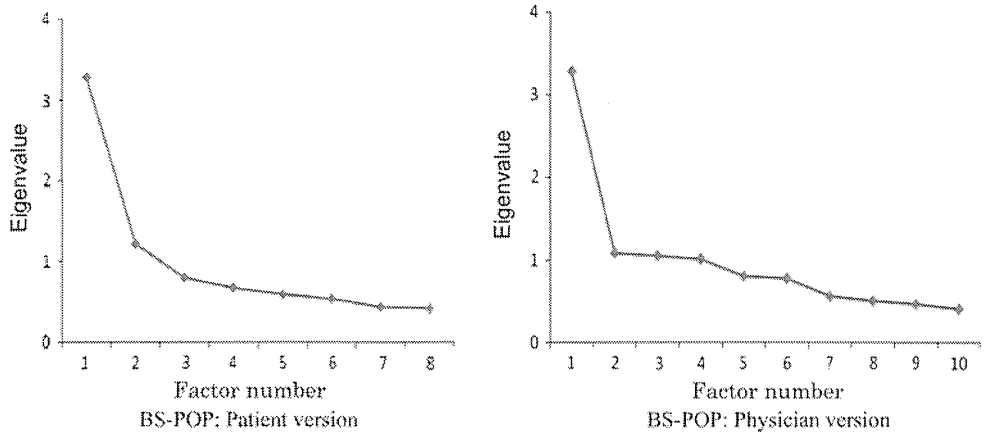

Fig. 2 Factor analysis shows that both the physician and patient versions of the Brief Scale for Psychiatric problems in Orthopaedic Patients (BS-POP) are unifactorial

Table 4 Pearson's correlation coefficients between subscales of MMPI and BS-POP

	MMPI												
	Pearson's correlation coefficient												
	L	F	K	1 Hs5K	2 D	3 Hy	4 Pd4K	5 Mf	6 Pa	7 Pt1K	8 Sc1K	9 Ma2K	0 Si
BS-POP for patients	−0.21*	0.35*	−0.29*	0.38*	0.62*	0.42*	0.30*	0.05	0.35*	0.49*	0.43*	0.12	0.37*
BS-POP for medical personnel	−0.01	0.14	−0.08	0.19*	0.29*	0.29*	0.14	−0.03	0.07	0.28*	0.21*	0.16*	0.06

1 Hs5K, hypochondriasis → scores greater than 70 are indicative of fixed somatic complaints and as ongoing concern about health; *2 D,* depression → scores between 50 and 65 are indicative of a personality that views life with the average amount of pessimism and optimism; *3 Hy,* hysteria → scores between 50 and 70 are indicative of an adventurous, outgoing, optimistic personality; greater than 70 is indicative of an immature and hysterical personality; *4 Pd4K,* psychopathic deviance → scores between 60 and 70 are indicative of some healthy nonconformism and rebelliousness; scores greater than 70 are indicative of nonconformism, acting out, and drug abuse; *5 Mf,* masculinity/femininity (Mf) → reflects cultural values; scores between 60 and 70 are indicative of sensitivity and aesthetic interests. This scale is of little clinical use; *6 Pa,* paranoia → scores greater than 70 are indicative of paranoia; *7 Pt1K,* psychasthenia → the tendency to worry over minor problems. Scores above 70 are indicative of obsessive, compulsive personalities, associated with depression; *8 Sc1K,* schizophrenia → indicative of untested beliefs, a sense of alienation, head in the clouds; scores greater than 70 tend to be pathologic; *9 Ma2K,* manic → indicative of energy level and tenacity; *0 Si,* social introversion → scores greater than 70 are indicative of a personality lacking in social skills

L lie scale → a naïve and deliberate evasive response reflecting the values of society, making a good appearance; *F* frequency scale → a deliberate distorted response, indicative of trying to look bad or a plea for help, or indicative of a disturbed thought process or a lack of understanding of questions; *K* defensiveness score → a denial of inadequacy, elevated scores are suggestive of good ego strength

* $p < 0.05$

were found with MMPI subscales L, F, K, 1, 2, 3, 4, 5, 7, 8, and 0. The correlation was particularly strong ($r = 0.62$) between the BS-POP for patients and MMPI scale 2, indicating depression. In the BS-POP for medical personnel, correlations were found with MMPI subscales 1, 2, 3, 7, 8, and 9.

With the BS-POP for patients, a strong correlation was found with all of the POMS subscales (Table 5). With the BS-POP for medical personnel, correlations were found with the POMS Depression–Dejection (D) Scale ($r = 0.22$), the Vigor (V) Scale ($r = -0.17$), the Fatigue (F) Scale ($r = 0.21$), and the Confusion (C) Scale ($r = 0.19$). With the BS-POP for medical personnel, no correlations were found with the Tension Anxiety (T-A) Scale and the Anger–Hostility (A-H) Scale, which are considered to have a particularly weak relationship with personality problems.

With the BS-POP for patients, correlations were found with all SF-36 v2 subscales (Table 6). Relatively strong correlations were found particularly between the BS-POP for patients and the Role Emotional (RE) Scale ($r = -0.44$) and the Mental Health (MH) Scale ($r = -0.52$), which are indicative of psychiatric problems. With the BS-POP for medical Personnel, correlations were found with all SF-36 v2 subscales.

With both the BS-POP for patients and the BS-POP for medical personnel, correlations were found with the scales indicating psychiatric problems. The above findings demonstrate that the BS-POP has criterion validity.

Reproducibility

The Pearson correlation coefficients of the first and second tests for the physician and patient versions were

Table 5 Pearson's correlation coefficients between subscales of POMS and BS-POP

	POMS					
	Pearson's correlation coefficient					
	Tension anxiety (T-A)	Depression dejection (D)	Anger hostility (A-H)	Vigor (V)	Fatigue (F)	Confusion (C)
BS-POP for patients	0.43*	0.49*	0.43*	−0.44*	0.55*	0.43*
BS-POP for medical personnel	0.14	0.22*	0.10	−0.17*	*0.21	0.19*

* $p < 0.05$

Table 6 Pearson's correlation coefficients between subscales of SF-36 v2 and BS-POP

	SF-36 v2							
	Pearson's correlation coefficient							
	Physical functioning (PF)	Role physical (RP)	Bodily pain (BP)	General health (GH)	Vitality (VT)	Social functioning (SF)	Role emotional (RE)	Mental health (MH)
BS-POP for patients	−0.42*	−0.40*	−0.28*	−0.46*	−0.54*	−0.41*	−0.44*	−0.52*
BS-POP for medical personnel	−0.34*	−0.38*	−0.26*	−0.31*	−0.33*	−0.33*	−0.34*	−0.36*

* $p < 0.05$

$r = 0.654$ and 0.719, respectively. In other words, reproducibility was confirmed for both the physician and the patient versions.

Discussion

In the present study, both the physician and patient versions were shown to possess reliability, factorial validity, criterion validity, and reproducibility.

Self-rated poor or fair overall health status is believed to be the biggest factor exacerbating low back pain [19]. In other words, patient self-assessment is important for predicting exacerbation of low back pain. However, patient self-assessment may include subjective or factitious responses in which patients make themselves appear better or worse than they really are. There are therefore limits to assessing psychiatric problems based solely on patient self-assessment, and objective assessments are also required. The BS-POP includes objective assessments by physicians, enabling more accurate evaluation of psychiatric problems.

In the present study, both physician and patient versions were unifactorial, demonstrating that each version has a single concept. This result suggests that total scores can be used for comparison purposes. The extracted factors were "personality problem" for the physician version and "mood problem" for the patient version.

When correlations between the BS-POP and the MMPI subscales, SF-36 v2 subscales, and POMS subscales were investigated, a greater overall correlation was found with the BS-POP for patients than with the BS-POP for medical personnel. Reasons other than the fact that the BS-POP for medical personnel assesses "personality problems" and the BS-POP for patients assesses "mood problems" may be involved. The MMPI, SF-36 v2, and POMS used in this study are questionnaires completed by the patients. It is possible that the BS-POP for patients shows a higher correlation with the MMPI subscales, SF-36 v2 subscales, and POMS subscales than the BS-POP for medical personnel, because the BS-POP for patients is completed by the patients, whereas an attending physician makes an evaluation and completes the BS-POP for medical personnel. These results suggested that it is a tool that can be easily used to assess the psychosocial problems of orthopedic patients.

In a study of LBP patients using the SF-36 v2, which is a generic QOL scale, the QOL declined in patients experiencing pain [20]. Psychological factors are considered to be deeply involved in the chronicity of LBP [7], and it is useful to perform psychosocial interventions early in the onset of LBP to prevent the condition from becoming chronic [21].

It has been reported that opioids [22], serotonin and norepinephrine reuptake inhibitors (SNRI), tricyclic antidepressants (TCAs) [23], radiofrequency procedures [24], and active physical treatment and cognitive behavioral treatment [25, 26] are effective in the treatment of chronic LBP. When treating patients with pain that has become chronic, BS-POP should be performed to screen for psychiatric problems.

A patient with $\geq$11 physician version points or $\geq$10 physician version points and $\geq$15 patient version points is considered to have psychiatric problems [17].

