Neurosteroid Allopregnanolone Suppresses Median Nerve Injury-induced Mechanical Hypersensitivity and Glial Extracellular Signal-regulated Kinase Activation through γ-Aminobutyric Acid Type A Receptor Modulation in the Rat Cuneate Nucleus

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ABSTRACT

Background: Mechanisms underlying neuropathic pain relief by the neurosteroid allopregnanolone remain uncertain. We investigated if allopregnanolone attenuates glial extracellular signal-regulated kinase (ERK) activation in the cuneate nucleus (CN) concomitant with neuropathic pain relief in median nerve chronic constriction injury (CCI) model rats.

Methods: We examined the time course and cellular localization of phosphorylated ERK (p-ERK) in CN after CCI. We subsequently employed microinjection of a mitogen-activated protein kinase kinase (ERK kinase) inhibitor, PD98059, to clarify the role of ERK phosphorylation in neuropathic pain development. Furthermore, we explored the effects of allopregnanolone (by mouth), intra-CN microinjection of γ-aminobutyric acid type A receptor antagonist (bicuculline) or γ-aminobutyric acid type B receptor antagonist (phaclofen) plus allopregnanolone, and allopregnanolone synthesis inhibitor (medroxyprogesterone; subcutaneous) on ERK activation and CCI-induced behavioral hypersensitivity.

Results: At 7 days post-CCI, p-ERK levels in ipsilateral CN were significantly increased and reached a peak. PD98059 microinjection into the CN 1 day after CCI dose-dependently attenuated injury-induced behavioral hypersensitivity (withdrawal threshold [mean ± SD], 7.4 ± 1.1, 8.7 ± 1.0, and 10.3 ± 0.8 g for 2.0, 2.5, and 3.0 mM PD98059, respectively, at 7 days post-CCI; n = 6 for each dose). Double immunofluorescence showed that p-ERK was localized to both astrocytes and microglia. Allopregnanolone significantly diminished CN p-ERK levels, glial activation, proinflammatory cytokines, and behavioral hypersensitivity after CCI. Bicuculline, but not phaclofen, blocked all effects of allopregnanolone. Medroxyprogesterone treatment reduced endogenous CN allopregnanolone and exacerbated nerve injury-induced neuropathic pain.

Conclusions: Median nerve injury-induced CN glial ERK activation modulated the development of behavioral hypersensitivity. Allopregnanolone attenuated glial ERK activation and neuropathic pain via γ-aminobutyric acid type A receptors. Reduced endogenous CN allopregnanolone after medroxyprogesterone administration rendered rats more susceptible to CCI-induced neuropathy. (Anesthesiology 2016; 125:1202-18)
primarily from dysfunction of neurons. However, emerging evidence suggests that posttraumatic neuronal plasticity and sensitization in the central nervous system are associated with activation of glial cells, which is engaged in the dorsal root ganglion sensory neuron–glia communication and contributes to the development of tactile allodynia.6–9

Median nerve injury evokes a barrage of injury-related discharges from damaged nerve fibers; afterward, these ectopic discharges facilitate the release of excitatory amino acids from damaged primary afferents, leading to glial and neuronal activation in the cuneate nucleus (CN).10–12 The CN then transmits this tactile hypersensitivity information to the thalamus; thus, the CN may influence ascending neuropathic pain signaling to the thalamus.13 Previous studies reported that extracellular signal-regulated kinase (ERK), a mitogen-activated protein kinase, may be involved in the regulation of nociceptive signaling in primary sensory pathways after a pathologic insult to the peripheral nervous system.14–16 Phosphorylated ERK (p-ERK) is selectively expressed in hyperaemic glia of the spinal dorsal horn after peripheral nerve injury and is essential for the ensuing glial activation.17 Intrathecal injection of a mitogen-activated protein kinase kinase (MeK) inhibitor, which specifically attenuates ERK activity, suppresses nerve injury-induced tactile allodynia, implying that persistent activation of this kinase is a prerequisite for sustaining allodynia.17,18

