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SHORT COMMUNICATION

Role of the neurosteroid allopregnanolone in the hyperalgesic behavior induced by painful nerve injury in rats

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Abstract The neurosteroid allopregnanolone (AP) influences the excitability of the central nervous system by acting as a positive allosteric modulator of γ-aminobutyric acid type A ($GABA_A$) receptors. Here, we investigated the role of AP and its therapeutic potential in rats that showed hyperalgesic behavior after undergoing spinal nerve ligation (SNL). AP levels measured in the spinal cord and brain of rats that underwent SNL were greater than the corresponding levels in control animals. More importantly, spinal AP levels in hyperalgesic rats were lower than those in the rats that did not develop hyperalgesia following SNL; in contrast, brain AP levels were comparable among these groups. No differences in serum AP levels were observed among the groups. In addition, intrathecal exogenous administration of AP showed the antihyperalgesic effects in hyperalgesic rats after SNL. These findings suggest that changes in spinal AP biosynthesis are involved in the pathogenesis of neuropathic pain following peripheral nerve injury, and pharmacological manipulation of this phenomenon may provide a potential therapeutic target for neuropathic pain.

Keywords Neurosteroid · Neuropathic pain · Hyperalgesia

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Neurosteroids, which are synthesized within the central nervous system (CNS), influence the excitability of the CNS via nongenomic mechanisms, such as allosteric modulation of γ-aminobutyric acid type A ($GABA_A$) receptors [1, 2]. Among the neurosteroids, allopregnanolone (AP) is considered the most potent modulator of GABAergic transmission, and hence can affect complex behaviors such as convulsion, depression, and anxiety [1–3]. Because GABA is thought to be the pain-related inhibitory transmitter within the CNS, AP may contribute to the mechanisms of antinociception [4]. In addition, previous studies demonstrated that the production of neurosteroids, including AP, is increased after traumatic brain and spinal cord injury [5] or injury caused by chronic constriction of the sciatic nerve [6]. On the basis of these earlier findings, we hypothesized that the pathogenesis of neuropathic pain after painful axotomy involves changes in AP biosynthesis. To test this hypothesis, we compared endogenous AP levels in the brain and spinal cord of hyperalgesic rats with those in rats that did not develop hyperalgesia after nerve injury.

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Kochi

Medical School. Rats, 6 weeks old and weighing 140–160 g, were randomly assigned to either a spinal nerve ligation (SNL) group or a control group that received skin sham surgery. SNL was performed according to the method originally described previously [7], but we did not excise the adjacent paraspinous muscles or the articular processes. Under anesthesia with isoflurane, the L5 and L6 spinal nerves were then tightly ligated with a 6-0 silk suture and transected distal to the ligature.

Behavioral testing was performed on the day before surgery and on the 3rd, 7th, and 14th days after surgery. A Quincke 22-gauge spinal needle was used for detecting elevated sensory responsiveness after surgery as previously described [8]. Briefly, the plantar surface of each hind paw was touched with the tip of the needle, which was applied with pressure adequate to indent but not penetrate the skin. Five needle applications were delivered in random order to each paw and repeated 5 min later for a total of 10 applications per session. These mechanical stimuli produced either a normal brief reflexive withdrawal or a hyperalgesia-type response that included sustained (>1 s) paw lifting, shaking, and grooming. The latter response occurs only after true SNL, and thus this may be accepted as an indication of a neuropathic pain [8]. The intensity of hyperalgesia was assessed by the number of hyperalgesia-type responses from ten trials. Animals with two or more hyperalgesic responses after SNL were considered hyperalgesic (SNL-H). In contrast, this level of hyperalgesia was not observed after SNL; the rats were considered to be the animals that did not develop hyperalgesia following axotomy (nonhyperalgesic, SNL-NH). Autopsy was conducted at the end of the experiments to confirm accurate sectioning and placement of ligatures in all SNL animals.

To measure endogenous AP levels in the spinal cord, brain, and serum, samples were collected after completion of behavioral testing on the 14th day after surgery. Animals were killed by decapitation under deep isoflurane anesthesia, and the whole brain and ipsilateral lumbar spinal cord (L4–L6) after SNL (or control) were harvested. AP levels were measured by a radioimmunoassay using previously published methods [9]. Results are presented as mean ± SEM. Statistical analysis was performed with one-way analysis of variance (ANOVA) or with Kruskal–Wallis ANOVA when assumptions of normality of the distribution were not verified. In both ANOVA and Kruskal–Wallis approach, in case of P less than 0.05, pairwise comparisons against the control were made. Corrections for multiple comparisons were made according to the Bonferroni method. Statistical significance was defined with $P < 0.05$.