Outcomes do not improve unless the patient's psychosocial problems are evaluated [27, 28]. Using the BS-POP enables earlier detection of psychosocial problems before therapy. By assessing latent psychosocial problems prior to the start of therapy, unnecessary surgical procedures can be avoided, and the most appropriate treatment can be selected for each patient. An orthopaedic surgeon should consult a psychiatrist when a patient has a high score using BS-POP.

In particular, multidisciplinary treatment is considered effective [29, 30]. Multidisciplinary therapy involving not only orthopedic surgery but also psychiatry and anesthesiology is needed.

The present study was conducted using the Japanese version of the BS-POP. Tables 1 and 2 show the English version of the BS-POP. The English version has been back-translated and compared to the original to ensure that the original intent is reflected. We hope that the BS-POP will be useful in orthopaedic surgery and primary care in English-speaking countries as well. For this purpose, further investigations are needed using the English version.

Conclusions

Computational psychological data demonstrated the reliability, factorial validity, criterion validity, and reproducibility of the BS-POP. The BS-POP is a useful tool for orthopedic surgeons who need to easily and quickly assess their patients' psychiatric problems. In the future, it will be necessary to investigate the responsiveness and to verify the reliability, validity, reproducibility, and responsiveness of the BS-POP with regard to healthy subjects.

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The effect of repeated restraint stress in pain-related behavior induced by nucleus pulposus applied on the nerve root in rats

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ORIGINAL ARTICLE

The effect of repeated restraint stress in pain-related behavior induced by nucleus pulposus applied on the nerve root in rats

Kazuhide Uesugi · Miho Sekiguchi · Shin-ichi Kikuchi · Shin-ichi Konno

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Abstract
Introduction Chronic pain has an impact on psychological and social factors. It is known that stress influences physiological and behavioral changes and affects several neurotransmitter and hormonal systems. It is also known that corticosterone is increased by stress. The role of chronic stress in sciatica in lumbar disc herniation (LDH) in rats has not been investigated. The aim of this study was to investigate the effect of the restraint stress (RS) on pain-related behavior induced by application of nucleus pulposus (NP) in rats.
Materials and methods Adult female Sprague–Dawley rats were divided into six experimental groups (naive group; naive + RS; sham group; sham + RS; autologous nucleus pulposus [NP] applied on the left L5 nerve root [NP group]; and NP + RS group). Von Frey tests were used to test pain-related behavior. Concentrations of plasma corticosterone were measured to assess changes in levels of endogenous corticosterone caused by RS. Expression of ATF-3 in the left L5 DRG was examined by immunohistochemical analyses in each group.
Results Mechanical withdrawal thresholds of the NP and NP + RS groups were significantly decreased after surgery compared with the naive group. Although the thresholds in the NP group recovered after 28 days, the thresholds in the NP + RS group were significantly decreased during the 42 days after surgery. RS increased the concentration of plasma corticosterone at 21 and 42 days after surgery. In the NP and the NP + RS groups, the expression of ATF-3 was significantly increased at 7 days after surgery. The expression of ATF-3 was sustained for 21 days by RS.
Conclusion Concentrations of plasma corticosterone were increased in three groups that underwent RS. The pain-related behavior persisted for the long term in the LDH model. The expression of ATF-3 in DRG neurons increased for 21 days by RS. These results suggest that RS plays a role in the chronicity of pain-related behavior in the LDH rats.

Keywords Repeated restraint stress · Corticosterone · Pain-related behavior · Nucleus pulposus · Lumbar disc herniation

K. Uesugi · M. Sekiguchi (✉) · S. Kikuchi · S. Konno
Department of Orthopaedic Surgery,
Fukushima Medical University School of Medicine,
1-Hikarigaoka, Fukushima City, Fukushima 960-1295, Japan
e-mail: miho-s@fmu.ac.jp

Introduction

Lumbar disc herniation (LDH) is a major cause of sciatica. The herniated disc induces sciatica by both mechanical and chemical means [17, 18, 25, 32–34]. Mechanical factors include compression of the nerve root and dorsal root ganglion (DRG) by the herniated disc [44, 45, 49]. Chemical irritation is caused by inflammatory mediators such as nucleus pulposus (NP) [27, 36, 37, 39, 40, 42], interleukin-1β, interleukin-6, 5-hydroxytryptamine, and tumor necrosis factor-alpha induced by herniated NP [2, 14, 16, 23, 34, 38]. Pain after injury to the nervous system (neuropathic pain) is a major chronic condition that remains difficult to treat. Chronic pain affects both psychological and social factors [13, 31]. The pain threshold decreases as a result of stress [47]. Thus, pain stemming from illness can be mitigated by reducing psychological stress [12]. In addition, the concentration of glucocorticoids in the blood increases in patients with chronic pain [24, 28]. Because psychosocial stress is often endured with

these conditions, and clinical observations suggest that stress increases susceptibility to developing pain and exacerbates existing pain, it is important to understand how stress affects the development and severity of neuropathic pain [6, 11, 31, 41, 46]. Clinical studies of the role of stress in the pathogenesis of chronic pain syndromes have implicated the hypothalamo-pituitary-adrenal axis [4, 29]. When an organism is exposed to stress, information about the stressful situation will reach an array of brain regions, including parts of the limbic system and areas involved in sensory processing. The output from these areas funnels through the nucleus paraventricularis of the hypothalamus, where it can give rise to activation of two hormone systems, that is, the rapid sympatho-adrenomedullar system and the slow-acting hypothalamo-pituitary-adrenal system. Activation of these systems leads to increased levels of adrenaline and corticosterone, respectively. Thus, increased plasma concentrations of corticosterone are a reflection of stress [15].

Although chronic stress might be regarded as an important factor of chronicity of pain in LDH, the influence of chronic stress in pain is unclear. The aim of this study was to investigate the effect of the restraint stress (RS) on pain-related behavior induced by the application of NP in rats. We also examined the release of endogenous corticosterone in plasma and the expression of activating transcription factor-3 (ATF-3) in DRG in a rat model of LDH.

Materials and methods

The experiment was carried out under the control of the Animal Care and Use Committee in accordance with the Guidelines for Animal Experiments of our institution and the Japanese Government Law Concerning the Protection and Control of Animals.

Animals and surgical procedure

A total of 210 adult female Sprague–Dawley rats (Japan SLC, Shizuoka, Japan) weighing 190–230 g were used. Animals were housed in plastic cages with free access to food and water. Rats were maintained under conditions of constant temperature (24 ± 2°C) and humidity (55 ± 15%) with a 12 h light–dark cycle (lights on at 7 h) for 7–8 days before starting the experiment.

Animals were anesthetized by intraperitoneal injection of 30 mg/kg sodium pentobarbital (Nembutal 50 mg/m; Abbott Laboratories, North Chicago, IL). Rats were placed in a prone position and an incision was made at the spinal midline at level L4–L6. Using a surgical microscope, the thoracolumbar fascia was incised along the left side of the supraspinous ligament for approximately 20 mm. The paraspinalis muscles were gently moved laterally to expose the left L5–L6 facet joint. The L5 nerve root, DRG, and spinal nerve were exposed by L5/6 partial laminectomy with great care taken to avoid trauma to tissue. In the NP group, autologous NP was harvested from the dorsal part of the tail. The harvested NP was 3 μL, and the mean weight of NP was 2.2 ± 0.3 mg ($n = 5$). NP was applied to the DRG and nerve root. Animals in the sham group underwent the same surgical procedure except for harvesting NP and application of autologous NP to the DRG.

The paraspinalis muscles of all rats were sutured and the skin was closed with metal clips. In the naive group, rats did not undergo surgery.

The surgical wound and physical condition of each animal was checked every day during the postoperative period. Postoperative analgesics were not used due to concerns about affecting the results.

Experimental groups

Rats were divided into six groups: NP group, NP + RS group, sham group, sham + RS group, naive group, and naive + RS group. RS commenced on the first day of the experiment. Rats receiving RS were placed individually into a wire mesh restrainer [26] (10 cm internal diameter, 20 cm length) for 6 h (08:00–14:00) daily from 1 day to 42 days after surgery. During RS, rats had the minimum amount of space in their restrainers to allow them to alter their posture. Rats that did not receive RS remained in the home cage.

Behavioral testing

Behavioral tests were performed in all groups ($n = 11$ in each group) during the day portion of the circadian cycle (16:00–18:00). All behavioral tests were performed by a technician who was unaware of the experimental groupings. Sensitivity to non-noxious mechanical stimuli was tested by the von Frey test. Baseline testing was performed 2 days before starting the experiment to accommodate animals with normal responses.

The hind paw withdrawal response to von Frey hair (North Coast Medical, Inc., Morgan Hill, CA) stimulation of the plantar surface of the footpads was determined at 1, 7, 14, 21, 28, 35, and 42 days after starting the experiment. The rats were placed individually into an acrylic cage with a mesh floor and allowed to acclimate for 15 min, until cage exploration and major grooming activities ceased. The lateral-plantar surface of the left hind paw, innervated by the L5 nerve [43], was stimulated with nine von Frey filaments (1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10.0, 15.0, and 26.0 g) threaded under the mesh floor. The grams for von Frey hairs were based on the manufacturer's ratings. Stimulation was initiated with the 1.0-g filament. The filament was

sequentially applied to the paw surface just until the filament bent, and was held for approximately 3 s. The response was considered positive if the hind limb indicated a lifting foot coupled with either licking or shaking of the foot as an escape response.