Allopregnanolone, a γ-aminobutyric acid (GABA) type A (GABA_A) receptor-positive modulator,19 is a natural neurosteroid derived from progesterone in the nervous system of many vertebrates, including rodents and humans.20–22 Several studies have revealed the analgesic, anesthetic, antidepressant, and neuroprotective effects of allopregnanolone.23–28 There is evidence that intrathecal or oral administration of allopregnanolone relieves allodynia in animal models of neuropathic pain.29,30

Based on these observations, we hypothesized that median nerve injury-induced glial ERK phosphorylation in the CN contributes to the development of behavioral hypersensitivity and that allopregnanolone supplementation will attenuate glial ERK activation and neuropathic pain through modulation of GABA_A receptor activity. To test the hypothesis, we studied the time course of ERK activation in the CN of a median nerve chronic constriction injury (CCI) rat model. We further employed an intra-CN microinjection of an MEK inhibitor, PD98059, to clarify the role of ERK phosphorylation in neuropathic pain development. In addition, we examined the effects of allopregnanolone alone or in combination with GABA_A or GABA type B (GABA_B) receptor antagonists on ERK activation and development of behavioral hypersensitivity after median nerve CCI. Finally, we investigated the impact of decreasing endogenous allopregnanolone levels on ERK activation and nerve injury-induced behavioral hypersensitivity by administration of medroxyprogesterone, a 3α-hydroxysteroid oxidoreductase (3α-HSOR) inhibitor that blocks the conversion of dihydroprogesterone to allopregnanolone.

Materials and Methods

Animal Preparations

The experimental protocol was approved by the Ministry of Science and Technology Committee and the Animal Center Committee, Fu Jen Catholic University, New Taipei City, Taiwan. Animal experiments adhered to the ethical guidelines of the International Association for the Study of Pain, Washington, D.C.,31 and all efforts were made to minimize animal suffering and to reduce the number of animals used. The experiments in this study were performed on male, Sprague–Dawley rats (200 to 250 g) housed under approved conditions with a 12-/12-h light/dark cycle and food and water available ad libitum.

Nerve Injury Surgery

The median nerve CCI model was modified from that established by Bennett and Xie.32 In brief, anesthesia was induced by an intraperitoneal injection of sodium pentobarbital (30 to 40 mg/kg). Under a dissecting microscope, the right median nerve was separated from the surrounding tissues at the elbow level and between the two heads of the pronator teres muscle. Four loose ligatures were tied around the nerve using a 4-0 chromic gut suture.10–13,33–35 The incision was then closed, and the animals were allowed to survive for 5 h and 1, 3, 5, 7, 14, and 21 days (n = 17 at each time point). Seventeen rats received a sham operation (median nerve exposed without ligation) in the same region. At predetermined time points, the animals were euthanatized and processed for immunohistochemistry, double immunofluorescence labeling, Western blotting, and high-performance liquid chromatography–mass spectrometry (HPLC-MS).

Delivery of the MEK Inhibitor in the CN

Given that ERKs (ERK1 and ERK2) are the only known substrates for MEK, MEK inhibition provides specific inactivation of ERK signaling.36 An MEK inhibitor, PD98059, was injected into the CN for targeted disruption of MEK activity.

The rats were anesthetized (30 to 40 mg/kg sodium pentobarbital, intraperitoneal) and placed in a stereotaxic head holder. The skull was exposed, and a brain infusion cannula was directed toward the CN. The injection site was defined using the stereotaxic coordinates provided by the rat brain atlas of Pellegrino et al.37 (coordinates: anteroposterior, mediolateral, and height: −6.4, 1.4, and −6.4; system A). The brain infusion cannula was cemented in place and secured to the skull with an instant adhesive gel. The animals were allowed to recover for 5 days after cannula insertion. The rats were then reanesthetized and subjected to CCI of the median nerve.