Taking the SNL animals together, AP contents in the spinal cord (8.3 ± 1.6 ng/g, $n = 18$) and brain (19.4 ± 2.2 ng/g, $n = 18$) were greater than those in control animals (2.9 ± 0.8 ng/g, $n = 10$; $p < 0.05$ and 5.0 ± 0.9 ng/g, $n = 10$; $P < 0.05$, respectively). Interestingly, AP levels in the brain were comparable between the SNL-H (18.9 ± 3.7 ng/g, $n = 10$) and SNL-NH groups (20.2 ± 2.9 ng/g, $n = 8$), whereas AP level in the spinal cord in the SNL-H group (5.4 ± 1.6 ng/g, $n = 10$) was approximately 2.2 times lower than that in the SNL-NH (12.1 ± 1.8 ng/g, $n = 8$; $P < 0.05$, Fig. 1a). No significant differences in serum AP levels were observed among the groups (Fig. 1a), suggesting that the high levels of spinal and brain AP after SNL may be independently synthesized from peripheral sources. The relationships between AP levels in different components and the number of hyperalgesia-type responses in SNL animals were assessed by linear regression. As shown in Fig. 1b, a significant relationship was found for the spinal cord AP levels ($R^2 = 0.68$, slope $= -0.18$; $P < 0.0001$), but not for the brain ($R^2 = 0.06$, slope $= 0.08$; $P = 0.81$) or serum AP levels ($R^2 = 0.01$, slope $= -0.005$; $P = 0.84$).

We next examined whether exogenous administration of AP (Sigma-Aldrich, St. Louis, MO, USA) had analgesic effects in SNL-H animals. Intrathecal administration of AP (1–100 μM, 30 min before testing) dose-dependently reduced the number of hyperalgesic responses ($P < 0.05$ vs. baseline) in SNL-H rats 14 days after surgery (Fig. 2). On the other hand, the same doses of AP did not show any effect in the SNL-NH group (data not shown). No adverse behavioral effects, such as motor effects, sedation, or agitation, were observed in either animal group.

Our novel finding is that spinal AP levels were lower in hyperalgesic rats than in animals that did not develop hyperalgesia following axotomy. In addition, spinal AP levels inversely correlated with the number of hyperalgesia-type responses after SNL. This negative correlation suggests that the changes in spinal AP contents are linked with mechanisms that mediate hyperalgesia after SNL. Specifically, inadequately low levels of spinal AP may contribute to increased nociceptive sensitivity, resulting in the development of hyperalgesia. On the other hand, although brain AP levels were increased after SNL, the levels were comparable between SNL-H and SNL-NH groups. This lack of correlation between the brain AP levels and the number of hyperalgesia-type responses after SNL implies that brain AP levels might be increased secondary to surgical stress per se rather than associated with the pathogenesis of neuropathic pain. However, we measured only whole-brain AP levels; thus, we cannot rule out that AP levels in specific brain regions may be related to neuropathic pain. Further study should be conducted to explore this possibility.

Our findings revealed that inappropriately low AP synthesis in the spinal cord is associated with the neuropathic pain after painful nerve injury. We also demonstrated that