Determination of plasma corticosterone concentrations

Radioimmunoassay (RIA) measurements of plasma were performed in all groups at 21 and 42 days after surgery ($n = 9$ in each group). Because concentrations of plasma corticosterone have a circadian rhythm [9], blood samples were collected at 14:00–15:00 to avoid the influence of the circadian rhythm. Rats were anesthetized using 99% diethyl ether (Wako Pure Chemical Industries, Osaka, Japan). Before rats were killed by decapitation, blood samples (4.0 mL) were collected by heart puncture through a polyethylene tube (coated with heparin) and mixed with 1/10 volume of 1.5% disodium dihydrogen ethylenediamine tetracetate dehydrate (EDTA-2Na). To obtain platelet-poor plasma (PPP), samples were centrifuged at 3,000*g* for 10 min at room temperature. The supernatants (1,500 μL) were stored at −20°C until assayed. Corticosterone in PPP was measured by RIA. The lower detection limit of the method was 0.1 ng/mL.

Immunohistochemistry

Immunohistologic examinations were performed in all groups at 7, 14, 21, 28, and 42 days after starting the experiment ($n = 5$ at each time point for each group). Rats were anesthetized using 99% diethyl ether (Wako Pure Chemical Industries), and perfused with fresh 4% paraformaldehyde in 0.1% M phosphate-buffered saline (PBS), and the L5 DRG were removed. They were postfixed briefly in 4% paraformaldehyde and subsequently embedded in paraffin. Sections (6 μm) of DRGs were cut from each sample and placed on slides. Sections were deparaffinized with xylem and rehydrated with 100% ethanol. Nonspecific binding sites were blocked with 2% normal goat serum in PBS/Triton X-100 applied for 1 h at room temperature. Rabbit antibody to ATF-3 (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA) was applied for 2 h at room temperature. Sections were rinsed in PBS and incubated for 1 h at room temperature with goat anti-rabbit Alexa 488 (green) fluorescent antibody (1:200; Molecular Probes Inc., Eugene, OR). After rinsing, sections were mounted on microscope slides with VECTASHIELD® Mounting Medium with DAPI (H-1200, Vector, Burlingame, CA). DAPI (4′,6-diamidino-2-phenilindole) stains nuclei specifically, with little or no cytoplasmic labeling. Its blue fluorescence stands out in vivid contrast to green or red fluorescent probes of other structures. Fluorescent staining was analyzed using an Olympus Optical BX50 microscope equipped with imaging software (Axio Vision, Carl Zeiss, Gottingen, Germany). Two slices of each DRG were used to determine the numbers of ATF-3-immunoreactive (IR) neurons. The numbers of ATF-3- and DAPI-positive neurons were counted in each section. The percentage of ATF-3-positive cells in DAPI-positive neurons was calculated.

Statistical analysis

All data were reported as mean ± SD. Data of behavioral tests, comparisons of the plasma corticosterone levels, and immunohistologic examinations were analyzed using the Bonferroni test. *P* values less than 0.05 were considered significant.

Results

Behavioral tests

Rats in all groups showed stable conditions at baseline in response to mechanical stimulation. In the NP group, the mechanical withdrawal thresholds were significantly decreased for 21 days after surgery compared with the naive group ($p < 0.01$) (Fig. 1). In the NP + RS group, the mechanical withdrawal thresholds were significantly decreased for 42 days after surgery compared with the naive group ($p < 0.01$). There was a significant difference in withdrawal thresholds from day 28 to day 42 between the NP and NP + RS groups ($p < 0.01$). There were no significant differences of the thresholds among all groups except for the NP and NP + RS groups at 42 days.

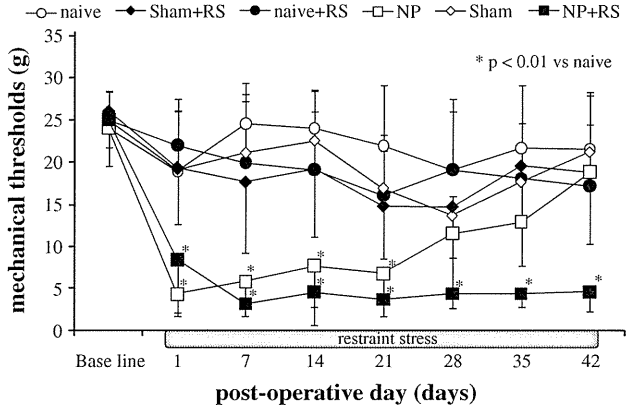

Fig. 1 Changes in mechanical withdrawal threshold of the footpad in rats. In the NP group, the mechanical withdrawal threshold was significantly decreased for 21 days after surgery compared with the naive group ($p < 0.01$). Significant differences between the NP + RS and naive groups can be observed for 42 days after surgery ($p < 0.01$). *$p < 0.01$ compared with the naive group

RIA analysis of plasma corticosterone

On day 21, concentrations of plasma corticosterone in the naive + RS, the sham + RS, the NP, and the NP + RS groups were significantly increased compared with the naive and sham groups ($p < 0.05$). There were no significant differences between the naive and sham groups (Fig. 2). Additionally, there were no significant differences between the NP and NP + RS groups. At 42 days after the start of the experiment, concentrations of plasma corticosterone in the three groups that underwent RS were significantly increased compared with the three groups that did not undergo RS ($p < 0.05$). Plasma corticosterone concentrations of the sham group and the NP group did not differ significantly from the naive group.

Immunohistochemical analysis of ATF-3

At 7 days after surgery, ATF-3-IR DRG neurons were observed in the NP and NP + RS groups. In contrast, there were few ATF-3-positive cells in the other four groups (Fig. 3). At 21 days after surgery, ATF-3-IR neurons were observed in the NP + RS group, but not in the NP group (Fig. 3). The ratio of ATF-3-IR neurons in the NP group was significantly increased compared with the sham and sham + RS groups at 7 days after surgery ($p < 0.01$). There were no differences of the ratio of ATF-3-IR cells among the NP, sham, and sham + RS groups from day 14 to day 42 (Fig. 4). In the NP + RS group, the ratio of ATF-3-IR neurons was significantly increased compared with the sham and sham + RS groups for 21 days after surgery ($p < 0.01$).

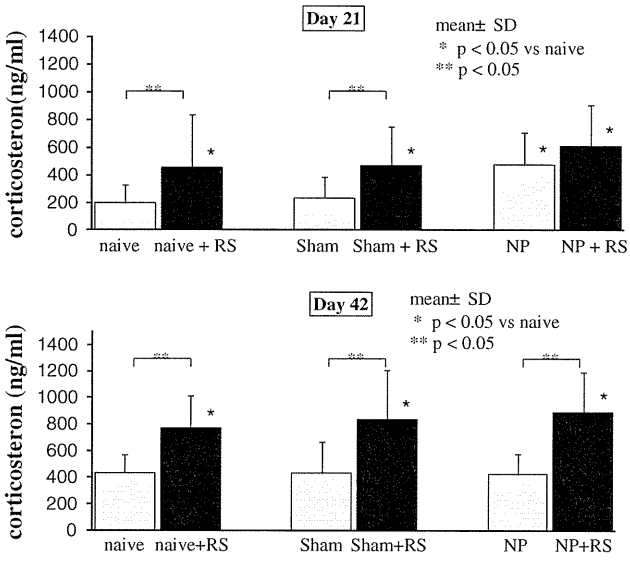

Fig. 2 Plasma corticosterone concentrations. At 21 and 42 days after surgery, concentrations of plasma corticosterone in the three groups that underwent restraint stress (RS) were significantly increased compared with the naive group ($p < 0.05$). *$p < 0.05$ compared with the naive group. At 42 days, concentrations of plasma corticosterone in the three groups that underwent RS were significantly increased compared with the three groups that did not undergo RS ($p < 0.05$)

Discussion

Rats subjected to RS alone showed no decrease in pain threshold, whereas NP rats subjected to RS showed a decrease in pain threshold that persisted for a long period. Plasma corticosterone concentrations increased in all groups that underwent RS. Plasma corticosterone concentrations also increased as a result of NP application, but this increase was temporary. In contrast, the increase in plasma corticosterone concentrations persisted for a long period with continual RS.

Corticosterone molecules reach all tissues, including the brain, readily penetrate the cell membrane, and interact with ubiquitous cytoplasmic/nuclear glucocorticoid receptors (GRs). Peripheral GRs play a significant role in the anti-inflammatory effects of corticosterone, which are mediated mainly through interactions between GRs and intracellular elements such as activating protein-1 at the site of tissue inflammation [30]. On the other hand, corticosterone could have neurotoxic effects contributing to neuronal damage [8]. Spinal neuronal GRs contribute to the development of neuropathic pain behaviors after chronic constriction injury [48]. It thus appears that rats experienced stress as a result of being restrained. In the present experiment, increased plasma corticosterone concentrations persisted and the pain threshold decreased during the same period as a result of RS in rats that received application of NP.