One day after nerve injury, all rats were implanted with an Alzet® osmotic pump (model 2004; DURECT Corporation, USA) with a reservoir volume of 200 μl and a 28-day delivery duration at 0.25 μl/h under anesthesia. PD98059 (2-amino-3'-methoxyflavone; Calbiochem, USA) was
dissolved in dimethyl sulfoxide (DMSO) and diluted in the Ringer’s solution (128 mM NaCl, 18 mM CaCl₂, 1.3 mM KCl, and 2.3 mM NaHCO₃ in distilled water) to achieve a final DMSO concentration of less than 1%. The osmotic pump was filled with the PD98059 solution at a dose of 2.0 (n = 6), 2.5 (n = 6), or 3.0 mM (n = 6) or with vehicle solution (DMSO; n = 6). Before implantation, the osmotic pumps were incubated in a 0.9% NaCl solution for 24 h at 37°C and filled according to the supplier’s instruction. The Alzet® osmotic pump was installed in the subcutaneous pocket of the back, and its catheter was connected to the brain infusion cannula. Animals in the control groups underwent median nerve CCI (n = 6) or sham operation (n = 5) but did not receive a microinjection of PD98059. Behavioral testing was performed on these animals at 1 day before nerve injury and again 5 h and 1, 3, 5, 7, 14, and 21 days after injury. Upon removal of the pump at the end of the experiment, the residual volume in the reservoir was measured to confirm the delivery of solutions.

Drug Treatment Protocols
Allopregnanolone (3α,5α-tetrahydroprogesterone; Steraloids, USA) was dissolved in absolute ethanol and diluted in the lactated Ringer’s solution to achieve a final ethanol concentration of less than 1%. Animals were given allopregnanolone at doses of 5, 10, or 20 mg/kg or equal volumes of vehicle (ethanol). Allopregnanolone or vehicle was fed through an orogastric tube in a volume of 1 ml/kg once a day on days 1, 2, 4, and 6 after median nerve CCI (n = 18 for each dose) or sham operation (n = 18 for each dose). 6-Medroxyprogesterone acetate (Provera; Steraloids) suspended in olive oil (15 or 20 mg/kg) or vehicle (olive oil) alone was injected subcutaneously in the subcapular area at 80 μl/kg. Medroxyprogesterone was administered once a day on days 1, 2, 4, and 6 after median nerve CCI (n = 18 for each dose) or sham operation (n = 18 for each dose). The allopregnanolone-treated and medroxyprogesterone-treated animals were subjected to the behavioral testing 7 days after median nerve injury or sham operation. Afterward, the rats were randomly assigned to one of the two experiments. In one experiment, the animals were processed for immunohistochemistry, and in the other, they were processed for Western blotting.

Immunohistochemistry
The rats were deeply anesthetized (sodium pentobarbital, 50 mg/kg, intraperitoneal) and perfused with 4% paraformaldehyde/0.1 M phosphate buffer (PB, pH 7.4). Medulla tissue blocks containing the CN were retrieved and stored in PB/30% sucrose overnight and then cut into 30-μm transverse slices with a cryostat (Leica, Germany).

Floating medulla sections were treated with 1% H₂O₂, blocked with 2% normal goat serum (NGS; GibcoBRL, USA) in PB for 1 h, and then incubated in a rabbit polyclonal anti-p-ERK antibody (1:200; Cell Signaling, USA), mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:800; Sigma-Aldrich, USA), or mouse monoclonal anti-OX-42 antibody (CD11b, 1:200; Serotec, USA) for 48 h at 4°C. In all cases, the primary antibody was diluted in 0.01 M phosphate-buffered saline (PBS, pH 7.4), containing 0.1% Triton X-100 and 5% NGS. After rinsing with PBS, the sections were incubated in Cy3-conjugated anti-rabbit immunoglobulin G (IgG; 1:200; Jackson ImmunoResearch, USA) or fluorescein isothiocyanate–conjugated anti-mouse IgG (1:200; Jackson ImmunoResearch) for 2 h at room temperature. Finally, the floating sections were mounted onto gelatinized slides and observed under a fluorescence microscope (Nikon, USA).

For GABA_A receptor immunohistochemistry, the sections were incubated in rabbit polyclonal anti-GABA_A receptor antibody (1:1,000; Sigma-Aldrich) for 48 h at 4°C, followed by incubation in 1:200 biotinylated anti-rabbit IgG (Vector, USA) for 2 h and avidin–biotin–horseradish peroxidase (HRP) complex (Vector) for 1 h at room temperature.
Peroxidase activity was visualized using the Vector® SG Substrate Kit (Vector). Finally, the floating sections were mounted onto gelatinized slides and examined under a light microscope (Zeiss Axiophot, Germany).

