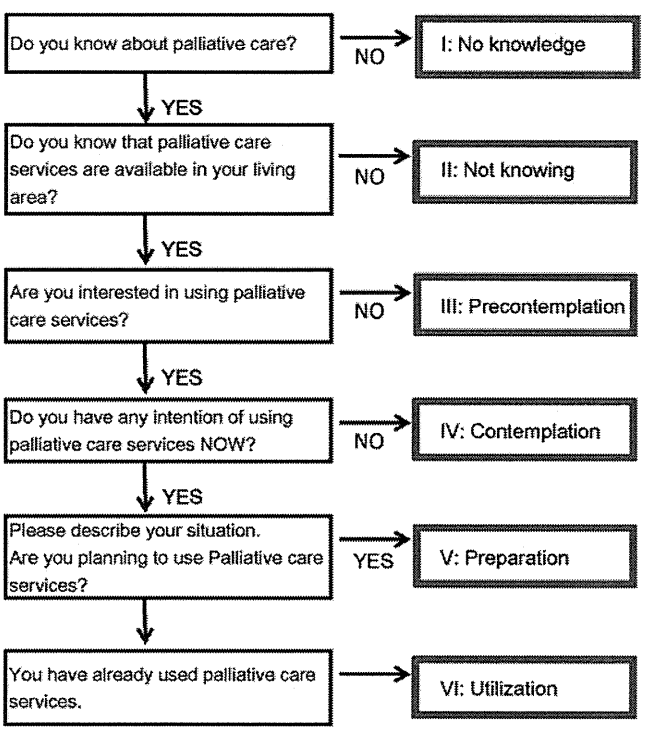

FIG. 1. Public awareness and readiness for palliative care services.

TABLE 1. DEMOGRAPHIC BACKGROUND OF THE RESPONDENTS

	Total		*General population*		*Those who have experienced cancer*	
	(n = 3190)		*(n = 1330)*		*(n = 1860)*	
	n	%	n	%	n	%
Age years						
40–49	705	22.1	302	22.7	403	21.7
50–59	1020	32.0	404	30.4	616	33.1
60–69	898	28.2	385	28.9	513	27.6
70-	567	17.8	239	18.0	328	17.6
Gender						
Male	1,426	44.7	666	50.1	760	40.9
Female	1,764	55.3	664	49.9	1100	59.1
Region (Prefecture)						
Chiba	945	29.6	413	31.1	532	28.6
Shizuoka	785	24.6	364	27.4	421	22.6
Nagasaki	733	23.0	274	20.6	459	24.7
Yamagata	727	22.8	279	21.0	448	24.1
Length of living in each area						
<1 year	38	1.2	22	1.7	16	0.9
1–5 year	131	4.1	60	4.5	71	3.8
>5 year	3,021	94.7	1,248	93.8	1,773	95.3

scale ranging from 1 to 6 points. Finally, three items related to palliative care beliefs/concepts ("Palliative care relieves pain and distress"; "Palliative care is used with chemotherapy and radiotherapy"; "Palliative care is for patients close to death.")[3,5] were presented, and responses were measured on a five point Likert-type scale from 1) strongly disagree to 5) strongly agree.

Analysis

Descriptive analyses were carried out summarizing the subjects' backgrounds, awareness of palliative care and utilization of such services, and scores for reliable media source opinions for total and each sampled area. Then we explored the distribution of knowledge and readiness of palliative care and utilization of the service associated with each sampled area and experiences of having cancer. These analyses were performed after dividing subjects into two groups (the general population and cancer patients/survivors) and we used the χ^2 test and Cramer's V to clarify relations between categorical variables and using coefficient correlation and relations between two categorical variables and ordered variables two-way analysis of variance (ANOVA). We conducted all statistical analyses using the Statistical Package for the Social Sciences (version 15.0.1.1J, SPSS Inc., Chicago, IL) software package. The significance level was set at $p < 0.05$ (two-tailed).

Results

Of the 8000 questionnaires delivered to the sampled subjects, 26 were returned as undeliverable and 3984 were returned (response rate, 49.8%). Of those returned, 3190 were considered valid for statistical analyses. The rest ($n = 794$) were invalid and were excluded from the analyses since major information was lacking. Thus, the final rate of valid replies was 39.9%.

A total of 1860 respondents (58.3% of all respondents) were identified as "those having experienced cancer" and the rest were identified as belonging to the "general population." Table 1 summarizes the background of respondents.

Public awareness, knowledge, and readiness for palliative care

A total of 63.1% of respondents admitting to having "no knowledge" of palliative care while 0.5% of respondents were actually using palliative care services. Respondents who knew about palliative care yet did not know about the availability of palliative care in their living area were 18.6% of all respondents. Female respondents were more likely to know about palliative care than male respondents ($\chi^2 = 55.09$, $df = 1$, $p < 0.001$, Cramer's V = 0.131), while age and length of living in each area were not significantly associated with

TABLE 2. PUBLIC AWARENESS AND READINESS FOR PALLIATIVE CARE SERVICES

	Total		*General population*		*Those who have experienced caner*		*Chiba*		*Shizuoka*		*Nagasaki*		*Yamagata*	
	(n = 3190)		*(n = 1330)*		*(n = 1860)*		*(n = 945)*		*(n = 785)*		*(n = 733)*		*(n = 727)*	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
I: No knowledge	2012	63.1	909	68.3	1,103	59.3	546	57.8	518	66.0	482	65.8	466	64.1
Having Knowledge	1178	36.9	421	31.7	757	40.7	399	42.2	267	34.0	251	34.2	261	36.0
II: Not knowing	593	18.6	230	17.3	363	19.5	201	21.3	96	12.2	115	15.7	181	24.9
III: Not interseted	24	0.8	13	1.0	11	0.6	5	0.5	12	1.5	5	0.7	2	0.3
IV: No Intention	499	15.6	167	12.6	332	17.8	171	18.1	142	18.1	116	15.8	70	9.6
V: Preparation	46	1.4	10	0.8	36	1.9	18	1.9	14	1.8	10	1.4	4	0.6
VI: Under Utilization	16	0.5	1	0.1	15	0.8	4	0.4	3	0.4	5	0.7	4	0.6

Cancer experience×Awareness (No knowledge vs. Having knowledge): $\chi^2 = 27.24$, $df = 1$.
$p < 0.01$, Cramer's V = 0.092.
Four areas×Awareness (No knowledge vs. Having knowledge): $\chi^2 = 16.83$, $df = 3$, $p < 0.01$, Cramer's V = 0.073.
Within People who Knew Palliative Care:
Cancer experience×Availability: $\chi^2 = 4.83$, $df = 1$, $p < 0.028$, Cramer's V = 0.064
Four areas×Availability: $\chi^2 = 61.88$, $df = 3$, $p < 0.01$, Cramer's V = 0.229.
Availability: No awareness of availability vs. awareness of availability.

either awareness, knowledge or readiness. Respondents who had cancer-related experiences (either themselves or via family members) were more likely to be aware of palliative care compared to the general population ($\chi^2 = 27.24$, $df = 1$, $p < 0.001$, Cramer's V = 0.092). Also among people who knew palliative care, there was a significant association between cancer experience and knowledge for availability or readiness ($\chi^2 = 4.83$, $df = 1$, $p = 0.028$, Cramer's V = 0.064). Table 2 also shows that awareness and knowledge of and readiness for palliative care was significantly different among each area ($\chi^2 = 16.84$, $df = 3$, $p < 0.001$, Cramer's V = 0.073). Particularly, respondents in Chiba-city have more knowledge about palliative care than individuals from the other three areas.

Typical images of palliative care

Table 3 indicates the results of two-way ANOVA for responses on three typical images of palliative care using awareness and cancer experiences as dependent variables, when age, gender, and area were controlled. First, the analysis revealed the differences in perception for three common images of palliative care between individuals having no knowledge of palliative care and those who had knowledge. Significant differences were observed between them in terms of images of palliative care in the following dimensions: "Palliative care relieves pain and distress" (general population; $F(1, 3186) = 33.02$, $p < 0.001$, Those having experienced cancer; $F(1, 3186) = 60.85$, $p < 0.001$) and "Palliative care is for patients close to death" (general population; $F(1, 3186) = 13.62$, $p < 0.01$, Those having experienced cancer; $F(1, 3186) = 13.00$, $p < 0.01$). People who know about palliative care have an overall positive image of it, tend to think that palliative care brings symptom control to the patients, and is specialized for terminally ill patients. There were no significant differences between the general population and cancer-experienced individuals on the three typical opinions of palliative care, and there were no significant interactions between cancer experience and knowledge of palliative care.

Discussion

This study is the first attempt to understand the public awareness of palliative care and utilization of services based on a nationwide sample in Japan. A clarification of these findings will hopefully contribute to understanding general perception of cancer palliative care and its variations by experiences related to cancer.

The primary aim of this study was to clarify the distribution of public awareness, knowledge of availability, and readiness for palliative care services. Per the results of the survey, 63.1% of all the participants had no knowledge of palliative care services. These results demonstrate a low public awareness of the Japanese palliative care services compared with other countries.[2,3] Moreover, among those who did possess knowledge about palliative care in general, 18% did not know about the specific availability of the service in their region. These results indicate that over 80% of people do not have sufficient knowledge of palliative care to take advantage of its services, and it is therefore important to promote a more comprehensive understanding of palliative care (including availability) to the general population.

Second, our data clarified that cancer experiences were related to a greater knowledge of and readiness for palliative

TABLE 3. MEAN SCORES OF IMAGES OF PALLIATIVE CARE BY AWARENESS AND EXPERIENCE OF CANCER

Awareness	*General population*						*Those who have experienced cancer*						*Main effect*						
Experience of Cancer	*Total*		*No knowledge*		*Having knowledge*		*Total*		*No knowledge*		*Having knowledge*		*Exp. Cancer*		*Awareness*		*Interaction*	
	M	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	F	p	F	p	F	p
Palliative care relieves pain and distress	3.81	0.80	3.72	0.83	4.00	0.70	3.88	0.85	3.76	0.88	4.06	0.76	2.75	.07	74.73	.00	0.08	.78
Palliative care is used with chemotherapy and radiotherapy	3.51	0.90	3.53	0.85	3.47	1.00	3.51	0.97	3.50	0.94	3.52	1.02	0.18	.68	0.04	.85	1.09	.30
Palliative care is for patients close to death	3.19	1.22	3.12	1.12	3.34	1.26	3.22	1.29	3.15	1.27	3.32	1.30	0.01	.91	15.30	.00	0.36	.55

The results by two-way analysis of variance (ANOVA) were shown when age, gender, and area were controlled as covariates.

care, but did not significantly relate to specific images commonly associated with palliative care. From our data it is difficult to strictly compare patients with cancer with the overall population since the sample surveyed in this study was from the general population, and therefore only a small number of patients with cancer were included. However, people who had experiences with cancer (either personally or via family members) recognized both the term and meaning of palliative care. Also, people who had knowledge of palliative care have an overall positive image of it, tend to think that palliative care brings symptom control to the cancer patients, and is specialized for terminally ill patients. Generally, as the images will be reinforced by actual experiences, those who experienced as patients with cancer or as family members might see or hear the care at late stage of the cancer process. This indicated current situation that palliative care for patients with cancer in general Japanese hospitals was mainly provided for late-stage cancer and that contributed to form the general opinions of palliative care. Also, the perception that palliative care is primarily for terminally ill patients care may cause late referrals to palliative care services.[5,6] These suggest that images derived from actual experiences will have strong impact for actual decision making for choosing or readiness for the services when the patients need. Therefore, it is important to provide proper and detailed information about palliative care services, as well as information regarding the availability of services, within areas of residence. We still have very big challenges to modify the general perception of cancer palliative care, because there is no known effective method to achieve this. Educational approaches in community may become one of the solutions, and will especially be needed to help people recognize that palliative care services accept even patients with early-stage cancer.