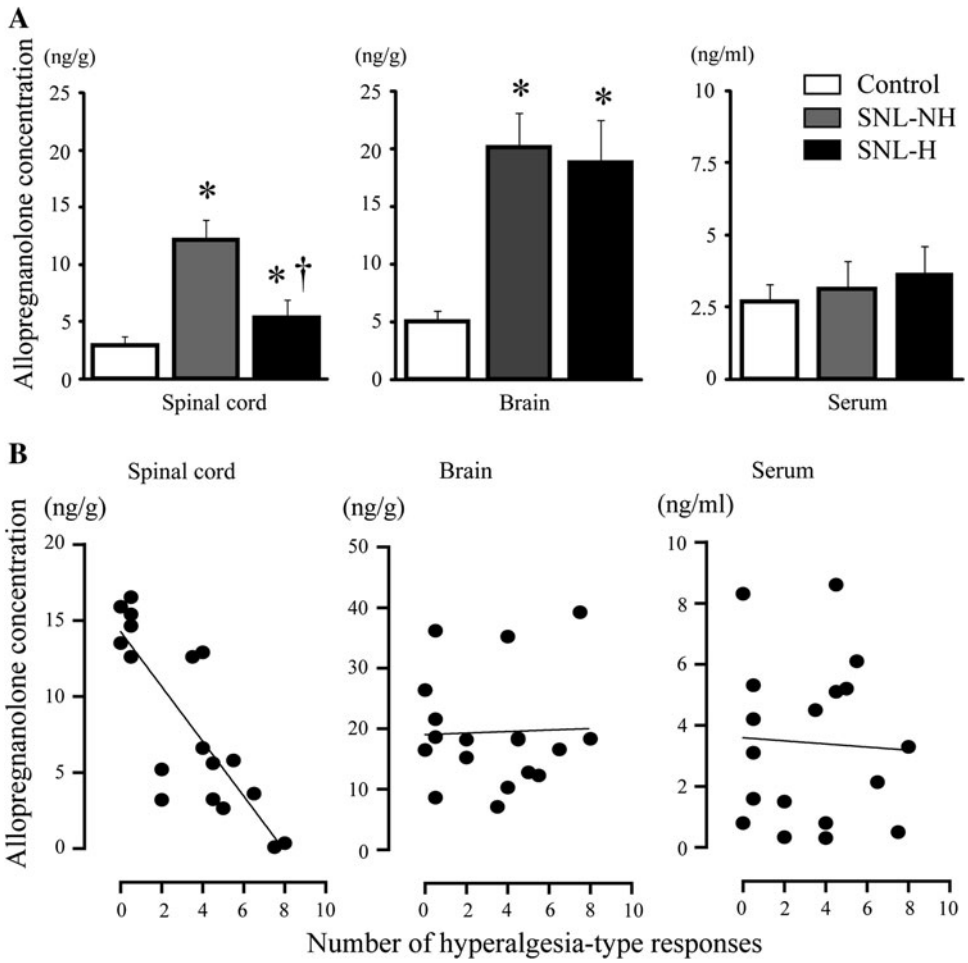

Fig. 1 Allopregnanolone levels in spinal cord, brain, and serum and correlation with the number of hyperalgesia-type responses. **a** Concentrations of allopregnanolone 14 days after surgery in skin sham surgery (SS, *control*) ($n = 10$), spinal nerve ligation nonhyperalgesic (*SNL-NH*) ($n = 8$), and spinal nerve ligation hyperalgesic (*SNL-H*) ($n = 10$) animals are shown as mean ± SEM. The symbol * indicates significantly higher allopregnanolone levels in comparison with the levels in the control (skin sham) group ($P < 0.05$); † indicates significantly lower allopregnanolone levels in comparison with those in the SNL-NH group ($P < 0.05$). **b** Correlation of allopregnanolone levels with the number of hyperalgesia-type responses from ten trials of needle stimulation after spinal nerve ligation. Simple linear regression curve and confidence intervals: *solid lines* represent simple linear regression

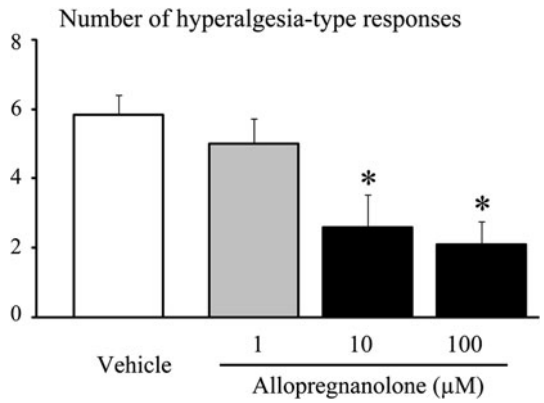

Fig. 2 Analgesic effects of exogenous treatment of allopregnanolone in neuropathic rats. Allopregnanolone (1–100 μM) or vehicle was administered intrathecally 30 min before testing in SNK-H rats 14 days after surgery. The analgesic effects were evaluated by the number of hyperalgesia-type responses out of ten trials of needle stimulation shown in response to ten touches of the right foot. Each column represents the mean with SEM ($n = 6$ per group). The symbol * indicates a statistically significant difference ($P < 0.05$) in comparison with the value for the vehicle treatment (control) group

intrathecal injection of exogenous AP can counteract the hyperalgesic behavior shown by hyperalgesic animals after axotomy. The data reported here suggest that a possible therapeutic approach for neuropathic pain may be represented by treatment with AP itself or with drugs that are capable of inducing in situ synthesis of AP.

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