Stress-induced activation of the hypothalamo-pituitary-adrenal and sympathoadrenal axes exacerbates pain by enhancing the pronociceptive effects of immune mediators produced in peripheral tissue [19].

Recent studies have shown that corticosterone exacerbates neurogenic pain via GRs and NMDA receptors [1]. GRs appear to be intimately involved in the transmission of pain, as they are distributed extensively across the central nervous system and are expressed abundantly in the posterior horn of the spinal cord [3, 7]. In addition, GR expression increases with nerve damage [48, 50]. It is therefore possible that nerve damage increases the effect on nerve cells of corticosterone originating from stress.

In this study, expression of ATF-3, a marker of nerve damage, became prolonged as a result of RS. This finding suggests the possibility that nerve disorders originating from nerve damage are prolonged by corticosterone, although the mechanism of corticosterone involvement in the nerve disorder is not clear. Administration of high

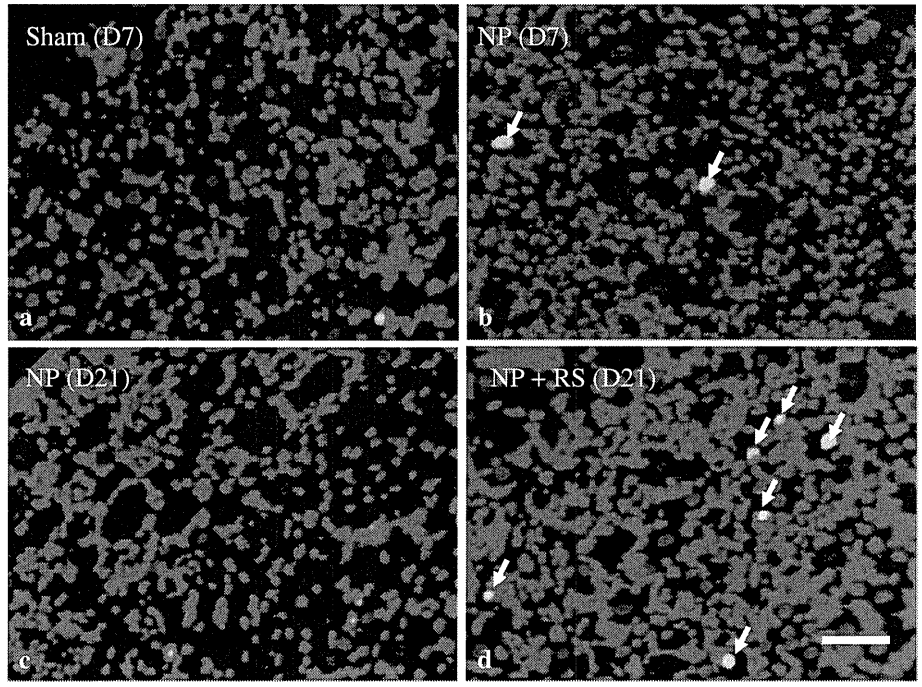

Fig. 3 Immunofluorescence analysis of ATF-3 in the left L5 DRG. Arrows indicate ATF-3–positive DRG neurons (*green*). In the sham group, few ATF-3-immunoreactive cells were seen in the L5 DRG (**a**). In the NP group, some ATF-3-positive cells were seen in the L5 DRG at 7 days after surgery (**b**). At 21 days after surgery, there were few ATF-positive cells in the NP group (**c**), but some ATF-3-positive cells were seen in the NP + RS group (**d**). *Scale bar* 25 μm

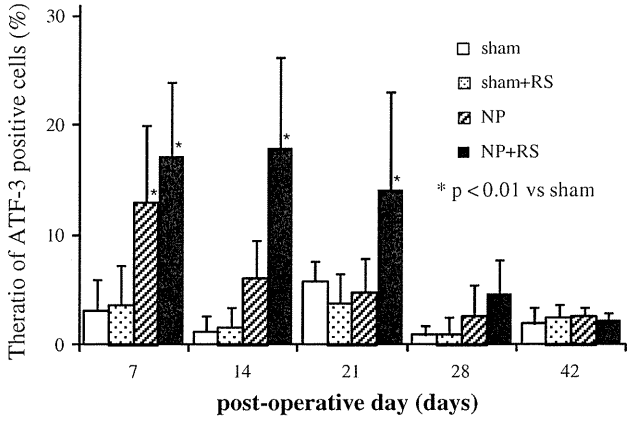

Fig. 4 The ratio of ATF-3-immunoreactive neurons to the total number of DRG neurons. In the NP + RS group, the ratio of ATF-3-positive cells was significantly increased during 21 days after surgery ($p < 0.01$). Data are mean ± SD ($n = 5$ for each group). *$p < 0.01$ compared with the sham group

concentrations of corticosterone during the acute phase of nerve damage is beneficial because corticosterone has anti-inflammatory and antiedematous actions on nerves [22]; however, acute phase administration of high concentrations of corticosterone were not investigated in the present study. These results imply that continued high plasma corticosterone concentrations play a major role in maintaining neurogenic pain during the chronic phase.

It has been reported that the pain threshold is reduced by stress in normal rats [5, 10, 20, 21]. We found no reduction in the pain threshold as a result of RS in either naive or sham rats, which had no damage to the nerves. However, reduction in the pain threshold persisted in rats in which neurogenic pain accompanying nerve damage was provoked by NP application. These results imply that stress can be a primary factor in pain-related changes becoming chronic.

The withdrawal threshold lasted longer after ATF-3 expression decreased in the NP with RS group. The nerve injury might be process for recovering at day 28, this time lag of ATF-3 expression and the reduction of threshold might show that a decrease of ATF-3 expression does not reflect the recovery of nerve function. Further studies are needed to investigate the effects on pain-associated substances such as substance P, CGRP, and cytokines, with and without RS. In addition, pain thresholds were examined for only 42 days during RS; however, pain-related changes over a longer period and after stopping RS were not investigated in the present study. In addition, this study focused on NP application as a chemical factor. Another nucleus pulposus application model included disc herniation without compression did not show allodynia [35]. On the other hand, the NP model in the present study showed allodynia [27, 37, 38, 40, 41, 43]. This model applied a consistent volume of NP and covered the DRG in each animal. Therefore, the two models are different and the disc incision model might have less of an influence on the nerve. I the model of this study, the same volume of NP was applied to the DRG, and has been done in other studies [27, 37, 38, 40, 41, 43]. Therefore, we consider our model to be that which investigates a chemical factor associated with disc herniation. Future studies should compare whether the impact of RS is affected by pain-related changes

induced by mechanical factors alone, chemical factors alone, or both as well as study the mechanism of corticosterone involvement in pain-related changes resulting from the application of NP. If the mechanism through which stress and corticosterone accompanying stress lead to chronicity were clarified, stress itself and corticosterone could possibly become targets for chronic pain therapy.

Conclusion

Increased plasma corticosterone concentrations persisted as a result of RS in naive, sham, and NP animals. In addition, a reduced pain threshold persisted as a result of RS in rats that received application of NP. These results suggest that chronic stress may prolong pain-related behavior.

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Conflict of interest None.

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CLINICAL REPORT

Use of rocuronium–sugammadex, an alternative to succinylcholine, as a muscle relaxant during electroconvulsive therapy

Hiroko Hoshi · Yuji Kadoi · Jiro Kamiyama · Akiko Nishida · Hiroyuki Saito · Masaki Taguchi · Shigeru Saito

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Abstract We compared the recovery time from neuromuscular blockade induced by rocuronium combined with sugammadex versus succinylcholine during electroconvulsive therapy (ECT). Anesthesia was induced using propofol, followed by succinylcholine (1 mg/kg) or rocuronium (0.6 mg/kg). Immediately after the seizure stopped, 16 mg/kg sugammadex was infused. Neuromuscular monitoring was performed and continued until recovery of the train-of-four ratio to 0.9. We compared the recovery time of T1 to 10 and 90% between groups. Patients were also assessed for clinical signs, such as time to first spontaneous breath from the administration of muscle relaxant and eye opening to verbal commands. Although recovery time of T1 to 10 and 90% in the rocuronium–sugammadex group was shorter than in the succinylcholine group, the difference was not statistically significant. Further, the seizure duration with succinylcholine (33 ± 8 s) was shorter than that with rocuronium–sugammadex (39 ± 4 s). In conclusion, this study demonstrates the potential benefit of use of rocuronium–sugammadex as an alternative to succinylcholine for muscle relaxation during ECT.

Keywords Electroconvulsive therapy · Muscle relaxant · Rocuronium · Sugammadex · Succinylcholine

H. Hoshi · Y. Kadoi (✉) · J. Kamiyama · A. Nishida · H. Saito · M. Taguchi · S. Saito
Department of Anesthesiology, Gunma University, School of Medicine, 3-39-22 Showa-Machi, Maebashi, Gunma 371-8511, Japan
e-mail: kadoi@med.gunma-u.ac.jp

Introduction

Succinylcholine is commonly used as a muscle relaxant during electroconvulsive therapy (ECT) because of its rapid onset and short duration of action [1]. However, succinylcholine has many side effects, such as myalgia, a small increase in plasma potassium and increase in intragastric and intra-ocular pressures [1].