This study has several limitations. First, this study did not include measurements for the effectiveness of each medium and we cannot discern which media sources and what kind of information directly led individuals to be more aware of palliative care and to use these services. Second, we did not explore the possible associations between the awareness of palliative care and amounts of actual cancer treatment undergone. A more detailed survey will need to be conducted in order to clarify the above items. Moreover, it would be useful to better explore the insights of specific populations. In future surveys, it should be possible to design more directed questionnaires to support hypothesis-based studies.

In conclusion, the public awareness of palliative care services and their availability is insufficient. Those with cancer experiences were more aware of palliative care and their availability than the general population. Only people who were aware of palliative care developed two typical images, while those with cancer-related experiences did not. Approaches to inform the general population (including those with cancer-related experiences) about palliative care have already been taken in Japan. However, more effective methods should be developed. We feel that it is possible to eliminate many existing barriers to the improvement of end-of-life quality, and the dissemination of knowledge related to such care and treatment in Japan should be a top priority.

Author Disclosure Statement

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Sleep disturbances in a neuropathic pain-like condition in the mouse are associated with altered GABAergic transmission in the cingulate cortex

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ABSTRACT

Insomnia is a common problem for people with chronic pain. Cortical GABAergic neurons are part of the neurobiological substrate that underlies homeostatic sleep regulation. In the present study, we confirmed that sciatic nerve ligation caused thermal hyperalgesia and tactile allodynia in mice. In this experimental model for neuropathic pain, we found an increase in wakefulness and a decrease in non-rapid eye movement sleep under a neuropathic pain-like state. Under these conditions, membrane-bound GABA (γ-aminobutyric acid) transporters (GATs) on activated glial fibrillary acidic protein-positive astrocytes were significantly increased in the cingulate cortex, and extracellular GABA levels in this area after depolarization were rapidly decreased by nerve injury. Furthermore, sleep disturbance induced by sciatic nerve ligation was improved by the intracingulate cortex injection of a GAT-3 inhibitor. These findings provide novel evidence that sciatic nerve ligation decreases extracellular-released GABA in the cingulate cortex of mice. These phenomena may, at least in part, explain the insomnia in patients with neuropathic pain.

1. Introduction

Several clinical reports on chronic pain of various etiologies have shown that it significantly interferes with sleep [33,39,53,59]. Most patients reported that their difficulties with sleep started after they began experiencing chronic pain [39,53]. Inadequate sleep due to chronic pain may contribute to the stressful negative consequences of living with pain (eg, interference with work, relationships, and hobbies).

Cumulative evidence from both human and animal studies demonstrates that the forebrain, including neurons in the cingulate cortex and insular cortex, plays an important role in pain-related perception and chronic pain. Neuroimaging studies in humans have further confirmed these observations and shown that the anterior cingulate cortex, together with other cortical structures, is activated by acute noxious stimuli, psychological pain, and social pain [8,14,31,49,50,55]. Recently, it has been reported that both the enhancement of glutamate release and postsynaptic glutamate receptor-mediated responses are changed in cortical synapses after nerve injury and inflammation [61,63], suggesting that chronic pain could lead to changes in excitatory synaptic transmission in the cingulate cortex. However, little, if any, is known about whether inhibitory GABAergic synaptic transmission in the cingulate cortex could change under neuropathic pain.

While GABAergic neurons are found in all cortical layers, they show a clear preference for layers IV and II–III. These cortical GABAergic neurons are implicated in sleep/wake control [19]. The magnitude and duration of GABA synaptic action are regulated by plasma membrane proteins, called GABA transporters (GATs), which mediate the high-affinity, Na^+/Cl^- dependent uptake of GABA into presynaptic axon terminals and glial processes [27–30,46]. To date, 4 subtypes of GATs (GAT-1, GAT-2, GAT-3, and BGT-1) have been isolated from the rodent and human nervous

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systems [3,4,9,21,36]. They exhibit different ionic dependencies and inhibitor sensitivities, and are differentially distributed within the central nervous system. GAT-1 [11,48] is highly selective and localized mainly in pre- and postsynaptic neurons and partly in glial cells. In contrast, GAT-3 has a lower density than GAT-1 in neurons, but is mostly distributed on astrocytes located close to neurons to uptake and metabolize released GABA [54].

We previously reported that chronic pain caused by sciatic nerve ligation caused a dramatic increase in glial fibrillary acidic protein (GFAP)-like immunoreactivity (IR), which is located in dendritic astrocytes, with an expanding distribution in the cingulate cortex of mice [32,40]. The morphological changes that occur in astrocytes produce what are collectively known as reactive astrocytes, which are characterized by specific changes such as the accumulation of intermediate-filament GFAP and hyperplasia [32,40]. These findings raise the hypothesis that neuropathic pain may after the reuptake of GABA in activated astrocytes of the cingulate cortex. This phenomenon leads to insomnia in patients with neuropathic pain.

In the present study, we evaluated the possible changes in GABA transmission accompanied by sleep disturbance under a neuropathic pain.

2. Materials and methods

2.1. Animals

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. This study was approved by the Animal Research Committee of Hoshi University. Institute of Cancer Research (ICR) mice (weighing 22–35 g) (Tokyo Laboratory Animals Science, Tokyo, Japan) and C57BL/6J mice (weighing 18–23 g) (CLEA Japan, Tokyo, Japan) were used for this study. Animals were kept in a room with an ambient temperature of 23 ± 1°C and a 12-h light-dark cycle (lights on 08:00 to 20:00 h). Food and water were available ad libitum during the experimental period. At the end of the experiments, animals were humanely killed by a rising concentration of ethyl ether.

2.2. Neuropathic pain model

We produced a partial sciatic nerve injury by tying a tight ligature with an 8-0 silk suture around approximately one-third to one-half the diameter of the sciatic nerve on the right side (ipsilateral side) under a light microscope (SD30, Olympus, Tokyo, Japan) as described previously [45]. In sham-operated animals, the nerve was exposed without ligation.

2.3. Measurement of thermal and tactile thresholds

Twelve C57BL/6J mice were used for the measurement of thermal and tactile thresholds, as described previously [45]. To assess the sensitivity to thermal stimulation, the right plantar surface of mice was tested individually by using a well-focused, radiant heat light source (model 33 Analgesia Meter; IITC/Life Science Instruments, Woodland Hills, CA). The intensity of the thermal stimulus was adjusted to achieve an average baseline paw-withdrawal latency of approximately 8–10 s in naive mice. The paw-withdrawal latency was determined as the average of 3 measurements per paw. Only quick hind paw movements (with or without licking of hind paws) away from the stimulus were considered to be a withdrawal response. Paw movements associated with locomotion or weight shifting were not counted as a response. The paws were measured alternating between the left and right with an interval of more than 3 min between measurements. Before the behavioral responses to the thermal stimulus were tested, mice were habituated for at least 30 min in a clear acrylic cylinder (15 cm high and 8 cm in diameter). Under these conditions, the latency of paw withdrawal in response to the thermal stimulus was tested. The data represent the average value for the withdrawal latency of the right hind paw.

To quantify the sensitivity to a tactile stimulus, paw withdrawal in response to a tactile stimulus was measured by using a bending force (0.02g and 0.16g) applied by von Frey filaments (North Coast Medical, Morgan Hill, CA). A von Frey filament was applied to the plantar surface of each hind paw for 3 s, and this was repeated 3 times with an intertrial interval of at least 5 s. Each of the hind paws was tested individually. Paw withdrawal in response to a tactile stimulus was evaluated by scoring as follows: 0, no response; 1, a slow and/or slight response to the stimulus; 2, a quick withdrawal response away from the stimulus without flinching or licking; 3, an intense withdrawal response away from the stimulus with brisk flinching and/or licking. The paw withdrawal in response to each filament was determined as the average of 2 scores per paw. Paw movements associated with locomotion or weight shifting were not counted as a response. The paws were measured alternating between the left and right with an interval of more than 3 min between measurements. Before the behavioral responses to tactile stimuli were tested, mice were habituated for at least 30 min on an elevated nylon mesh floor. Under these conditions, paw withdrawal in response to a tactile stimulus was tested.

2.4. Electroencephalogram and electromyogram recordings

Under 3% isoflurane anesthesia, mice were implanted with electroencephalogram (EEG) and electromyogram (EMG) electrodes for polysomnographic recordings (Pinnacle Technology, Lawrence, KS). Briefly, to monitor EEG signals, 2 stainless steel EEG recording screws were positioned 1 mm anterior to the bregma or lambda, both 1.5 mm lateral to the midline. EMG activity was monitored by stainless steel, Teflon-coated wires placed bilaterally into both trapezius muscles. Sleep–wake states were then monitored for a period of 24 h at 2 days after placement of the EEG recording screws, encompassing both the baseline and the experimental day. The EEG/EMG signals were amplified, filtered (EEG, 0.5–30 Hz; EMG, 20–200 Hz), digitized at a sampling rate of 128 Hz, and recorded by SLEEPSIGN software (Kissei Comtec, Nagano, Japan). Vigilance was automatically classified off-line under 4-s epochs into 3 stages, ie, wakefulness, rapid eye movement (REM) and non-REM (NREM) sleep, by SLEEPSIGN according to the standard criteria [24,25,56]. As a final step, defined sleep–wake stages were examined visually and corrected, if necessary. For each epoch, the EEG power density in the delta (0.75–4.0 Hz) and theta bands (6.25–9.0 Hz) and the integrated EMG value were displayed on a PC monitor. Three vigilance states—(1) waking (high EMG and low EEG amplitude and high theta activity concomitant with highest EMG values), (2) NREM sleep (low EMG and high EEG amplitude, high delta activity), and (3) REM sleep (low EMG and low EEG amplitude, high theta activity)—were determined for 4-s epochs, and the scores were entered into a PC via a keyboard. EEG and EMG activities were monitored for 24 h at 7 days or 28 days after sciatic nerve ligation. Vehicle (1% dimethyl sulfoxide) or the GAT-3 inhibitor (*S*)-1-[2-[tris(4-methoxyphenyl) methoxy] ethyl]-3-piperidine carboxylic acid (SNAP-5114) (1 nmol/mouse) in a volume of 100 nL/mouse was injected into the cingulate cortex (from the bregma: anterior, +2.00 mm; lateral, +0.30 mm; ventral, −2.00 mm; angle, 30°) via an infusion cannula with a Hamilton

syringe at an infusion rate of 20 nL/min. Recordings were started from 8:00 PM. Vehicle or SNAP-5114 was injected twice at 8:00 PM and 2:00 AM. Baseline recordings were taken for each animal for 24 h, beginning at 10 AM, and vehicle (saline), midazolam, or zolpidem was injected at 1 pm on that day.

2.5. Immunohistochemistry

Six ICR mice at 7 days after surgery were deeply anesthetized by the inhalation of 3% isoflurane with oxygen as the carrier gas and intracardially perfusion-fixed with freshly prepared 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). After perfusion, the brains were quickly removed and thick coronal sections of the frontal cortex including the cingulate cortex were rapidly dissected and postfixed in 4% paraformaldehyde for 2 h. They were then permeated with 20% sucrose in 0.1 M PBS for 1 day and 30% sucrose in 0.1 M PBS for 2 days with agitation, and finally frozen in an embedding compound (Sakura Finetechnical, Tokyo, Japan). Transverse sections 8 μm thick were cut with a cryostat (Leica CM1510; Leica Microsystems, Heidelberg, Germany). The cingulate cortex sections were blocked in 10% normal horse serum in 0.01 M PBS for 1 h at room temperature. The primary antibodies were diluted in 0.01 M PBS containing 10% normal horse serum [1:350 GAT-3 (Chemicon International, Temecula, CA, for double staining; Santa Cruz Biotechnology, Santa Cruz, CA, for single staining) and 1:100–150 GFAP (Chemicon International for double staining; Santa Cruz Biotechnology for single staining)] and incubated for 2 days overnight at 4°C. The samples were then rinsed and incubated with the appropriate secondary antibody conjugated with Alexa 488 and Alexa 546 (Molecular Probes, Eugene, OR) for 2 h at room temperature. The slides were then coverslipped with PermaFluor Aqueous mounting medium (Immunon, Pittsburgh, PA). The fluorescence of immunolabeling was detected with a light microscope (Olympus AX-70; Olympus Optical, Tokyo, Japan) and photographed with a digital camera (Polaroid PDMC II/OL; Olympus Optical).