**Double Immunofluorescence Labeling**

Rats were perfused with 4% paraformaldehyde in 0.1 M PB (pH 7.4) at 7 days after median nerve CCI. Medulla tissue blocks were retrieved, and 30-μm transverse slices were cut with a cryostat (Leica). Medulla sections were treated with 1% H2O2, blocked with 2% NGS, and incubated for 48 h at 4°C with a mixture of p-ERK antibody (1:200; Cell Signaling) and one of the following cell-specific markers: mouse monoclonal antineuronal nuclei (NeuN, 1:1,000; Millipore, USA) for identifying neurons; mouse monoclonal anti-GFAP (1:800; Sigma-Aldrich) for astrocytes; or mouse monoclonal anti-OX-42 (CD11b, 1:200; Serotec) for microglial cells. After rinsing with PBS, the sections were incubated for 2 h at room temperature with a mixture of Cy3-conjugated anti-rabbit IgG (1:200; Jackson Immunoresearch) and fluorescein isothiocyanate–conjugated anti-mouse IgG (1:200; Jackson Immunoresearch). The stained sections were mounted onto gelatinized slides and examined under a fluorescence microscope (Nikon). Images were captured with a charge-coupled device spot camera (Nikon). Twenty nonadjacent medulla sections from five rats were randomly selected, and p-ERK-labeled cells, p-ERK/GFAP double-labeled cells, and p-ERK/OX-42 double-labeled cells were counted. The percentage of cells with colocalized antigens was calculated as the number of double-labeled cells divided by the total number of p-ERK–labeled cells.

**Sample Preparation and Western Blot Analysis**

Under sodium pentobarbital anesthesia (intraperitoneal), the rats were sacrificed by decapitation. The brainstem containing the CN was removed and cut into 150-μm slices with a vibratome (TPI, Series 1000, USA). The sections were stained with a 0.05% toluidine blue solution (Wako, Japan) for 1 min, washed three times in PBS, mounted onto glass slides, and air dried for 10 min. Under a dissecting stereomicroscope (Leica), the CN was microdissected with a blunt-ended microdissection needle. The punched tissues were weighed, and stored at −80°C. Endogenous allopregnanolone was measured using a highly sensitive and specific HPLC-MS method after solid-phase extraction purification, as previously described with minor modifications. Briefly, 60 mg brain tissues were spiked with an internal standard (methyltestosterone) and homogenized in 0.5 ml of PB using a glass homogenizer. The homogenate was then extracted three times using 1.5 ml ethyl acetate. The dried samples were resuspended in 1.5 ml MeOH/H2O (50:50, v/v) and filtered through an SPE cartridge (Supelco, USA), previously activated with MeOH (3 ml) and H2O electrophoresis and transferred to nitrocellulose membranes (GE Healthcare, USA). Equal protein loading was confirmed by Ponceau S staining. Membranes were blocked with 5% nonfat dry milk in PBS/0.1% Tween-20 for 1 h and then incubated overnight at 4°C with an antibody against p-ERK (rabbit, 1:1,000; Cell Signaling, ERK (rabbit, 1:1,000; Cell Signaling), GFAP (mouse, 1:1,000; Sigma-Aldrich), OX-42 (CD11b, mouse, 1:1,000; BD Biosciences, USA), and β-actin (mouse, 1:5,000; Sigma-Aldrich). Immunolabeled membranes were washed three times in PBS/0.1% Tween-20 and incubated with HRP-conjugated sheep anti-rabbit IgG (1:5,000; GE Healthcare) or HRP-conjugated sheep anti-mouse IgG (1:5,000; GE Healthcare) for 1 h at room temperature. Peroxidase activity was detected by incubating the membrane with enhanced chemiluminescence Western blotting detection reagents (GE Healthcare) for 1 min and exposure to hyperfilm (GE Healthcare) for 5 min. The optical densities of specific p-ERK, ERK, GFAP, OX-42, and β-actin bands were measured using a computer-assisted image analysis system (Gel-Pro Analyzer software; Media Cybernetics, Inc., USA).