Sugammadex has recently been introduced as a fast-acting, selective relaxant-binding agent that was specifically designed to rapidly reverse rocuronium-induced neuromuscular blockade. Lee et al. [2] reported that reversal of profound, high-dose rocuronium-induced neuromuscular block with 16 mg/kg sugammadex was significantly faster than spontaneous recovery from succinylcholine.

We compared recovery from neuromuscular blockade induced by rocuronium–sugammadex versus that induced by succinylcholine in five patients presenting for ECT.

Case description

Informed consent was obtained from patients or their families. All protocols were approved by the local institutional clinical study committee and the institutional review board. Five patients who were scheduled to undergo ECT were studied. None of the patients had a history of cardiovascular disease.

Anesthetic management

The patients underwent at least 10 sessions of ECT (three times per week at 1- or 2-day intervals). To avoid induction of the parasympathetic reflex, the patients received

atropine sulfate (0.01 mg/kg IM) 30 min prior to the ECT procedure.

Measured parameters during the procedure included blood pressure (BP), heart rate, oxygen saturation (SpO_2; measured by pulse oximetry on the left index finger), end-expiratory partial pressure of carbon dioxide (end-tidal CO_2) at the nostrils (Capnomac UltimaTM; Datex Co, Ltd., Helsinki, Finland) and electrocardiogram (ECG; lead II). Measurements were initiated prior to ECT and were terminated at the end of the procedure.

Anesthesia was induced using propofol (1.0 mg/kg intravenously over 5 s), followed by either succinylcholine (SCC) (1 mg/kg intravenously) or rocuronium (0.6 mg/kg intravenously) over 5 s, followed by a 10-ml saline bolus. Assisted mask ventilation was initiated with 100% oxygen. After T1 was assessed as being zero by neuromuscular monitoring, an electroshock stimulus was applied bilaterally for 5 s.

End-tidal CO_2 was maintained at 30–35 mmHg and the SpO_2 value was maintained above 98% by manual mask assistance throughout the therapy. For patients who were given rocuronium, 16 mg/kg sugammadex was infused with a 10-ml saline bolus immediately after the seizure stopped.

During the first and second ECT sessions, we confirmed that 1 mg/kg of propofol and 1 mg/kg of SCC could provide adequate anesthetic conditions and muscle relaxation to all patients. In addition, the intensity of the ECT stimulus required to achieve a minimum seizure duration of more than 20 s was determined during these sessions.

Patients received 1 mg/kg of SCC as the muscle relaxant agent for three of the subsequent ECT sessions and 0.6 mg/kg of rocuronium during the remaining four ECT sessions. Muscle relaxant selection was made in a non-blinded manner. Electroencephalographic (EEG) seizure duration was recorded by a two-channel EEG after the electrical stimulus.

Neuromuscular assessment

Neuromuscular monitoring was performed using the TOF-watch SX (Organon, Roseland, NJ, USA). The tibial nerve in the leg was supramaximally stimulated at the inferolateral aspect of the medial malleolus with square pulses of 0.2-ms duration, delivered as train-of-four pulses, at intervals of 15 s. The resulting contractions of the great toe muscles were quantified by an acceleromyographic monitor. Calibration was performed and baseline responses were recorded after propofol administration and before muscle relaxant administration. Neuromuscular monitoring was continued until recovery of the train-of-four ratio to 0.9. Following the protocol of Lee et al. [2], we compared the time to recovery of T1 to 10 and 90% between relaxants. T1 was zero in all patients when sugammadex was administered.

Patients were also assessed for clinical signs such as the time interval between the first spontaneous breath and administration of muscle relaxant and the time to opening of eyes to verbal commands.

All data are expressed as means ± standard deviation (SD). Paired *t* test was used for the comparisons. Calculations were performed by Stat View 5.0 software (Abacus, Concepts, Berkeley, CA, USA).

Table 1 shows the comparison between the effects of succinylcholine and rocuronium–sugammadex in terms of time from the start of administration of neuromuscular blocking agent to T1 zero, to recovery of T1 to 10% and to recovery of T1 to 90%. Although there was a tendency to a shorter time of onset of muscle relaxant action with succinylcholine compared with that with rocuronium, no significant differences in T1 0% were found between the two relaxants. Further, although there was a tendency to shorter time to recovery of T1 to 10 and 90% with rocuronium–sugammadex as compared to succinylcholine, the difference was not statistically significant.

Table 1 Time from commencement of administration of neuromuscular blocking agent to T1 zero, recovery of T1 to 10% and recovery of T1 to 90%

	Height (cm)	Weight (kg)	T1 0% (s)		Recovery of T1 to 10% (s)		Recovery of T1 to 90% (s)	
			SCC	Ro + Sugam	SCC	Ro + Sugam	SCC	Ro + Sugam
Case 1 (62 years, F)	152	40.9	155	277	507	638	565	664
Case 2 (64 years, M)	165	52.0	67	97	305	173	532	387
Case 3 (53 years, F)	149	57.5	105	135	285	221	540	428
Case 4 (67 years, M)	165	51.0	85	112	484	213	694	278
Case 5 (68 years, M)	165	65.5	148	171	537	267	549	390
Means ± SD	159 ± 8	53.3 ± 9.0	112 ± 38	158 ± 71	423 ± 119	302 ± 190	576 ± 67	429 ± 142
P value			0.07		0.26		0.07	

SCC Succinylcholine, *Ro + Sugam* Rocuronium + Sugammadex

Table 2 Measures of seizure duration, time to first spontaneous breath and eye opening to verbal commands

	Seizure duration (s)		Time to first spontaneous breath (s)		Eye opening to verbal commands (s)	
	SCC	Ro + Sugam	SCC	Ro + Sugam	SCC	Ro + Sugam
Case 1	40	43	234	410	535	675
Case 2	38	41	262	374	471	549
Case 3	39	44	250	208	480	330
Case 4	22	33	189	131	697	224
Case 5	27	36	472	257	543	529
Means ± SD	33 ± 8	39 ± 4	281 ± 110	276 ± 115	545 ± 90	461 ± 181
P value	0.01		0.94		0.48	

SCC Succinylcholine, *Ro + Sugam* Rocuronium + Sugammadex

Table 2 compares the effects of succinylcholine and rocuronium–sugammadex administration in terms of seizure duration, time to first spontaneous breath and time to eye opening in response to verbal commands. Seizure duration with succinylcholine was shorter than that with rocuronium–sugammadex. No significant differences in time to first spontaneous breath and eye opening in response to verbal commands were found with the two muscle relaxants.

Table 3 shows the time from administration of sugammadex to recovery of T1 to 10%, to 90% and time to first spontaneous breath with administration of rocuronium–sugammadex. Quick recovery from the muscle relaxant effect was found with rocuronium–sugammadex.

All patients included in this study had normal renal function, as shown by normal ranges of BUN and plasma creatinine. No adverse effects, such as nausea, vomiting or headache, were found with either relaxant. In addition, no recurarization was seen in any of the patients treated with rocuronium–sugammadex.

Table 3 Time from administration of sugammadex to recovery of T1 to 10%, T1 to 90% and time to first spontaneous breath

	Recovery of T1 to 10% (s)	Recovery of T1 to 90% (s)	Time to first spontaneous breath (s)
Case 1	308	334	43
Case 2	23	237	19
Case 3	30	150	30
Case 4	57	122	47
Case 5	50	173	47
Means ± SD	93 ± 120	203 ± 84	37 ± 12

Discussion

We showed similar efficacy with rocuronium–sugammadex as compared to succinylcholine for muscle relaxation during ECT.

Use of succinylcholine is associated with a variety of adverse events and contraindications [3]. In addition, succinylcholine is thought to be a potent trigger for malignant hyperthermia (MH) [3]. Although several ultrashort acting non-depolarizing muscle relaxants have been developed, none of these have a shorter duration of action than succinylcholine [1, 4–7].

While Trollor et al. [8] reviewed the possible safety of the use of succinylcholine in cases with neuroleptic malignant syndrome, some researchers examined other neuromuscular agents, such as vecuronium [9, 10], mivacurium [4, 7, 11], rapacuronium [6] and rocuronium [5] during ECT. Kelly et al. [11] showed the safety of mivacurium as an alternative to succinylcholine. In contrast, Cheam et al. [4] reported that a low dose of mivacurium was less effective than succinylcholine. Another report [9] that examined the safety of vecuronium reported that vecuronium shortened the seizure duration and prolonged anesthetic time.

There is only one report evaluating the effects of rocuronium versus succinylcholine on clinical recovery from ECT [5]. Turkkal et al. [5] reported that although the time to first spontaneous breath was longer in the rocuronium group than in the succinylcholine group, no significant differences were detected between the two groups in terms of eye opening, head lift or tongue depressor testing. However, the dosage of rocuronium used in the study of Turkkal et al. [5] was relatively small (0.3 mg/kg), which is thought to be inadequate for muscular paralysis. Rocuronium (0.6–1.2 mg/kg) typically produces complete neuromuscular block within 2 min, as compared with an average of 1 min with 1 mg/kg succinylcholine [12]. However, at this dose, rocuronium has a longer duration of action, making it inappropriate for use in ECT where rapid recovery of neuromuscular function is required. Hence, we selected a clinically more commonly used dosage of rocuronium of 0.6 mg/kg.