To evaluate the specificity of GFAP and GAT-3 antibodies, we prepared preabsorption controls of these antibodies. The peptides (for GFAP at 1:100, Santa Cruz Biotechnology for GAT-3 at 1:100, Santa Cruz Biotechnology) were incubated with the antibodies overnight at 4°C. The preabsorbed antibodies were then used on the cingulate cortex sections.

For semiquantitative analysis, the density of GFAP or GAT-3 labeling was measured with a computer-assisted imaging analysis system (ImageJ, free-download software developed by the National Institutes of Health) as previously described [41]. Brain slices for immunohistochemistry corresponded approximately to Fig. 22 in the atlas of Franklin and Paxinos [16]. A standardized rectangle was positioned over the images for the sham group. We calculated the area and density of pixels within the threshold value representing immunoreactivity, and the integrated density was the product of the area and density. The same box was then dragged to the corresponding position for the ligation group, and the integrated density of pixels within the same threshold was calculated again.

To evaluate co-localization, the analysis was performed following the protocols established by Li et al. [35]. Only images in which there was no pixel saturation were analyzed. Background fluorescence was first subtracted in ImageJ by selecting an unstained area of each image and running the background subtraction plug-in available at http://www.uhnresearch.ca/facilities/wcif/fdownload.html. The intensity correlation quotient (ICQ) was then determined by running the intensity correlation analysis plug-in for ImageJ developed by Tony Collins and Elise Stanley (Toronto Western Research Institute, Toronto, ON, Canada), which is also available at the above link. The ICQ indicates whether the intensity of staining for 2 proteins varies in synchrony over space. An ICQ value of +0.5 means that for any pixel with a certain intensity of staining for one protein, the intensity of staining for the other protein studied will be exactly the same, whereas an ICQ value of 0 signifies no relationship between the 2 staining patterns.

2.6. Western blot test

One, 3, 7, and 28 days after the surgery, each ICR mouse brain ($n = 6$ for each group) was removed from the skull. The frontal cortex region was then dissected on an ice-cold metal plate. The procedures used for sample preparation and Western blot testing were performed as described previously [42]. The protein concentration in the samples was assayed by the method of Bradford [6]. For immunoblot detection, membranes were blocked in Tris-buffered saline (TBS) containing 2% nonfat milk (Bio-Rad Laboratories, Hercules, CA) and 0.1% Tween 20 (Research Biochemicals, Natick, MA), and 5% nonfat dried milk for 1 h at room temperature with agitation. The membrane was incubated with primary antibody diluted in TBS [1:1000 GAT-1 (Chemicon International), 1:1000 GAT-2 (Chemicon International), 1:1000 GAT-3 (Chemicon International), 1:20,000, 400,000, and 600,000 glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon International)] containing 5% nonfat dried milk overnight at 4°C. The membrane was washed in TBS containing 0.05% Tween 20 (TTBS), followed by 2 h of incubation at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL) diluted 1:10,000 in TBS containing 2% nonfat milk containing 0.1% Tween 20 and 5% nonfat dried milk. After this incubation, the membranes were washed in TTBS. The antigen–antibody peroxidase complex was then finally detected by enhanced chemiluminescence (Pierce, Rockford, IL) according to the manufacturer's instructions and visualized by exposure to Amersham Hyperfilm (Amersham Life Sciences, Arlington Heights, IL).

2.7. RNA preparation and semiquantitative analysis by reverse transcription-polymerase chain reaction

The levels of mRNA were determined following the methods described previously [43–45]. One, 3, 7, and 28 days after the surgery, each C57BL/6J mouse brain ($n = 6$ for each group) was removed from the skull. The frontal cortex region was then dissected on an ice-cold metal plate and homogenized in ice-cold lysis buffer containing β-mercaptoethanol following the manufacturer's instructions. First-strand cDNA was prepared as described elsewhere [45] and GAT-1, GAT-3, GFAP, AQP4, Slc15a2, and Acsbg1 were amplified in 50 μL of a polymerase chain reaction (PCR) solution containing $MgCl_2$, dNTP mix, and DNA polymerase with either synthesized primer (Table 1). Samples were heated to 94°C for 2 min, 55°C for 2 min, and 72°C for 3 min and cycled 34 times through 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The final incubation was at 72°C for 7 min. The mixture was applied to 2% agarose gel for electrophoresis with the indicated markers and primers for the internal standard (GAPDH). Each sample was applied to more than 2 lanes in the same gel. The agarose gel was stained with ethidium bromide and photographed with ultraviolet transillumination. The intensity of the bands was analyzed and quantified by computer-assisted densitometry by ImageJ software. For the control, the different intensities of each band obtained from sham-operated mice were analyzed, and the average intensity was calculated. Each control intensity was then compared again with the average intensity to calculate the standard error. Under these conditions, the intensities of bands for samples obtained from nerve-ligated mice were analyzed and compared with the average intensity for mice that were sham-operated with saline. Finally, the percentage of the control with the standard error for each sample was quantified.

Table 1
Primers used for RT-PCR.

Primer	Sense	Antisense
GAT-1	5′-TAACAACAACAGCCCATCCA-3′	5′-GGAGTAACCCTGCTCCATGA-3′
GAT-3	5′-ACAGTCAGTTTGTGTGCGTG-3′	5′-CAACTTCTGTAATTTCTCGG-3′
GFAP	5′-ACAACTTTGCACAGGACCTC-3′	5′-CGATTCAACCTTTCTCTCCA-3′
AQP4	5′-CTGGAGCCAGCATGAATCCAG-3′	5′-TTCTTCTCTTCTCCACGGTCA-3′
Slc15a2	5′-ACCATGCCTTCAGCAGCCTCT-3′	5′-CGCTAGGATCATACCAACAGC-3′
Acsbg1	5′-ACCCCCTCCAAAGAAGATGGC-3′	5′-CGGATCTGCAAAGCAGACCTG-3′
GAPDH	5′-CCCACGGCAAGTTCAACGG-3′	5′-CTTTCCAGAGGGGCCATCCA-3′

2.8. In vivo microdialysis study and quantification of GABA

Six or 28 days after sciatic nerve surgery, C57BL/6J mice ($n = 4$–7 for each group) were implanted with a microdialysis probe (D-I-4-01; 1-mm membrane length; Eicom) into the cingulate cortex according to a brain atlas [16]. At 24 h after implantation, mice were placed in experimental cages (30 cm wide × 30 cm deep × 30 cm high). The probes were perfused continuously at a flow rate of 2 mL/min with artificial cerebrospinal fluid containing 0.9 mM $MgCl_2$, 147.0 mM NaCl, 4.0 mM KCl, and 1.2 mM $CaCl_2$. Outflow fractions were collected every 20 min. After 3 baseline fractions were collected in the cingulate cortex, 4-aminopyridine (4-AP) was injected into the mouse cingulate cortex with a 1-mL Hamilton syringe and a motorized syringe pump. For these experiments, dialysis samples were collected for 120 min after treatment with 4-AP. GABA was determined on a high-performance liquid chromatography (HPLC) (Eicom) system with precolumn derivatization with *o*-phthaldialdehyde/2-mercaptoethanol (OPA) reagent with electrochemical detection (ECD) (Eicom). Briefly, 30 μL of dialysate and 10 μL of OPA derivation fluid were allowed to react for 150 s at room temperature. One hundred fifty seconds later, 30 μL of this mixture was injected. The mobile phase containing NaH_2PO_4 (7.8 g/L), EDTA (2Na; 5 mg/L), and 50% methanol was delivered at a flow rate of 500 μL/min. GABA was identified according to the retention time of the GABA standard, and the amounts of GABA were quantified by calculations using peak areas.

2.9. Assessment of loss of righting reflex

To assess the effect of GABAergic drugs on sedative actions, which could partly explain the "insomnia" under the present condition, loss of righting reflex (LORR) was evaluated [58] and assessed between 13:00 and 18:00. C57BL/6J mice were injected

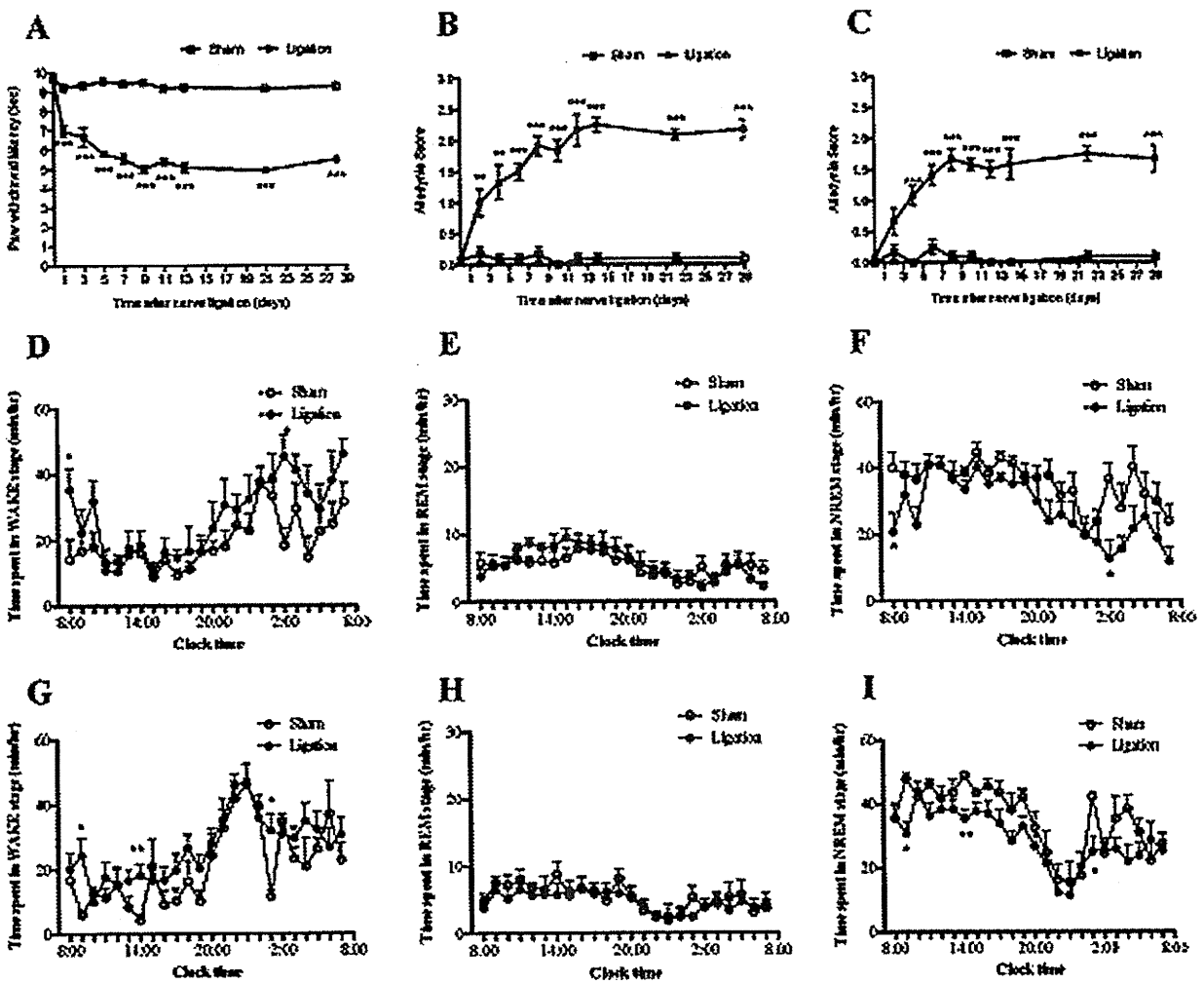


Fig. 1. (A–C) Effects of sciatic nerve ligation on thermal hyperalgesia (A) and tactile allodynia (B and C) in sham-operated or nerve-ligated mice for 28 to 29 days. A tactile stimulus was applied using filaments with 2 different bending forces [0.02g (B) and 0.16g (C)]. Two-way ANOVA was performed, followed by Bonferroni testing. Each point represents the mean ± SEM of 8 to 10 mice. **$P < .01$ and ***$P < .001$ vs sham group. (D–I) Changes in the sleep/wake pattern as determined by EEG/EMG at 7 days or 28 days after sciatic nerve ligation. The WAKE time was prolonged in mice at 7 days (D) or 28 days (G) after sciatic nerve ligation. There were no changes in REM sleep at 7 days (E) or 28 days (H) after sciatic nerve ligation. NREM sleep was suppressed by sciatic nerve ligation after 7 days (F) or 28 days (I). Each point represents the mean ± SEM of 6 to 8 mice. *$P < .05$ and **$P < .001$ vs sham group.

with a drug (midazolam, zolpidem or pentobarbital) or saline ($n = 7–12$ for each group), and then ataxic mice were carefully placed in the supine position in a V-shaped plastic plate until they were able to right themselves 3 times within 30 s. The onset time of LORR was defined as the time when the righting reflex was lost after drug injection. LORR time was defined as the time from when mice were placed in the supine position in the V-shaped plastic plate until the righting reflex was regained. In the combination study, animals were microinjected with SNAP-5114 (1 nmol/mouse) in a volume of 100 nL/mouse into the cingulate cortex (from the bregma: anterior, +1.00 mm; lateral, +0.30 mm; ventral, −1.40 mm) via an infusion cannula with a Hamilton syringe at an infusion rate of 20 nL/min. Three hours after the microinjection of SNAP-5114, the mice were injected with midazolam.