**ELISA**

The concentrations of proinflammatory cytokines in the CN were determined by ELISA. The samples were prepared as described in the first paragraph of Sample Preparation and Western Blot Analysis. The supernatant was collected, and the levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) were quantified using the TNF-α immunoassay kit (R&D Systems, Inc., USA), IL-1β immunoassay kit (R&D Systems, Inc.), and IL-6 immunoassay kit (R&D Systems, Inc.), respectively, on a microplate reader (Molecular Devices Corporation, USA). All procedures were performed following the manufacturer’s instructions. The lower detection limits of the assays were 5 (TNF-α), 5 (IL-1β), and 21 (IL-6) pg/ml. All cytokine assays had intra- and interassay coefficients of variation lower than 9% and 10%, respectively.

**HPLC-MS**

Under deep anesthesia by sodium pentobarbital (intraperitoneal), the rats were euthanized via decapitation. The CN was microdissected, weighed, and stored at −80°C. Endogenous allopregnanolone was measured using a highly sensitive and specific HPLC-MS method after solid-phase extraction purification, as previously described with minor modifications. Briefly, 60 mg brain tissues were spiked with an internal standard (methyltestosterone) and homogenized in 0.5 ml of PB using a glass homogenizer. The homogenate was then extracted three times using 1.5 ml ethyl acetate. The organic phase from each extraction was combined and dried with a gentle stream of nitrogen in a 55°C water bath. The dried samples were resuspended in 1.5 ml MeOH/H2O (50:50, v/v) and filtered through an SPE cartridge (Supelco, USA), previously activated with MeOH (3 ml) and H2O.
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(5 ml). The SPE cartridge was first perfused with 1 ml of 80% MeOH/H2O (80:20, v/v), and the eluent was discarded. Finally, allopregnanolone was eluted in 2 ml of MeOH and dried again with a gentle stream of nitrogen in a 55°C water bath. The dry residue was resuspended in 2 ml ethyl acetate/methanol (80:20, v/v) and filtered through an NH2 column (Supelclean LC-NH2, 500 mg; Supelco, USA), preconditioned with 3 ml ethyl acetate and 3 ml ethyl acetate/methanol (80:20, v/v), that allows neuroactive allopregnanolone to pass unretained. The NH2 column was further rinsed with 2 ml more of the solvent mixture, and the combined eluates were evaporated with a gentle stream of nitrogen. Purification with the NH2 column was necessary to improve the accuracy of the assay. Purified allopregnanolone was derivatized according to the method described by Higashi et al.41 After purification, the dried samples were dissolved in 100 μl dehydrated alcohol and derivatized with 200 μl 2-nitro-4-trifluoromethylphenylhydrazine, followed by vortex mixing. Samples were allowed to react for 1 h at 65°C in a water bath and then dried under a gentle stream of nitrogen. Derivatized samples were resuspended in 100 μl acetonitrile/H2O (84:16, v/v), and 10 μl of each sample was loaded onto the HPLC-MS. The estimated recovery of allopregnanolone was 88%. The lower limit of detection for allopregnanolone was 4 pg/ml using this method.

HPLC-MS analysis was conducted on an Agilent 1100 HPLC-MS (Agilent Technologies, Inc., USA). An SB-C18 column (2.1 × 75 mm ID, 3.5 μm; Agilent Technologies, Inc.) equilibrated with 90% methanol was used to separate the derivatives (10-μl samples) of allopregnanolone at a flow rate of 0.5 ml/min. The mass spectrometer (Agilent Technologies, Inc.) was operated in the negative ion mode with the atmospheric-pressure chemical ionization source using nitrogen as a sheath and auxiliary and sweep gas flow rates of 5 l/min. Other ion-source parameters were as follows: vaporizer temperature, 250°C; drying gas temperature, 300°C; ion-source collision energy, 180 V; and capillary pressure, 3.5 kV. Allopregnanolone was analyzed by multiple reaction monitoring.