Lee et al. [2] compared the time required for sugammadex reversal of profound rocuronium-induced neuromuscular block with time to spontaneous recovery after succinylcholine. In their study, 1.2 mg/kg rocuronium or

1 mg/kg succinylcholine was used for blockade of neuromuscular transmission and facilitation of tracheal intubation. Sugammadex (16 mg/kg) was administered 3 min after rocuronium administration. Mean times to recovery of T1 to 10 and 90% were significantly faster in the rocuronium–sugammadex group as compared with the succinylcholine group. Hence, they concluded that reversal of profound high-dose rocuronium-induced neuromuscular block (1.2 mg/kg) with 16 mg/kg sugammadex was significantly faster than spontaneous recovery from 1 mg/kg succinylcholine. This report implies that the rocuronium–sugammadex combination may be useful for inducing muscle paralysis during ECT. Our study indicated possible equipotent effects of rocuronium- and succinylcholine-induced neuromuscular block for muscle relaxation.

Certain other factors should be considered while analyzing our results.

First, we previously showed that the onset and duration of muscle relaxants were greatly influenced by cardiac output before injection [13]. Hence, the onset and duration of muscle relaxation in each of our patients may have been affected by their individual cardiac outputs.

Second, in this study, sugammadex was infused after the end of the seizure. Puhringer et al. [12] reported that the timing of administration of sugammadex might influence the reversal of profound rocuronium-induced neuromuscular blockade. Thus, it is possible that our results were affected by the timing of administration of sugammadex.

The mechanism of the longer seizure duration with ECT following rocuronium–sugammadex administration as compared to that with SCC administration is unknown. Small differences in the hyperventilation status before electrical stimulation between the two groups might have some effect on seizure duration [1]. Another possibility is that the number of sessions of ECT might have affected the seizure duration because of improvement in the depressive condition resulting from ECT [1].

The dose of 0.6 mg/kg of rocuronium used in this study was half the dosage used by Lee et al. [2]. However, a dose of 16 mg/kg of sugammadex was used as the neuromuscular antagonist in this study. Reportedly, a dose of at least 4–8 mg/kg of sugammadex is needed for recovery from deep neuromuscular blockade, indicated as a post-tetanic count of 1–2 on the TOF monitor [14]. We believe that more profound neuromuscular blockade was induced in our study group by administration of sugammadex as compared to this previous study. In addition, we were afraid of the risk of recurarization with use of a small dose of sugammadex. Hence, a dose of 16 mg/kg of sugammadex was used as the neuromuscular antagonist in this study.

We measured variables only twice during ECT. With subsequent ECT sessions, patients require larger doses of propofol to achieve unconsciousness due to improvement in the depressive condition induced by ECT. Hence, the propofol dosage could have greatly influenced seizure duration and hemodynamic changes induced by ECT in this study. In addition, improvement in the depressive condition by ECT could have led to the anti-depressant agent being changed, which could also have affected the hemodynamic changes induced by ECT.

For unknown reasons, there were some differences in the time to recovery of T1 to 10 and 90% and the time to the first spontaneous breath between case one and the others. One possible speculation for this difference is that case 1 exhibited differential effects to the non-depolarizing neuromuscular agents because of undetectable degeneration, demyelination or axon loss in the motor nerve ending of the neuromuscular junction and infarction or atrophy of the skeletal muscle. This could partly explain why almost twice the time was needed for the recovery of T1 to 10 and 90% in this patient. Another possibility is that the onset and duration of muscle relaxants are greatly influenced by cardiac output before injection [13] be responsible for these differences observed in case 1.

Although the cost of sugammadex may preclude its routine use for ECT, rocuronium–sugammadex may be useful for muscle relaxation during ECT in patients in whom the use of succinylcholine is contraindicated, such as those with severe osteoporosis, amyotrophic lateral sclerosis and a history of neuroleptic malignant syndrome [7].

In conclusion, we demonstrated the potential efficacy of rocuronium–sugammadex as an alternative to succinylcholine for muscle relaxation during ECT.

Conflict of interest None.

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The influence of manipulations to alter ambient GABA concentrations on the hypnotic and immobilizing actions produced by sevoflurane, propofol, and midazolam

Koichi Nishikawa [a,*], Kazuhiro Kubo [a], Hideaki Obata [a], Yuchio Yanagawa [b], Shigeru Saito [a]

[a] Department of Anesthesiology, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi City 371-8511, Japan
[b] Department of Genetic and Behavioral Neuroscience, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi City 371-8511, Japan

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ABSTRACT

Recent studies have suggested that extrasynaptic $GABA_A$ receptors, which contribute tonic conductance, are important targets for general anesthetics. We tested the hypothesis that manipulations designed to alter ambient GABA concentrations (tonic conductance) would affect hypnotic (as indicated by loss of righting reflex, LORR) and immobilizing (as indicated by loss of tail-pinch withdrawal reflex, LTWR) actions of sevoflurane, propofol, and midazolam. Two manipulations studied were 1) the genetic absence of glutamate decarboxylase (GAD) 65 gene (GAD65−/−), which purportedly reduced ambient GABA concentrations, and 2) the pharmacological manipulation of GABA uptake using GABA transporter inhibitor (NO-711). The influence of these manipulations on cellular and behavioral responses to the anesthetics was studied using behavioral and electrophysiological assays. HPLC revealed that GABA levels in GAD65−/− mice were reduced in the brain (76.7% of WT) and spinal cord (68.5% of WT). GAD65−/− mice showed a significant reduction in the duration of LORR and LTWR produced by propofol and midazolam, but not sevoflurane. NO-711 (3 mg/kg, ip) enhanced the duration of LORR and LTWR by propofol and midazolam, but not sevoflurane. Patch-clamp recordings revealed that sevoflurane (0.23 mM) slightly enhanced the amplitude of tonic GABA current in the frontal cortical neurons; however, these effects were not strong enough to alter discharge properties of cortical neurons. These results demonstrate that ambient GABA concentration is an important determinant of the hypnotic and immobilizing actions of propofol and midazolam in mice, whereas manipulations of ambient GABA concentrations minimally alter cellular and behavioral responses to sevoflurane.

1. Introduction

The GABAergic system in the central nervous system (CNS) is a key target of general anesthetics (Mihic et al., 1997; Sonner et al., 2003; Rudolph and Antkowiak, 2004; Hemmings et al., 2005; Franks, 2006). Two types of GABAergic inhibition are known; a phasic form (phasic inhibition) regulating neural excitability via the activation of postsynaptic $GABA_A$ receptors by intermittent GABA release from presynaptic terminals, and a persistent tonic form (tonic inhibition) generated by continuous activation of extrasynaptic $GABA_A$ receptors by low concentrations of ambient GABA (Brickley et al., 1996). Growing evidence suggests that tonic inhibition mediated by extrasynaptic $GABA_A$ receptors might contribute to the actions of intravenous anesthetics such as propofol (Bai et al., 2001; Bieda and MacIver, 2004). These extrasynaptic $GABA_A$ receptors have different pharmacological and kinetic properties compared with synaptic $GABA_A$ receptors, as a result of the distinct subunit compositions (Glykys and Mody, 2007). Given that extrasynaptic $GABA_A$ receptors respond to low ambient levels of GABA, manipulations of ambient GABA concentrations may affect cellular and behavioral responses to general anesthetics.

Two manipulations studied were 1) the genetic absence of glutamate decarboxylase (GAD) 65 gene (GAD65−/−), and 2) the pharmacological manipulation of GABA uptake using GABA transporter inhibitor. GAD is the only synthetic enzyme responsible for the conversion of L-glutamic acid to GABA. The brain contains two forms of GAD, which differ in molecular size, amino acid sequence,

Abbreviations: ACSF, artificial cerebrospinal fluid; GABA, γ-aminobutyric acid; mIPSC, miniature inhibitory postsynaptic current; GAD, glutamate decarboxylase; GAT, GABA transporter; LORR, loss of righting reflex; LTWR, loss of tail-pinch withdrawal response; NO-711, 1-[2-[[(Diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride ($C_{21}H_{22}N_2O_3 \cdot HCl$).

* Corresponding author. Tel.: +81 27 220 8454; fax: +81 27 220 8473.
E-mail address: nishikaw@med.gunma-u.ac.jp (K. Nishikawa).