2.10. Drugs

The drugs used in this study were midazolam (Dormicum, 5 mg/mL, Astellas Pharma, Tokyo, Japan); zolpidem (Myslee, Astellas Pharma, Tokyo, Japan); SNAP-5114 (Sigma-Aldrich) pentobarbital (Tokyo Chemical Industry, Tokyo, Japan); GABA (Wako, Osaka, Japan); and 4-AP (Sigma-Aldrich). Undiluted clinical-grade midazolam stock solution (5.0 mg/mL) was injected at 0.15 mL/10 g body weight.

2.11. Statistical analysis

Data are expressed as the mean with SEM. Two-way ANOVAs with independent and repeated measures, as well as planned comparisons or Student's *t*-tests, were used as appropriate. Multiple comparisons were performed by the Bonferroni post hoc test, where appropriate. All statistical analyses were performed with Prism version 5.0a (GraphPad Software, San Diego, CA).

3. Results

3.1. Thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation in mice

Sciatic nerve ligation caused a marked decrease in the latency of paw withdrawal in response to a thermal and tactile stimuli only on the ipsilateral side. Such a persistent painful state caused by partial ligation of the sciatic nerve lasted for more than 28 days after nerve ligation (Fig. 1A: thermal hyperalgesia, $F_{1,90} = 28.10$, $P < .001$; 1B: tactile allodynia (0.02 g), $F_{1,90} = 110.1$, $P < .001$; 1C: tactile allodynia (0.16 g), $F_{1,90} = 263.7$, $P < .001$ vs sham group).

3.2. Changes in vigilance under a neuropathic pain-like state with EEG/EMG

By means of this experimental model for neuropathic pain, we investigated the changes in sleep patterns in mice with sciatic nerve ligation. Frontal cortical activity and postural muscle tone, as monitored by EEG/EMG, are useful for identifying sleep/wake abnormalities. Vigilance was classified offline into 3 stages: wakefulness, REM sleep, and non-REM (NREM) sleep. Sciatic nerve ligation for 7 or 28 days was associated with statistically significant increases in wakefulness (7 days; Fig. 1D: $F_{1,184} = 9.195$, $P < .05$ vs sham-operated mice, 28 days; Fig. 1G: $F_{1,299} = 4.868$, $P < .05$ vs sham-operated mice). No difference in REM sleep was found between sham operation and sciatic nerve ligation for 7 and 28 days (Fig. 1E and H). In NREM sleep, sciatic nerve ligation for 7 days or 28 days was associated with statistically significant decreases (7 days; Fig. 1F: $F_{1,184} = 11.37$, $P < .01$ vs sham-operated mice, 28 days; Fig. 1I: $F_{1,299} = 11.56$, $P < .01$ vs sham-operated mice).

3.3. Increase in levels of GABA transporters in the frontal cortex of mice with sciatic nerve ligation

It has been widely accepted that extracellular levels of GABA are regulated by GABA transporters (GATs), which mediate GABA uptake from the synaptic cleft in the brain. In the present study, the expression of GAT-1 (mouse GAT1) was observed in the frontal cortex area including the cingulate cortex from sham-operated mice (Fig. 2A). Furthermore, abundant GAT-3 (mouse GAT4) protein was observed in the same area (Fig. 2C). In contrast, a low expression of GAT-2 (mouse GAT3) was found in the mouse frontal cortex (Fig. 2B). In nerve-ligated mice, the protein levels of GAT-1 and GAT-3 at 7 days after sciatic nerve ligation were significantly increased compared to those in sham-operated mice ($P < .001$) (Fig. 2A and C). Furthermore, the significant increase in the level of membrane-located GAT-3 lasted for at least 28 days after sciatic nerve ligation (Fig. 2C). On the other hand, the lower immunoreactivity of GAT-2 in nerve-ligated mice was at the same level as that in sham-operated mice (Fig. 2B).

3.4. Localization of GAT-3 immunoreactivity on activated astrocytes in the cingulate cortex with sciatic nerve ligation

In the present study, we evaluated the specificity of GFAP and GAT-3 antibodies. Incubation of these antibodies that had been preabsorbed with their immunogen peptides did not show any immunoreactivities in the mouse cingulate cortex (Fig. 3B-i–E-ii). Next, we demonstrated that the number of GFAP-positive astrocytes in the mouse cingulate cortex was markedly increased at 7 days after sciatic nerve ligation (Fig. 3H-ii). Each individual astrocyte labeled by GFAP was hypertrophied with an enlarged cell body in sciatic nerve-ligated mice. According to a semi-quantitative analysis, GFAP IR was significantly increased by about 3-fold after nerve ligation (sham; 100.0 ± 27.0%, ligation; 307.5 ± 35.8% of control, $P < .001$ vs sham-operated mice).

Double-immunolabeling with antibodies specific to GAT-3 and GFAP showed that increased GAT-3 IR was predominantly found in the dendrites of astrocytes stained with GFAP in the cingulate cortex of sciatic nerve-ligated mice, as shown by apparent co-localization with GFAP IR (Fig. 3I-iii). These apparent sciatic nerve ligation-induced increases in the co-localization of GAT-3 and GFAP were quantified by the intensity correlation analysis/ICQ method [35,37] as described in the Section 2. The increased GAT-3 by sciatic nerve ligation was co-localized with GFAP in the cingulate cortex, and was significantly enhanced compared to that with a sham operation (from 0.041 ± 0.004 (ICQ) to 0.108 ± 0.015 (ICQ) for ligation, $P < 0.01$).

3.5. No change in the expression of GABA transporter and astrocyte-specific marker mRNA under a neuropathic pain-like state

In the reverse transcriptase-PCR (RT-PCR) assay, there were no differences between sham-operated and ligated mice with regard to mRNA levels of GAT-1 and GAT-3 in the frontal cortex (Fig. 4). Likewise, mRNA levels of GFAP and other astrocyte-specific markers (Slc15a2, AQP4, and Acsbg1) in the frontal cortex were not changed at any time after nerve ligation (Fig. 5).

3.6. Extracellular GABA levels were significantly decreased in the cingulate cortex of mice with sciatic nerve ligation

Fig. 6A-i and ii shows the placement of microdialysis probes within the mouse cingulate cortex. The basal level of GABA release in the cingulate cortex of mice with neuropathic pain was comparable to or slightly less than that in sham-operated mice after 7 or

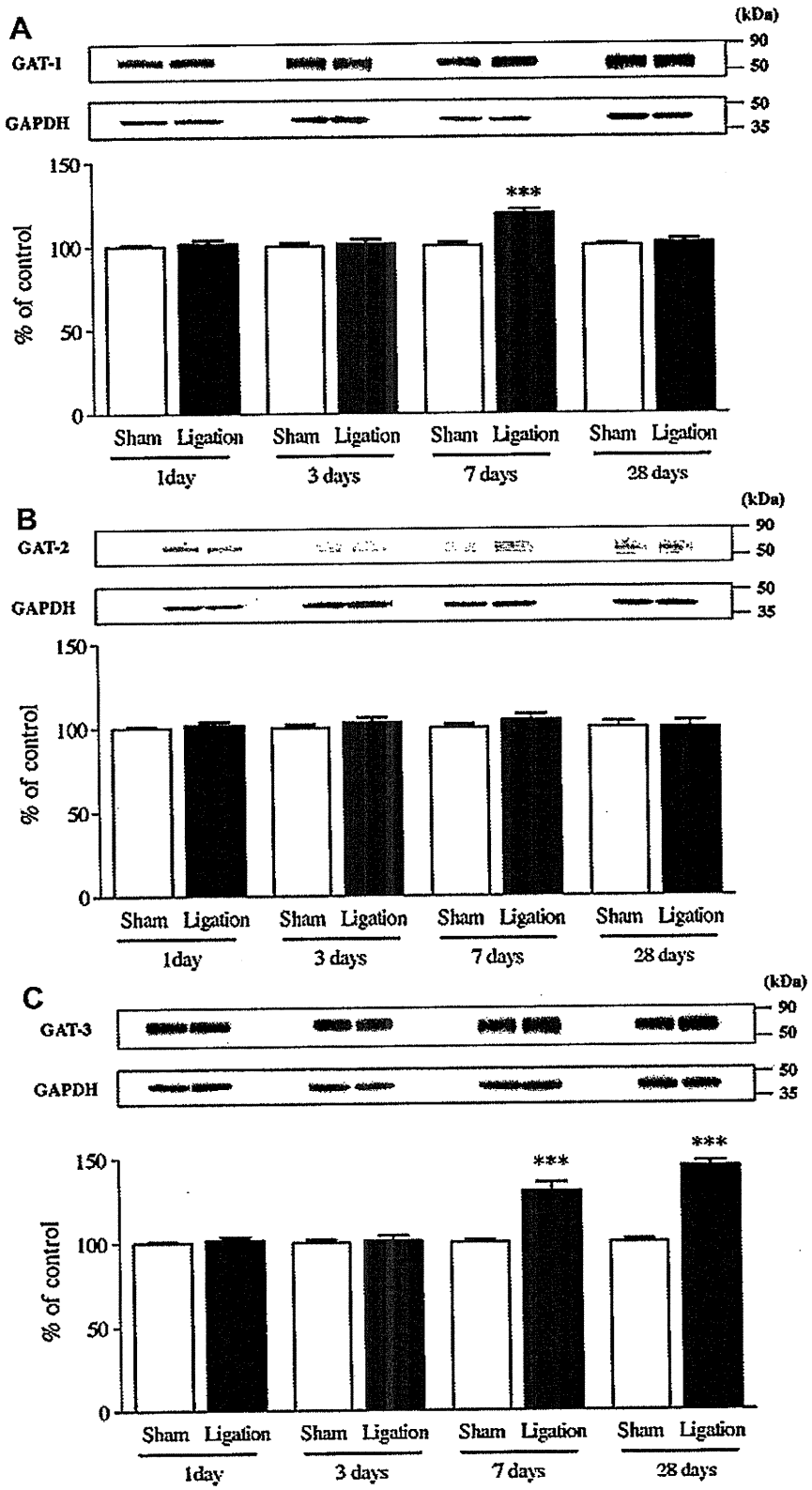


Fig. 2. Changes in the levels of GABA transporters in the frontal cortex under neuropathic pain. The membrane-bound levels of GABA transporter-1 (GAT-1) (A), GABA transporter-2 (GAT-2) (B) and GABA transporter-3 (GAT-3) (C) in the mouse frontal cortex at 1, 3, 7, or 28 days after sciatic nerve ligation. The protein levels of GAT-3 at 7 and 28 days after sciatic nerve ligation were significantly increased. Upper: Representative Western blot of GAT-1, GAT-2, and GAT-3 levels. Each column represents the mean ± SEM, ***$P < .001$ vs sham group.

28 days (Fig. 6B-i or C-i, respectively). Under these conditions, GABA released in the cingulate cortex of sham-operated mice after the administration of 4-aminopyridine (4-AP), a K^+ channel blocker that leads to electrical discharges of neurotransmitters, was significantly decreased in nerve-ligated mice (Fig. 6B-ii: $F_{1,36} = 5.412$, $P < .05$; C-ii: $F_{1,40} = 5.606$, $P < .05$ vs sham group).

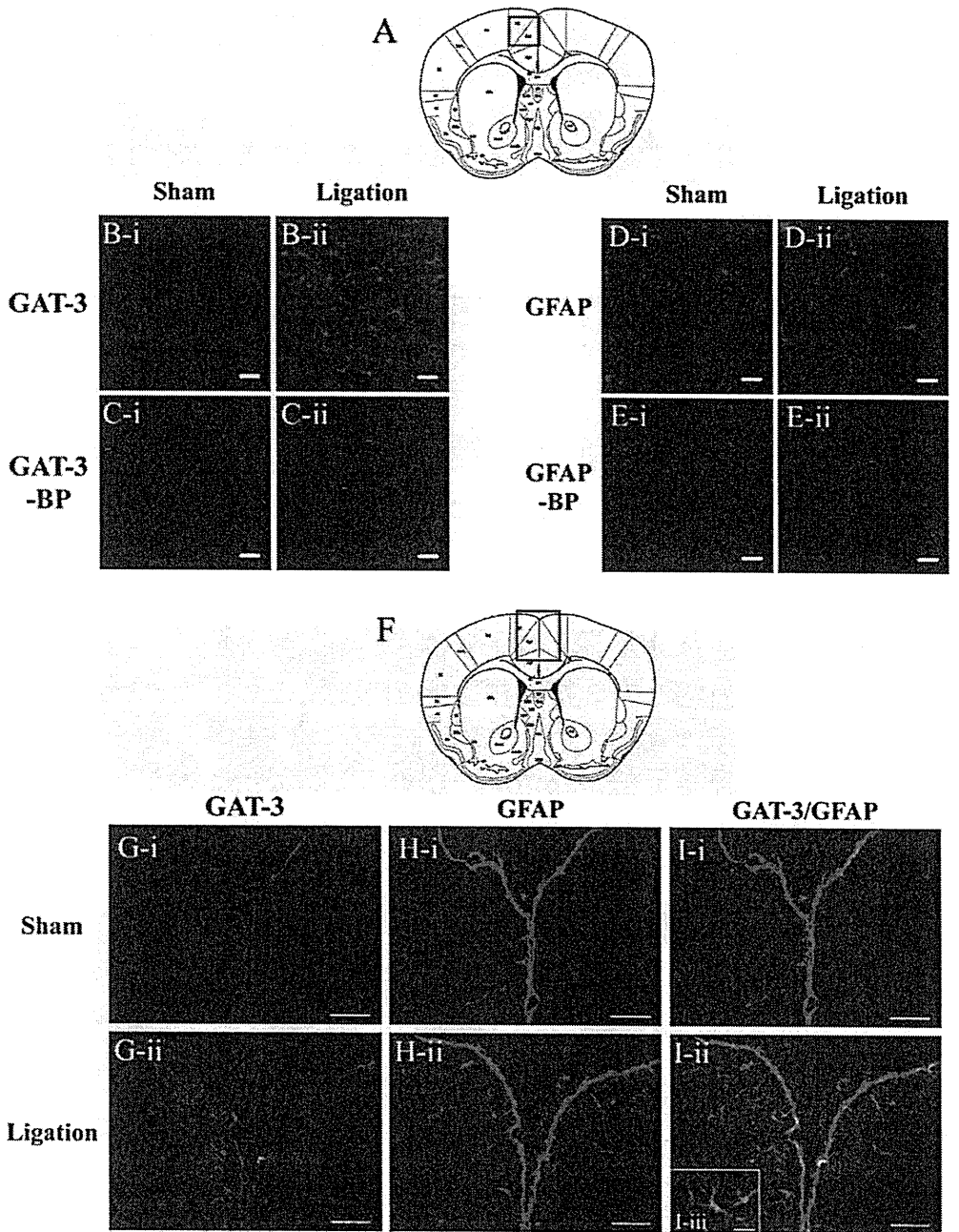


Fig. 3. Immunofluorescent staining for GAT-3 IR and GFAP IR in the cingulate cortex of sham-operated and nerve-ligated mice. The mouse brain sample was prepared 7 days after nerve ligation. The mouse brain atlas shows the region of the cingulate cortex after incubation with GAT-3 (B-i and B-ii) or GFAP (D-i and D-ii) antibodies and GAT-3 or GFAP antibodies preabsorbed with their immunogen peptide (C-i, C-ii, E-i, and E-ii). GAT-3 IR in the cingulate cortex of nerve-ligated mice (G-ii) was dramatically increased compared to that in sham-operated mice (G-i). GFAP IR was dramatically increased with morphologic differentiation in the cingulate cortex of nerve-ligated mice (H-ii) compared to that in sham-operated mice (H-i). Apparent co-localization of GAT-3 with GFAP in the cingulate cortex of sham-operated mice (I-i) and nerve-ligated mice (I-ii). GAT-3 IR was exclusively observed on GFAP IR cells in the cingulate cortex of nerve-ligated mice (I-iii, magnification). Scale bars = 50 μm.

3.7. Effect of intracingulate cortex injection of the selective GAT-3 inhibitor SNAP-5114 on sleep disturbance under a neuropathic pain-like state

As previously mentioned, sciatic nerve ligation (with intravesical injection into the cingulate cortex) caused significant increases in the WAKE stage (Fig. 7A-i: $F_{1,230} = 18.23$, $P < .01$, Fig. 7A-ii: $P < .001$), and decreases in the NREM stage at 7 days after the operation (Fig. 7C-i: $F_{1,230} = 6.821$, $P < .05$, 7C-ii: $P < .05$). There was no difference in REM sleep between sham operation and sciatic nerve ligation for 7 days (Fig. 7B). The increased WAKE stage and decreased NREM stage were significantly attenuated by intracingulate cortex injection of SNAP-5114 in nerve-ligated mice, compared to those in sham-operated mice (WAKE stage; Fig. 7A-i: $F_{1,230} = 4.968$, $P < .05$, Fig. 7A-ii: $P < .05$, NREM stage; Fig. 7C-i: $F_{1,230} = 9.522$, $P < .05$, Fig. 7C-ii: $P < .05$).

3.8. Evaluation of $GABA_A$ receptor agonist-induced LORR under a neuropathic pain-like state

In the present study, the onset time of LORR induced by i.p. administration of the full benzodiazepine site agonist midazolam was significantly prolonged in mice with sciatic nerve ligation, whereas LORR time with midazolam was significantly shortened

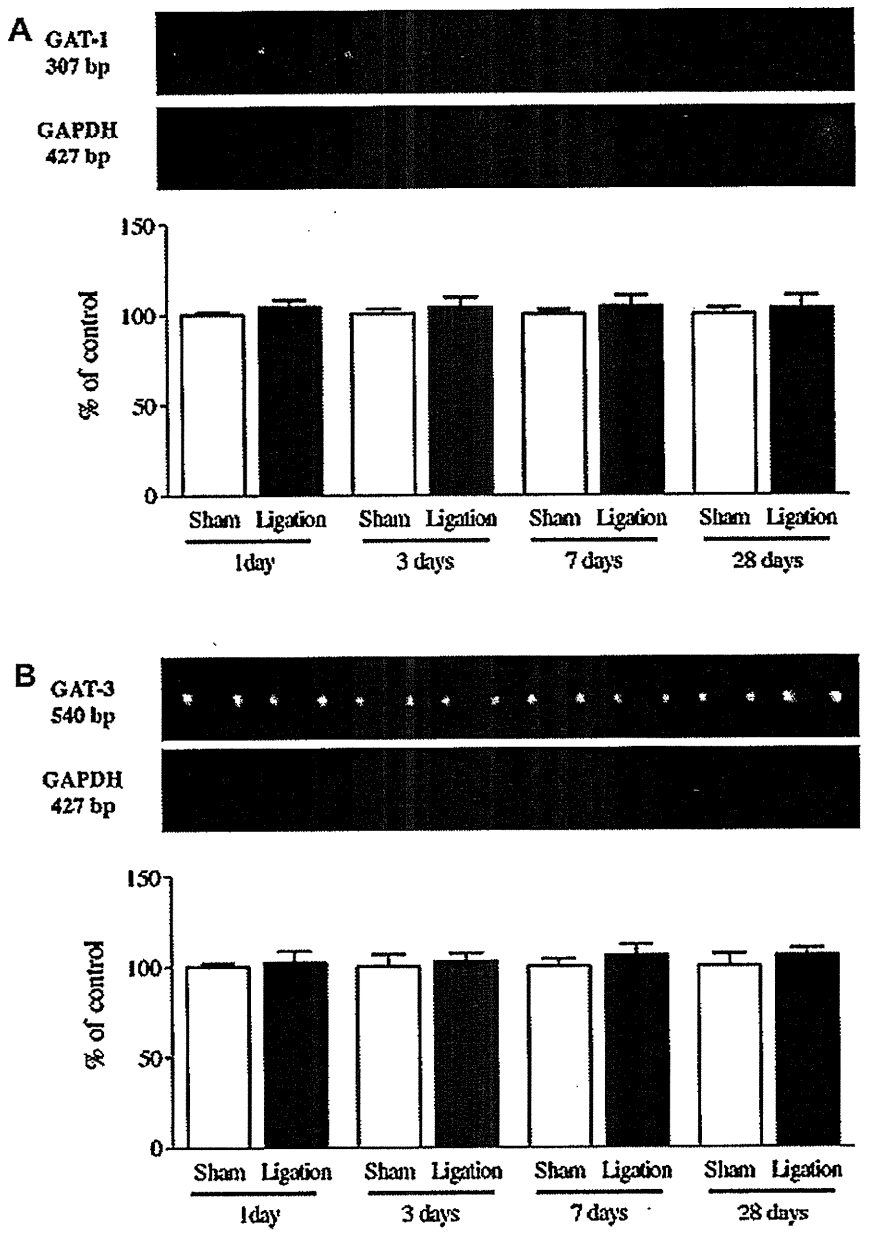


Fig. 4. No change in the expression of GABA transporters mRNA under a neuropathic pain-like state. Representative RT-PCR for mRNAs of GAT-1 (A) or GAT-3 (B) and the internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the frontal cortex of mice at 1, 3, 7, or 28 days after sciatic nerve ligation. The intensity of the bands was determined in a semiquantitative manner using ImageJ software. Values for GAT-1 and GAT-3 mRNA in sciatic nerve-ligated mice were normalized by the value for the respective GAPDH mRNA. The values in nerve-ligated mice are expressed as a percentage of the increase in sham-operated mice. Each column represents the mean ± SEM.

in mice with sciatic nerve ligation (Fig. 8A and B, $^{*}P < .05$, $^{**}P < .01$ vs sham group). Furthermore, sciatic nerve ligation also significantly prolonged the onset time of LORR and shortened the LORR-time induced by i.p. administration of zolpidem, which is a nonbenzodiazepine hypnotic that interacts at the benzodiazepine modulatory site of the $GABA_A$ receptor (Fig. 8C and D, $^{*}P < .05$, $^{**}P < .01$ vs sham group). On the other hand, nerve ligation did not alter the time of onset or the duration of pentobarbital-induced LORR (Fig. 8E and F). Further pharmacological evidence for the possible involvement of activated GAT-3 in the attenuation of the hypnotic effects induced by zolpidem under neuropathic pain was obtained in the present in vivo analysis. The prolonged onset time of LORR and shortened LORR-time induced by zolpidem 7 days (Fig. 8G-i; $P < .01$, Fig. 8G-ii; $P < .05$) or 28 days (Fig. 8H-i; $P < .05$, Fig. 8H-ii; $P < .05$) after nerve ligation were almost completely recovered by the premicroinjection of SNAP-5114 into the cingulate cortex of sciatic nerve-ligated mice (7 days: Fig. 8G-i; $P < .01$, Fig. 8G-ii; $P < .05$, 28 days: Fig. 8H-i; $P < .05$, Fig. 8H-ii; $P < .05$).