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antigenicity, cellular and subcellular locations, and interaction with the GAD cofactor pyridoxal phosphate (Erlander et al., 1991). The 67-KDa isoform (GAD67) is found mainly in the cell body, whereas GAD65 is localized to the nerve terminal and is reversibly bound to the membrane of synaptic vesicles (Namchuk et al., 1997). GAD65−/− mice remain viable without apparent anatomical deficits and postsynaptic $GABA_A$ receptor density is unchanged (Kash et al., 1997), although the survival rate of GAD65−/− mice was slightly reduced with age, largely due to spontaneous seizures (Stork et al., 2000). As a result of reduced GABAergic tone, GAD65−/− mice appear to show increased anxiety levels (Kash et al., 1999; Kubo et al., 2009a), different sensitivity to pentobarbital (Kash et al., 1999), and hyperalgesia to thermal, but not chemical, stimulation (Kubo et al., 2009b). On the other hand, inhibition of GABA uptake and/or metabolism is a strategy for enhancing ambient GABA concentrations. GABA is cleared from the synaptic cleft by specific, high-affinity, sodium- and chloride-dependent transporters, which are thought to be located on presynaptic terminals and surrounding glial cells, i.e., four distinct GABA transporters, GAT-1, GAT-2, GAT-3 and BGT-1 (Borden, 1996). NO-711, a potent and selective GAT-1 inhibitor, was used because GAT-1 is responsible for the majority of neuronal GABA transport.

We have reported that sevoflurane enhances GABAergic inhibition (Nishikawa and MacIver, 2001; Nishikawa and Harrison, 2003; Nishikawa et al., 2005), suggesting that $GABA_A$ receptor is one of the plausible molecular targets. In addition, several targets have been also proposed for inhalational general anesthetics; glycine receptors (Mascia et al., 1996), two-pore-domain potassium channels (Sirois et al., 2000), NMDA receptors (Sonner et al., 2003), HCN channels (Chen et al., 2005), and some subtypes of sodium channels (Wu et al., 2004), whereas a specific point mutation in $GABA_A$ receptor is critical for propofol and etomidate (Jurd et al., 2003). These data suggest that the relative contributions of GABAergic inhibition to *in vivo* anesthetic actions are different between sevoflurane and intravenous anesthetics. We first tested the hypothesis that genetic and pharmacological manipulations to alter ambient GABA concentrations would affect loss of righting reflex (LORR), a surrogate measure of hypnosis, and loss of tail-pinch withdrawal reflex (LTWR), a measure of immobilization, produced by sevoflurane, propofol, and midazolam. We then studied the influence of these manipulations on *in vitro* sevoflurane actions on membrane properties of frontal cortical layer V neurons using patch-clamp methods. The present study provides evidence that genetic and pharmacological manipulations to alter ambient GABA concentrations (tonic conductance) affect the response to propofol and midazolam, but minimally affect the actions of sevoflurane.

2. Materials and methods

2.1. Mice

All animal procedures and protocols used in this study were approved by the Animal Care Committee of Gunma University Graduate School of Medicine (protocol # 05-71) and performed through NIH guidelines for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

The generation of glutamate decarboxylase 65 (GAD65) knockout mice used in the present study was described by Yanagawa et al. (1999) and Yamamoto et al. (2004). In brief, we designed a targeting vector to disrupt most of the open reading frame by inserting an in-frame stop codon in the exon 3. The linearized targeting vector was introduced by electroporation into E14.1 embryonic stem (ES) cell derived from strain 129/Ola mice, and we obtained ES cell clones carrying the GAD65 targeted mutation through homologous recombination. The correctly targeted ES cells were injected into C67BL/6J mouse (CLEA Japan, Inc., Tokyo, Japan) blastocysts to make chimeras. The chimeric male mice were mated with female C57BL/6J, and germ-line transmission was achieved. The resultant GAD65 heterozygous (+/−) mice were backcrossed for more than ten generations onto the C57BL/6J background. Wild-type (+/+) and knockout (−/−) littermates were produced from heterozygous mating pairs. GAD65−/− mice were viable and fertile and gross behaviors appeared to be normal without apparent anatomical deficits. Adult (12–16-weeks old) male WT mice and GAD65−/− mice weighing 23–27 g were used for experiments. Mice were group-housed in a pathogen-free transgenic facility, and water and food were available *ad libitum*. None of the animals were used for more than two experiments and at least 1 week was allowed for the mice to recover.

2.2. Measurement of neurotransmitter contents

For analysis of neurotransmitter tissue content, WT mice and GAD65−/− mice at 12 weeks of age were sacrificed by decapitation under deep sevoflurane anesthesia. Tissue samples of the whole brain and the whole spinal cord were removed quickly and tissue weight was measured. The tissue was added to 3–5 ml of saline (saline volume was approximately ten folds of tissue weight), and then homogenized in phosphate-buffered saline (PBS) containing 0.2% protease inhibitor using a polytron homogenizer (24,000 rpm, 15 s, 2–3 times). Following removal of cell debris by centrifugation at 3000 rpm (20 min, 4 °C), the supernatant (500 μl), which was added to sulfosalicylic acid (750 μl), was centrifuged again at 3000 rpm (20 min, 4 °C). The supernatant after pH adjustment was analyzed using high-performance liquid chromatography (HPLC) and fluorescence detection. HPLC was performed by the company (SRL, Tokyo, Japan). Neurotransmitter content (nmol/g) was calculated as follows: measured neurotransmitter concentration (nmol/ml) × saline volume added (ml)/tissue weight (g).

2.3. Behavioral assays for intravenous drugs

Loss of righting reflex (LORR) was used as a surrogate measure for hypnosis. Each animal was received an intraperitoneal (ip) injection of propofol (Maruishi Pharmaceuticals Co, Ltd., Osaka, Japan) or midazolam (Astellas Pharma Inc., Tokyo, Japan) with a volume of 10 μl/g of body weight, and then placed on their backs in a chamber (20 × 28 × 15 cm). The ability to right themselves was evaluated as described (Kubo et al., 2009a). Because we have previously reported that GAD65−/− mice showed altered responses to propofol (100 mg/kg, ip) (Kubo et al., 2009a), propofol (125 mg/kg, ip) was tested in the present study. Midazolam (50 mg/kg, ip) was used as described previously (Quinlan et al., 1998). Mice were judged to have lost this reflex when unable to right itself within 10 s. The time from ip injection of the drug to LORR was considered as the latency, and the time between the LORR and the time mice regained the ability to right themselves within 2 s was considered the duration of LORR. Loss of tail-pinch withdrawal response (LTWR) was used as a surrogate measure for immobilization (Quasha et al., 1980). A surgical spring clip (6 mm in size, Applied Medical, CA, USA) was placed at the base of an animal's tail for 5 s.

Vehicle solutions for behavioral studies were as follows: propofol, lipofundin MCT/LCT 10% (B. Braun Melsungen AG, Melsungen, Germany); midazolam, saline. An intraperitoneal injection of lipofundin MCT/LCT 10% (10 μl/g) alone had no hypnotic/analgesic effect on mice behavior ($n = 5$). NO-711 hydrochloride (Sigma-Aldrich Japan, Tokyo, Japan), a potent and selective GAT-1 inhibitor that cross the blood–brain barrier (Borden et al., 1994), was diluted in sterile saline and injected 20-min prior to experiments (a volume of 10 μl/g of body weight). Other drugs were purchased from Sigma–Aldrich Chemicals (Tokyo, Japan).

2.4. Behavioral studies of sensitivity to sevoflurane

Mice were placed into a sealed Plexiglas chamber (32 × 32 × 22 cm), warmed by heating pads from below. After 20-min equilibration period with a chosen concentration of sevoflurane (0.5–5.0% atm, Maruishi, Osaka, Japan) delivered via an anesthetic-specific vaporizer (Sevotec 5, Ohmeda, UK) with fresh air flow at a rate of 3.0 l/min, a blinded observer scored the mice for LORR and LTWR in a quantal fashion. Sevoflurane concentration was continuously controlled by the infrared gas analyzer (BP-508, Nippon Colin Co. Ltd., Tokyo, Japan). In LORR assays, mice were judged to have lost righting reflex when unable to right itself within 10 s. In LTWR assays, movement to tail-pinch was tested by the placement of the surgical clip at the base of an animal's tail for 5 s. If any movement to tail-pinch was detected, the concentration of sevoflurane was increased for another 20-min equilibration period, and the response was tested again. The concentration at which the mouse lost its tail-pinch reflex was noted. Sevoflurane concentration also was confirmed by gas chromatograph analysis (GC-4000, GL Sciences Inc., Tokyo, Japan) of samples drawn from the chamber.

2.5. Electrophysiology

The methods of brain slice electrophysiology were described previously (Nishikawa et al., 2005; Ishizeki et al., 2008). Briefly, mice were decapitated under deep isoflurane anesthesia, and the brain was then removed and immediately immersed in a cold (1–4 °C) modified Ringer solution, comprised of 234 mM sucrose; 2.5 mM KCl; 1.25 mM NaH_2PO_4; 10 mM $MgSO_4$; 0.5 mM $CaCl_2$; 26 mM $NaHCO_3$; and 11 mM glucose saturated with 95% O_2 and 5% CO_2. A block of tissue

containing the frontal cortex was quickly dissected out and glued to a DTK-1000 vibratome tray (Dosaka EM, Tokyo, Japan) using oxygenated cold modified Ringer solution. Slices (500 μm) were cut from the brain and then kept in the pre-chamber (Brain Slice Chamber System; Harvard Apparatus, Holliston, MA) filled with artificial cerebrospinal fluid (ACSF) consisting of (in mM), 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 11 glucose, bubbled with 95% O_2 and 5% CO_2 at room temperature (22–24 °C). Slices were allowed 1 h for recovery in the pre-chamber, which was designed to keep 8–12 slices viable for several hours.