3.9. Changes in the hypnotic effects of midazolam and zolpidem under a neuropathic pain-like state as determined by EEG/EMG recording

To confirm the changes in the hypnotic effects of midazolam and zolpidem 7 days after nerve ligation, we performed EEG/EMG recording. When midazolam was administered i.p. to sham-operated mice, it decreased wakefulness for about 4 h. This reduction in wakefulness occurred at the same time as increases in NREM. In contrast, when midazolam was administered i.p. to nerve-ligated mice, its effects, such as decreased wakefulness and increased NREM, were significantly attenuated (7 days WAKE: Fig. 9A; $F_{1,40} = 18.78$, 7 days NREM: Fig. 9C; $F_{1,40} = 6.771$, $P < .05$, 28 days WAKE: Fig. 9D; $F_{1,40} = 29.56$, $P < .01$, 28 days NREM:

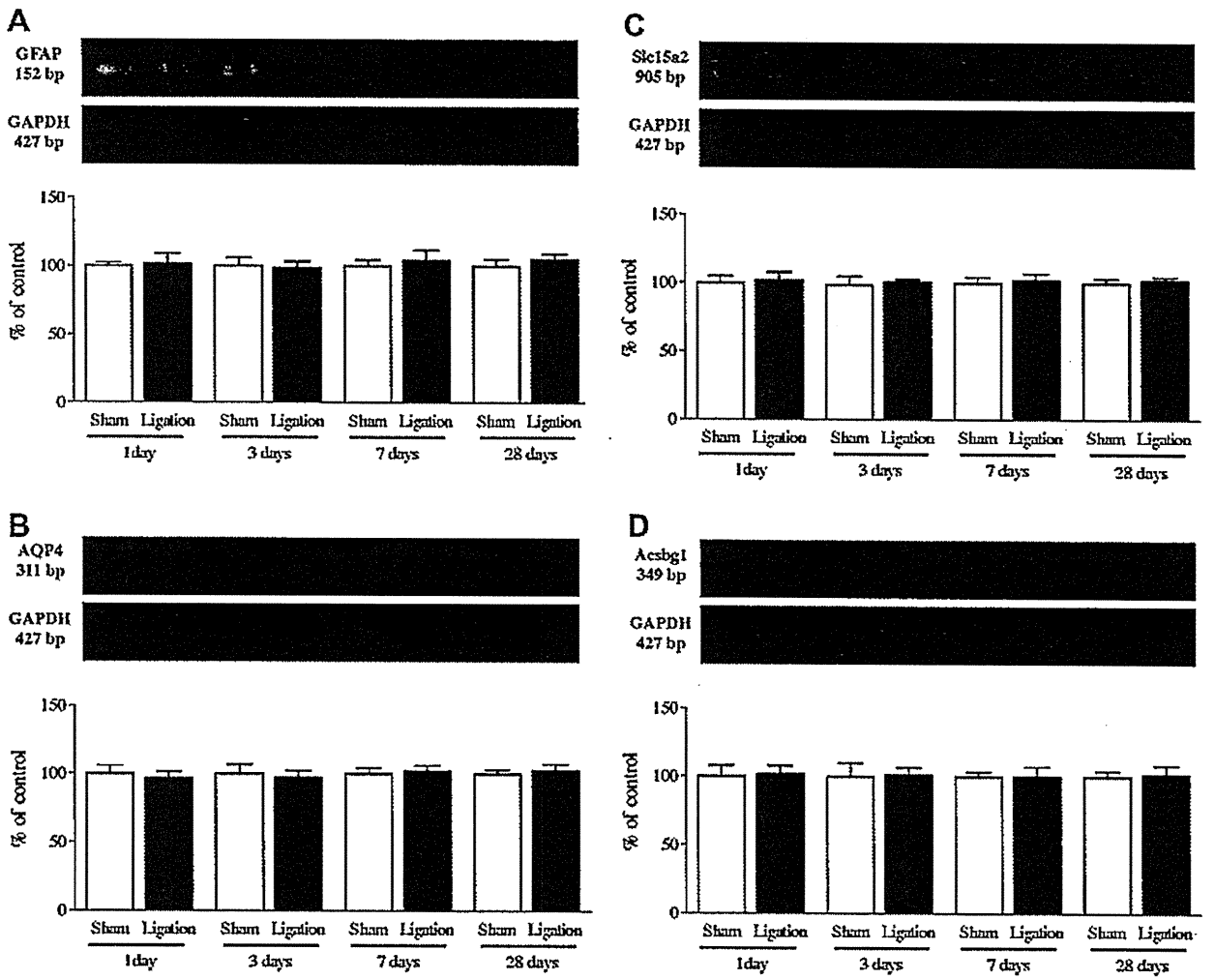


Fig. 5. No change in the expression of astrocyte-specific marker mRNA under a neuropathic pain-like state. Representative RT-PCR for mRNAs of GFAP (A), AQP4 (B), Slc15a2 (C), Acsbg1 (D) and the internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the frontal cortex of mice at 1, 3, 7, or 28 days after sciatic nerve ligation. The intensity of the bands was determined in a semiquantitative manner using ImageJ software. Values for GFAP, AQP4, Slc15a2, and Acsbg1 mRNA in sciatic nerve-ligated mice were normalized by the value for the respective GAPDH mRNA. The values in nerve-ligated mice are expressed as a percentage of the increase in sham-operated mice. Each column represents the mean ± SEM.

Fig. 9F; $F_{1,40} = 6.827$, $P < .05$). When zolpidem was administered i.p. to sham-operated mice, it decreased wakefulness for about 1.5 h. This reduction in wakefulness occurred at the same time as an increase in NREM. When zolpidem was administered i.p. to nerve-ligated mice, its effects were significantly suppressed (7 days WAKE: Fig. 9G; $F_{1,84} = 6.970$, $P < .05$, 7 days NREM: Fig. 9I: $F_{1,84} = 12.67$, $P < .01$, 28 days WAKE: Fig. 9J; $F_{1,84} = 11.56$, $P < .05$, 28 days NREM: Fig. 9L: $F_{1,84} = 8.442$, $P < .05$).

4. Discussion

From a clinical point of view, the relationship between insomnia and chronic pain seems to be straightforward: pain causes emotional arousal and arousal interferes with the ability to initiate and maintain sleep [33,34]. In the present study, we demonstrated that sciatic nerve-ligated mice exhibit an equivalent dysregulation of wakefulness and NREM sleep. These findings are consistent with clinical reports that sleep deprivation can be particularly devastating in patients with chronic pain [8,15,17,18,22,38,47]. It was previously reported that sciatic nerve constriction induced poor sleep quality with disrupted sleep in rats, particularly during the first week of that condition [1]. In the present study, we demonstrated that sleep dysregulation was observed after sciatic nerve ligation in mice. These findings suggest that continuous peripheral noxious stimuli may lead to the development of chronic insomnia.

In a previous study, we found that chronic pain induced by sciatic nerve ligation caused a morphological change in GFAP-labelled astrocytes in the cingulate cortex of mice [40]. In support of these findings, in the present study we further confirmed that GFAP-positive IR was significantly increased in the cingulate cortex of nerve-ligated mice. Each individual astrocyte labeled by GFAP was branched, indicating the activation of astrocytes in the cingulate cortex of nerve-ligated rodents. Under these conditions, the mRNA levels of GFAP and other astrocyte-located molecules in the frontal cortex including the cingulate cortex were not altered by sciatic nerve ligation.

The key finding in this study was that membrane-bound GAT-1 and GAT-3 in the frontal cortex of sciatic nerve-ligated mice were significantly increased 7 days after nerve ligation compared to those in sham-operated mice. The significant increase in GAT-3, but not GAT-1, lasted for at least 28 days. Under these conditions, there were no differences between sham-operated and ligated mice with regard to mRNA levels of GAT-1 and GAT-3 in the frontal cortex at any time after nerve ligation. On the other hand, there was no significant difference in the levels of GAT-2 between sham-operated and nerve-ligated

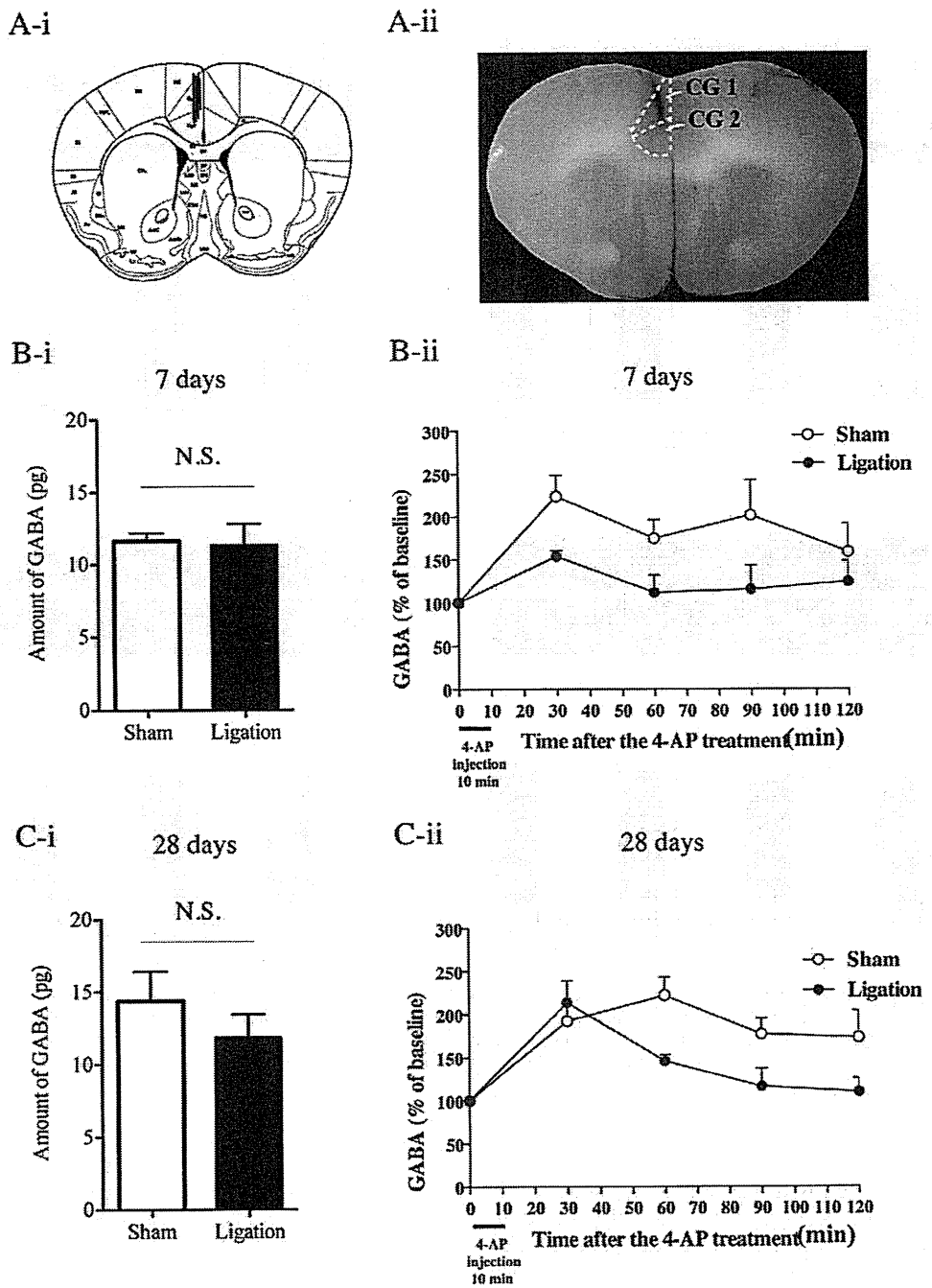


Fig. 6. Changes in 4-aminopyridine (4-AP)-induced extracellular GABA release under a neuropathic pain-like state. Schematic reconstruction of microdialysis probe (vertical bars) placements within the cingulate cortex was shown in (A-i). Length of vertical bars corresponds to the length of the active portion of the dialysis membrane (1.0 mm). Photomicrograph showed a vestige of microdialysis probe. Blue-ink was microinjected into the same position as microdialysis probe after the experiment (A-ii). There were no changes in the basal level of GABA in sham-operated and sciatic nerve-ligated mice at 7 days (B-i) or 28 days (C-i) after surgery (N.S.; not significant). Effects of treatment with 4-AP on the extracellular level of GABA in the cingulate cortex of sham-operated and sciatic nerve-ligated mice at 7 days (B-ii) or 28 days (C-ii) after surgery. 4-AP (1 mM) was injected into the cingulate cortex for 10 min. GABA release in the cingulate cortex was significantly decreased in nerve-ligated mice at 7 days after surgery. Two-way ANOVA was performed, followed by Bonferroni testing. Each point represents the mean ± SEM of 6 to 8 mice.

mice, indicating that neuropathic pain-like stimuli may predominantly promote the long-lasting membrane translocation of GAT-3 in the frontal cortex. In the present double-staining approach, we found that GAT-3 IR in the cingulate cortex of nerve-ligated mice was clearly co-localized with activated GFAP-positive astrocytes, which is supported by the finding that GAT-3 is mainly located on astrocytes [54]. These findings support the idea that neuropathic pain may increase GABA reuptake via increased membrane-bound GAT-3 in activated astrocytes of the cingulate cortex.

To clarify whether the extracellular level of GABA in the cingulate cortex could be altered under a long-lasting pain-like state, an in vivo microdialysis study was performed. The GABA level at the synaptic cleft in the cingulate cortex was significantly decreased in nerve-ligated mice after the administration of 4-AP. These results suggest that neuropathic pain can decrease endogenously released GABA at the synaptic cleft associated with an increase in the reuptake of GABA via increased GAT-3 located on activated astrocytes, which diminishes the efficiency of GABAergic neurotransmission in the cingulate cortex.

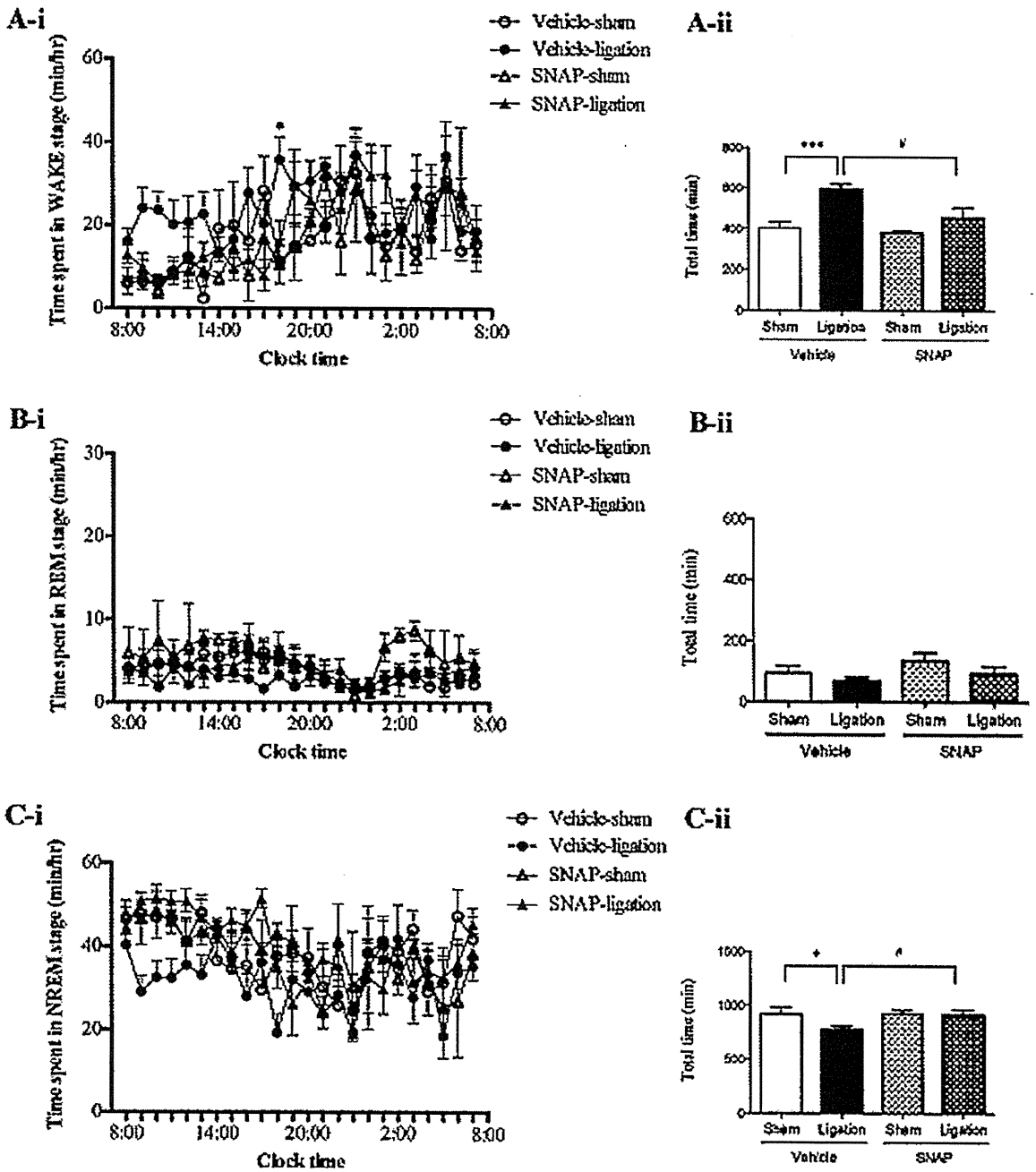
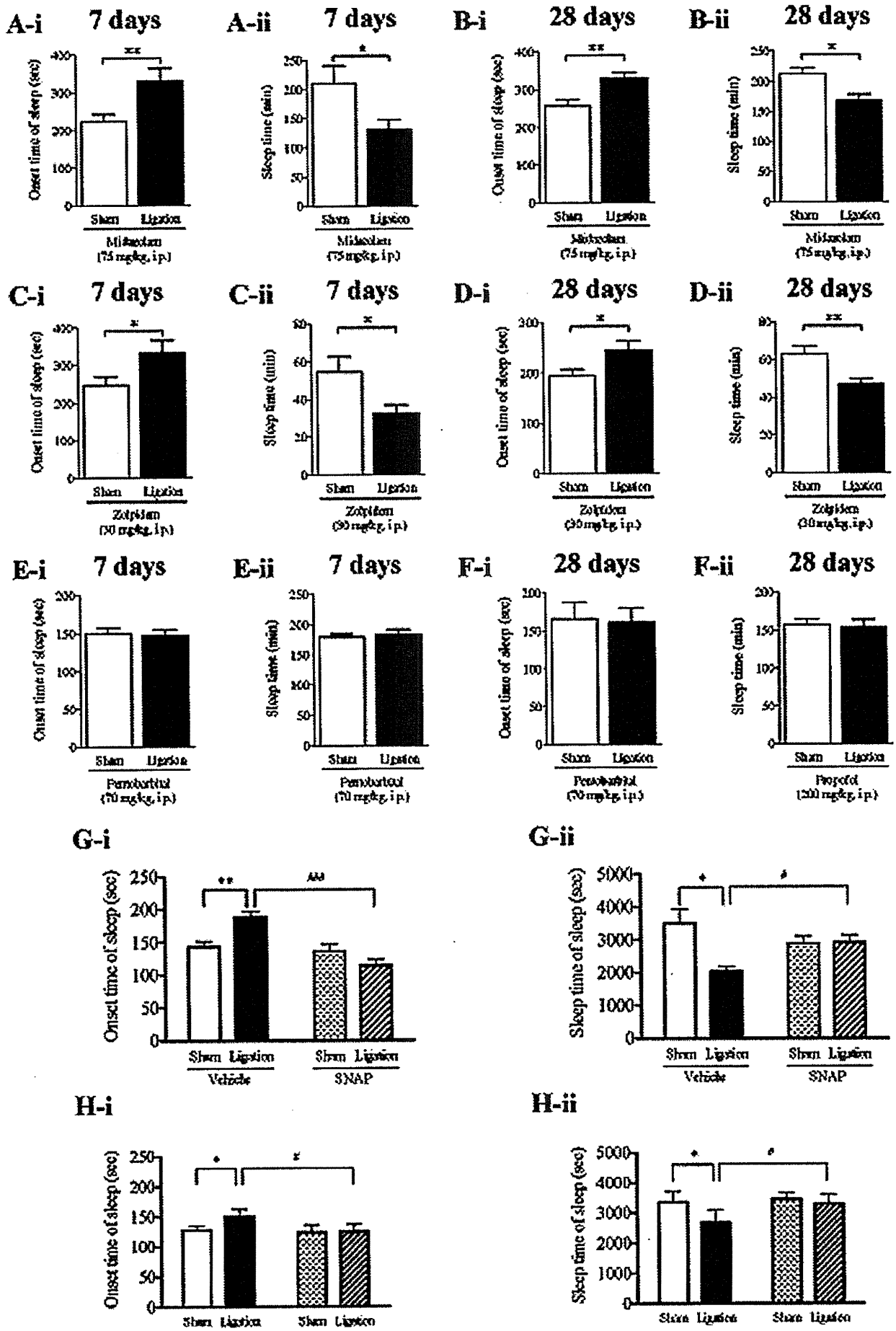


Fig. 7. Changes in sleep vigilance after the intracingulate cortex injection of SNAP-5114 under a neuropathic pain-like state as determined by EEG/EMG recordings. Sleep–wake states after the microinjection of vehicle or SNAP-5114 into the cingulate cortex at 7 days after sciatic nerve ligation. Vehicle or SNAP-5114 (1 nmol/mouse) was injected at 8:00 AM. Time spent in the WAKE stage (A-i, ii), REM sleep stage (B-i, ii) and NREM sleep stage (C-i, ii) was determined by EEG/EMG recording. The increased WAKE stage (A-ii) and decreased NREM stage (C-ii) were significantly attenuated by the intracingulate cortex injection of SNAP-5114 in nerve-ligated mice compared to those in sham-operated mice (A-i, B-i, and C-i). The statistical analysis was performed by 2-way ANOVA and the Bonferroni test (unless otherwise indicated). (A-ii, B-ii, and C-ii) The statistical analysis was performed by Student's *t*-test. Each bar represents the mean ± SEM of 5 to 6 mice. $^{*}P < .05$ and $^{***}P < .001$ vs sham group, $^{\#}P < .05$ vs ligation group.

In the present EEG/EMG analysis, we found that the increased WAKE stage and decreased NREM stage under a neuropathic pain-like state were almost reset by microinjection of the GAT-3 inhibitor SNAP-5114 into the cingulate cortex. These findings indicate that the increase in membrane-translocated GAT-3 on activated astrocytes, which can uptake and metabolize GABA released at the synaptic cleft, in the cingulate cortex area may be accompanied by sleep disturbance under a neuropathic pain-like state.

In general, it has been considered that excitatory and inhibitory transmission maintain a physiological balance in the normal state. A recent study by Xu et al. [61] suggests that both presynaptic and postsynaptic excitatory glutamatergic transmission are enhanced in the anterior cingulate cortex of mice with neuropathic pain. Although further experiments are still needed, these phenomena may explain the loss of the balance between excitatory and inhibitory transmission in the cingulate cortex under neuropathic pain.