Slices were transferred to a recording chamber (1.0 ml in volume, Brain Slice Chamber System, Harvard Apparatus, Inc., Holliston, MA) perfused with an oxygenated ACSF at a rate of approximately 3 ml/min. Patch electrodes were made from borosilicate thin-walled capillaries (GDC-1.5, Narishige Co., Ltd., Tokyo, Japan). Recording electrodes (5–7 MΩ) were filled with Cs_2SO_4-based solution (Cs_2SO_4 110 mM, TEA 5 mM, $CaCl_2$ 0.5 mM, $MgCl_2$ 2 mM, EGTA 5 mM, HEPES 5 mM and MgATP 5 mM; pH 7.2) to investigate spontaneous IPSCs at a holding potential of 0 mV. The liquid junction potential in these conditions was approximately 9 mV, and all data presented were corrected using this value. K-gluconate solutions (K-gluconate 100 mM, EGTA 10 mM, HEPES 40 mM, $MgCl_2$ 5 mM, ATP 2 mM, GTP 0.3 mM, pH 7.25.) were used for current clamp recordings, so the impermeable ion (gluconate) would not contribute to anesthetic-induced changes in resting membrane potential or current–voltage relations. Whole cell patch-clamp recordings were made from visualized layer V pyramidal neurons in the frontal cortex using an upright Axioskop2 FS plus microscope (Zeiss, Jena, Germany). The magnified image was collected by an intensified CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) with contrast enhancement. The image of neurons was displayed on a video monitor, and glass patch pipettes were visually advanced using a micromanipulator (MWO3, Narishige Co., Ltd., Tokyo, Japan) through the slice to the surface of the neuron. Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA) was used for whole cell recordings. Whole cell currents were filtered at 2–5 kHz and digitized at 10 kHz (Digidata 1322A, Axon Instruments Inc.) and stored on a Pentium-based PC and paper recorder for later analysis. The GABAergic nature of the synaptic currents was verified by applying the $GABA_A$ receptor antagonist, picrotoxin (PIC). Series resistances were between 10 and 25 MΩ, and were then compensated approximately 80%. Recordings were performed at room temperature (22–24 °C).

In some experiments using WT mice, NO-711 (3 mg/kg) was injected intraperitoneally 20 min before decapitation. It was confirmed, 10 min later, that NO-711 prolonged the latency to jumping or licking responses in the hot-plate test (53 °C) (Kubo et al., 2009b), and then brain slices were made as described above. The effects of NO-711 in the slice remained effective for several hours, since the amplitude of tonic inhibition was larger than that of WT mice without NO-711 injection ($n = 5$). Thus, *in vitro* experiments with NO-711 were performed within 4 h after making slice preparations. In separate experiments, NO-711 was directly added to the ACSF solution that was bathing the slice in the recording chamber. However, in this case, it was difficult to determine the concentration that should be used to match the dose injected in behavioral studies. Judging from the influence on tonic inhibition, we estimated that 1–2 μM NO-711 were relevant concentrations.

2.6. Data analysis

Data acquisition and analysis were performed with pCLAMP software version 8.1 (Axon instruments Inc., Union City, CA) and IGOR Pro version 5.0 (WaveMetrics, Lake Oswego, OR). Synaptic currents were defined as current deflections with a fast rising phase and a relatively slower decay phase. The rise time was defined as time interval between 10% and 90% of the peak amplitude and synaptic currents having the rise time <2 ms were included for analysis. The amplitude of synaptic current was measured from the initial inflection point (not from the baseline) to the peak, to avoid the effects of summation on amplitude distribution. Threshold-level crossing were set at approximately three folds of baseline noise, which was measured during the period of no detectable events. As a result, synaptic currents larger than 6 pA in the amplitude were counted for analysis. This definition eliminated the infrequently observed single channel events or synaptic currents with slow rise time, but successfully detected most IPSCs. The decay phase was fitted with a single exponential curve and a time from peak to 1/e was defined as the decay time.

2.7. Application of sevoflurane to slices and concentration measurement

Artificial cerebrospinal fluid solution at room temperature was bubbled with a carrier gas (95% O_2, 5% CO_2) passing through a calibrated commercial vaporizer (Sevotec 5; Ohmeda, BOC Health Care, West Yorkshire, United Kingdom) at the designated concentration, and was applied to the recording chamber using a gravity-feed and vacuum system. High-quality polytetrafluorethylene was used for tubing and valves to minimize loss of volatile anesthetic and drug binding. Sevoflurane (Maruishi Pharmaceutical, Osaka, Japan) concentrations used for this study were 2.8% (clinically relevant concentration for mice) and 5.0% (high dose for mice). To determine the actual aqueous concentrations of sevoflurane in the submerged recording chamber for each concentration used, aliquots of the solution were taken from the recording chamber and filled into airtight glass containers for gas chromatographic measurements as described previously (Ishizeki et al., 2008). In brief, aliquots of the solution were directly taken from the recording chamber for gas chromatographic measurements. The peak of sevoflurane was observed approximately 3 min after injection, and the area under the curve was measured. The aqueous sevoflurane concentration was calculated by comparing to that of sevoflurane standard solution (1.0 mM), in which 20 μl of sevoflurane was dissolved in ethanol (100 ml). The final aqueous concentration of 2.8% sevoflurane was 0.23 ± 0.01 mM ($n = 5$), and 5.0% was 0.41 ± 0.01 mM ($n = 5$).

Although sevoflurane is administered to humans in the gas phase at body temperature (37 °C), *in vitro* electrophysiological experiments using sevoflurane were carried out at room temperature. In general, gas-phase potencies are reported to be temperature-dependent, increasing markedly with decreasing temperatures (Franks and Lieb, 1998). Procedures using gas-phase EC_{50} concentrations for room temperature experiments can thus result in overdosing with the *in vitro* preparations. Franks and Lieb (1996) have reported that the upper estimate of mammalian minimum alveolar concentration values for sevoflurane expressed as free aqueous concentration in saline is 0.33 mM. Taking these data into considerations, sevoflurane 0.23 mM was used as a clinically relevant concentration and 0.41 mM as a relatively high concentration.

2.8. Statistics

Results are expressed as mean ± SD. The results were analyzed by using Student's *t*-test or one way analyses of variance (ANOVA). Post hoc comparisons between the individual groups were performed by means of the Tukey test. Statistical significance between curves fitted to LORR and LTWR data was performed by comparing EC_{50} values via *t*-test. The level of statistical significance was set at $P < 0.05$ in all tests.

3. Results

3.1. GABA levels in the brain and the spinal cord are reduced in GAD65−/− mice

We measured the GABA content in the whole brain and spinal cord in GAD65−/− mice at 12–16-weeks old. GAD65−/− mice showed a significant reduction in GABA levels in the brain (76.7% of WT, $P < 0.001$, $n = 6$ each, Fig. 1A) and the spinal cord (68.5% of WT, $P < 0.001$, $n = 5$ for WT and $n = 6$ for GAD65−/− mice, Fig. 1B). Although a compensatory mechanism involving the balance between inhibitory and glutamatergic excitatory neurotransmission might have been expected, the difference in glutamate and glycine levels did not reach statistical significance when calculating mean values from experiments on five or six animals, respectively.

3.2. Ambient GABA levels are altered in GAD65−/− mice and in WT mice following blockade of GAT-1 transporter

Because the major anesthetic targets are believed to be cortical neurons and thalamic neurons (Franks, 2006), $GABA_A$ receptor-mediated miniature IPSCs (mIPSCs) were recorded from the frontal cortex layer V pyramidal neurons at 0 mV, close to the reversal potential of EPSCs, using Cs_2SO_4-based internal solutions (Fig. 2A). Tetrodotoxin (TTX, 1 μM) was used to block sodium channels that give rise to action potentials. Under these conditions, glutamate-mediated EPSCs were negligible. These observations were further confirmed by applying a GABAergic antagonist, picrotoxin (PIC, 50 μM or 100 μM, Pitler and Alger, 1992). Because both doses produced a similar baseline shift, PIC (50 μM) was used in following experiments. These neurons were identified using IR-DIC microscopy by their large (>20 μm diameter) pyramidal shaped cell bodies with long apical dendrites extending toward the pial surface. The mean amplitude of mIPSCs was unchanged in GAD65−/− mice or in WT with NO-711 injection (12.9 ± 3.5 pA in WT mice, 11.9 ± 4.7 pA in GAD65−/− mice, and 13.9 ± 3.5 pA in WT mice with NO-711, $n = 10$ each). The rise time of mIPSCs was also similar among groups (WT mice, 0.8 ± 0.2 ms, $n = 10$; GAD65−/− mice, 0.9 ± 0.3 ms, $n = 10$, WT mice with NO-711 (ip), 0.8 ± 0.3 ms, $n = 10$).

Ambient GABA levels were then evaluated by measuring the amplitude of tonic conductance of layer V pyramidal neurons in the