It has been established that benzodiazepines decrease wakefulness through the enhanced affinity of endogenous GABA binding to $GABA_A$ receptors [2,20]. Considerable evidence indicates that benzodiazepines, such as midazolam and zolpidem, cannot independently elicit the influx of Cl^- ions through the $GABA_A$ receptor,


A-i
7 days
A-ii
7 days
B-i
28 days
B-ii
28 days
C-i
7 days
C-ii
7 days
D-i
28 days
D-ii
28 days
E-i
7 days
E-ii
7 days
F-i
28 days
F-ii
28 days
Onset time of sleep (sec)
Sleep time (min)
Sham
Ligation
Midazolam (75 mg/kg, i.p.)
Zolpidem (30 mg/kg, i.p.)
Pentobarbital (70 mg/kg, i.p.)
Propofol (200 mg/kg, i.p.)
G-i
G-ii
H-i
H-ii
Onset time of sleep (sec)
Sleep time of sleep (sec)
Vehicle
SNAP

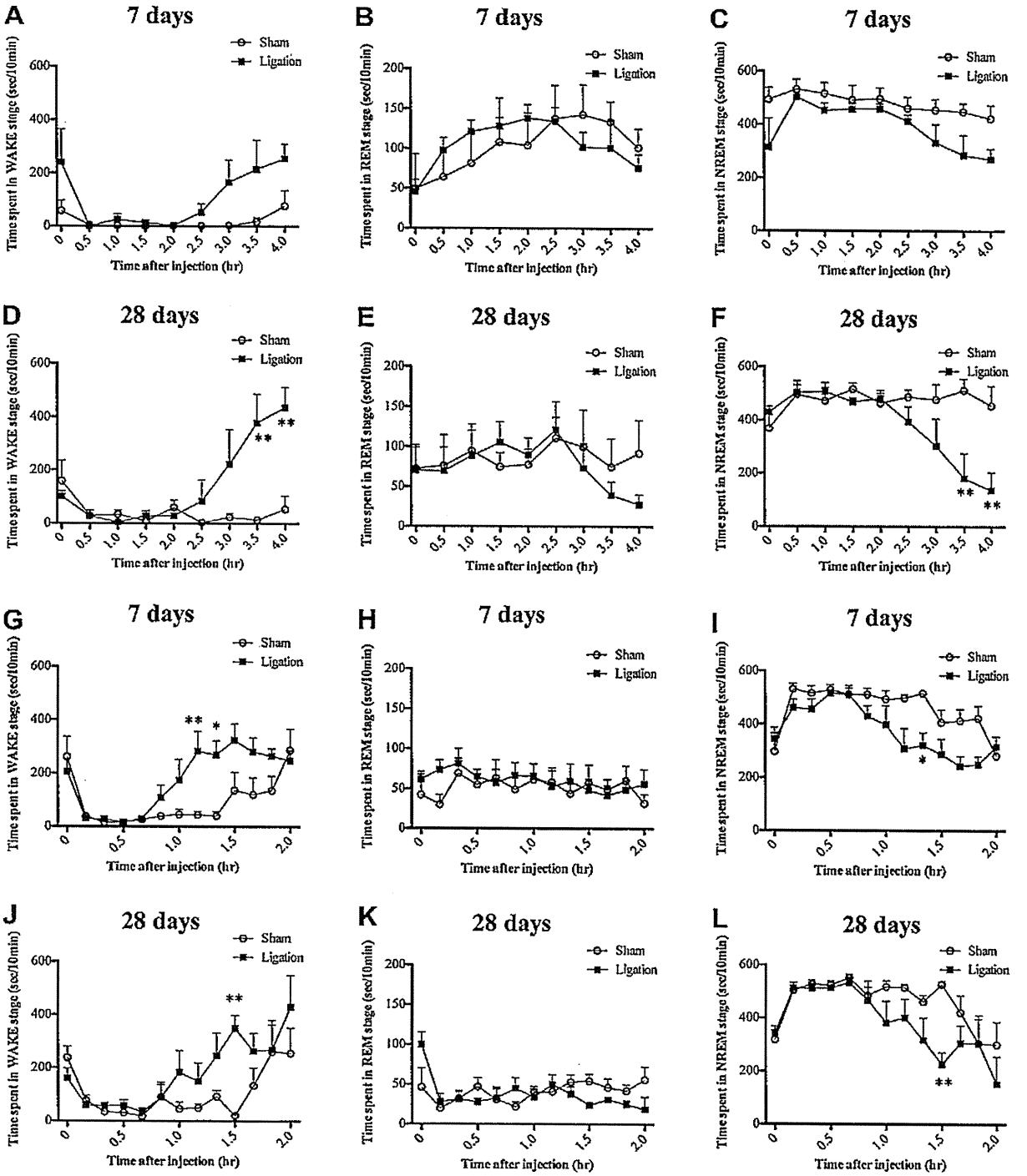


Fig. 9. Sleep–wake states after an i.p. injection of midazolam or zolpidem under a neuropathic pain-like state. At 7 days (A–C) or 28 days (D–F) after sciatic nerve ligation, mice were injected with midazolam (75 mg/kg, i.p.). The wake time was prolonged in mice with midazolam at 7 days (A) or 28 days (D) after sciatic nerve ligation. There were no changes in REM sleep induced by midazolam between sham-operated and nerve-ligated mice at 7 days (B) and 28 days (E). NREM sleep induced by midazolam was suppressed at 7 days (C) and 28 days (F) after sciatic nerve ligation. At 7 days (G–I) or 28 days (J–L) after sciatic nerve ligation, mice were injected with zolpidem (30 mg/kg, i.p.). The wake time was prolonged by zolpidem at 7 days (G) or 28 days (J) after sciatic nerve ligation. There were no changes in the REM sleep induced by zolpidem between sham-operated and nerve-ligated mice at 7 days (H) and 28 days (K). NREM sleep induced by zolpidem was suppressed at 7 days (I) or 28 days (L) after sciatic nerve ligation. One-way ANOVA was performed, followed by Bonferroni testing (unless otherwise indicated). Each point represents the mean ± SEM of 6 to 8 mice.

Fig. 8. Effects of midazolam, zolpidem and pentobarbital on the onset time of LORR and the LORR-time under a neuropathic pain-like state. At 7 days (A, C, and E) or 28 days (B, D, and F) after sciatic nerve ligation, mice were injected with midazolam (75 mg/kg, i.p.), zolpidem (30 mg/kg, i.p.) or pentobarbital (70 mg/kg, i.p.). The onset time of LORR induced by midazolam or zolpidem was significantly prolonged, whereas the LORR-time with midazolam or zolpidem was significantly shortened in mice at 7 or 28 days after sciatic nerve ligation. Each column represents the mean ± SEM, $^{*}P < .05$ and $^{**}P < .01$ vs sham group. The onset time of LORR and the LORR-time induced by zolpidem after the microinjection of SNAP-5114 (SNAP) into the cingulate cortex of nerve-ligated mice. At 7 days (G) or 28 days (H) after nerve ligation, mice were injected with zolpidem (30 mg/kg, i.p.) 3 h after the microinjection of vehicle or SNAP (1 nmol/mouse) into the cingulate cortex. Each bar represents the mean ± SEM of 6 to 9 mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs vehicle-sham group, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs vehicle-ligation group.

but rather facilitate the action of endogenous GABA by increasing the frequency of channel-opening, whereas barbiturates can directly open $GABA_A$ receptor-associated chloride channels in the absence of GABA [5,26]. The LORR has been considered to be useful as a marker of the hypnotic state to investigate the sleep effects induced by benzodiazepines. In the present study, we documented that the LORR effects induced by i.p. administration of midazolam and zolpidem, both of which require endogenous GABA to produce their hypnotic effects, were significantly attenuated by sciatic nerve ligation. Furthermore, on the basis of the results of EEG/EMG, midazolam and zolpidem reduced the amount of wakefulness and increased the amount of NREM sleep in sham-operated mice. These effects were dramatically suppressed in mice with sciatic nerve ligation. To further ascertain the specific involvement of GAT-3 in the reduction of GABAergic transmission under a neuropathic pain-like state, we next investigated the effects of the microinjection of SNAP-5114 on the hypnotic effects of zolpidem in mice with sciatic nerve ligation. In this study, the prolonged onset time of LORR and shortened LORR-time by midazolam in nerve-ligated mice was completely reversed to the normal level by the intracingulate cortex injection of SNAP-5114. In contrast, the onset time of LORR and the duration of LORR induced by the i.p. injection of pentobarbital, which can induce its effect without GABA, in sciatic nerve-ligated mice were similar to those in sham-operated mice. These findings support the idea that nerve injury may decrease inhibitory GABAergic transmission in the cingulate cortex, possibility leading to suppression of the hypnotic effects induced by benzodiazepines in mice with sciatic nerve ligation.

It is well recognized that the GABAergic system plays an important role in nociceptive processing in the spinal cord. A large proportion of GABA-A receptor-mediated neural inhibition occurs via postsynaptic action on spinal dorsal horn neurons [7,12,23,51,57,62]. An important factor that could lead to the development of neuropathic pain is the reduced inhibition, or disinhibition, of spinal cord neurons [13,52,60]. Changes in GABA receptor disinhibition in the spinal cord may arise from the reduced expression of potassium-chloride cotransporter 2 (KCC2) after peripheral nerve injury [10]. Considering these findings, we cannot deny the possibility that, like released GABA, postsynaptic GABA receptor function in the cingulate cortex could also be changed by neuropathic pain, which might be responsible for the insomnia and lower sensitivity to benzodiazepines as a result of chronic pain.

In conclusion, we have demonstrated that neuropathic pain may decrease the level of released GABA at the synaptic cleft in the mouse cingulate cortex associated with an increase in GABA uptake through increased GATs on astrocytes, which results in the suppression of GABAergic transmission in the cingulate cortex region. Although further clarification is needed, we propose that this phenomenon may at least partly explain sleep dysregulation under a neuropathic pain.

Conflict of interest statement

The authors have no conflicts of interest to report.

Acknowledgments

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Enhancement of Glutamatergic Transmission in the Cingulate Cortex in Response to Mild Noxious Stimuli Under a Neuropathic Pain-Like State

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KEY WORDS glutamate; chronic pain; heat stimuli; brain activation; hyperalgesia

ABSTRACT Pain is evoked by noxious body stimulation or through negative emotional events and memories. There are several caveats to the simple proposition that pain and emotion are linked in the cingulate cortex (CG). In this study, we investigated whether mild noxious heat stimuli could affect the neuronal activity in the CG of rats with sciatic nerve ligation. We produced a partial sciatic nerve injury by tying a tight ligature in rats. Seven days after sciatic nerve ligation, rats received mild noxious heat stimuli. Mild noxious heat stimuli produced flinching behaviors in sciatic nerve-ligated rats, but not sham-operated rats. In addition, the mild noxious heat stimuli caused a significant increase in the release of glutamate in the CG of nerve-ligated rats compared with that of sham-operated rats. Furthermore, phosphorylated-NR1-positive cells in this area significantly increased after mild noxious heat stimuli under a neuropathic pain. Under this condition, there were no significant changes in the levels of immediate-early genes such as c-fos, c-jun, JunB, and Fra1 in the CG between nerve-ligated and sham-operated rats. However, mild noxious heat stimuli under a neuropathic pain-like state produced a marked increase in the phosphorylated-c-jun (p-c-jun) immunoreactivity, which is commonly used to map neurons in the brain that can be activated after *N*-methyl-D-aspartate receptor activation. These findings raise the possibility that mild noxious heat stimuli under a peripheral nerve injury may increase the release of glutamate and promote its related postneuronal activity in the CG. **Synapse 65:424–432, 2011.**

INTRODUCTION

Patients with neuropathic pain exhibit a wide range of symptoms, including spontaneous and stimulus-evoked pain (Woolf and Mannion, 1999). Persistent primary afferent inputs are believed to cause a state of central (i.e., "spinal") sensitization, which enhances responses to sensory inputs and thus maintains an enhanced pain state with some similarities to long-term potentiation (Chapman et al., 1998; Dickenson et al., 2001; Ren, 1994; Suzuki and Dickenson, 2000; Ziegler et al., 1999). Although many studies have focused on the long-term changes in the functions of spinal cord dorsal horn neurons containing some receptors, protein kinases, and peptides after nerve injury, it has been recognized that patients with chronic pain often suffer affective disorders such as anxiety, depression, and poor sleep. Therefore, it has been suggested that brain mechanisms may also

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