Behavioral Testing

Von Frey Filament Test. The testing was conducted on the PD98059-treated, allopregnanolone-treated, medroxyprogesterone-treated, and corresponding vehicle-treated rats. Rats were placed in individual plexiglas chambers (25 × 40 × 18 cm) with wire-mesh bottoms and allowed to acclimatize to the environment for 30 min. Mechanical withdrawal threshold of the rat forepaw was determined using a series of von Frey filaments (Semmes-Weinstein Monofilaments; North Coast Medical, Inc., USA; bending force: 0.16, 0.4, 0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10.0, 15.0, and 26.0 g). The measurements were conducted based on the method described by Tal and Bennett.52 Quantitative mechanical stimuli were applied to the medial plantar surface of each forepaw in an ascending order to determine the withdrawal threshold. Each von Frey filament was applied five times, and each stimulus lasted 5 s. The minimal time interval between two successive stimuli was 10 min. The lowest bending force of the filament to evoke at least two withdrawal responses was defined as the withdrawal threshold.

Rotarod Test. The motor performance of allopregnanolone-treated rats was evaluated using an accelerating rotarod (Ugo Basile, Italy). The rats were placed on a rotating drum with speed increasing from 4 to 40 rpm over 5 min, and the latency to fall (in seconds) was measured as an index of performance. Training sessions were conducted 1 and 2 days before the experiments, with three 5-min trials on each day to allow animals to acclimatize to the rotarod. All studies were performed in a random, blinded manner to avoid expectation bias.

Data Presentation and Statistical Analysis

Estimation of the number of rats needed for this study was based on the assumption of an SD of 1.2 g in the mean forepaw withdrawal threshold to detect a 40% difference between vehicle- and drug-treated groups.11 Thus, to obtain a power of 80% at a two-sided significance level of 0.05, a minimum of six rats were required for each group.

Data are presented as mean ± SD. For analysis of the behavioral testing results in PD98059- and vehicle-treated CCI rats, two-way repeated-measures ANOVA followed by Tukey post hoc testing was applied. Otherwise, mean values were compared using one-way ANOVA followed by Tukey post hoc testing as appropriate. There were no missing data in this study. P < 0.05 denoted statistical significance. All statistical analyses were performed using the SPSS software (version 19.0; SPSS, Inc., USA).

Results

ERK Phosphorylation in CN Glial Cells and Effects of the MEK Inhibitor on Behavioral Expression in CCI Rats

Very low p-ERK immunoreactivity was detected in the ipsilateral CN of sham-operated rats and the contralateral CN of CCI rats (fig. 1, A and B). Immunoreactivity for p-ERK increased in the ipsilateral CN as early as 1 day post-CCI (fig. 1, C and D) and continued to increase until day 7 (fig. 1E–G), followed by a gradual decrease during the next 14 days (fig. 1, H and I). Western blot analysis showed that p-ERK levels in the ipsilateral CN were significantly higher in CCI rats as early as 1 day after nerve injury compared to sham-operated rats and peaked on day 7 (fig. 1J).

Von Frey filament testing showed that the mechanical withdrawal threshold decreased from a baseline of 15.7 ± 1.0 g to 4.7 ± 1.0 g on day 7 postinjury in CCI rats (fig. 1K). Model rats developed mechanical hypersensitivity 1 day after CCI, which persisted throughout the experimental period. An antinociceptive effect was observed with microinjection of the MEK inhibitor, PD98059 (2.0, 2.5, or 3.0 mM), into the CN of CCI rats 1 day after nerve injury.
As illustrated in figure 1K, mechanical hypersensitivity was attenuated after administration of PD98059 in a dose-dependent manner, while microinjection of vehicle did not alter the mechanical withdrawal threshold.

To identify the cell types expressing p-ERK immunoreactivity after nerve injury, we performed double immunofluorescence labeling for p-ERK and cell-specific markers: NeuN for neurons, GFAP for astrocytes, and OX-42 for microglia. We found that p-ERK was colocalized with GFAP and OX-42 but not with NeuN (fig. 2), indicating that phosphorylation of ERK in the CN was restricted to astrocytes and microglial cells. Quantitative analysis showed that about 42% of p-ERK immunoreactive cells were astrocytes and 57% were microglia. Little GFAP immunoreactivity and OX-42 immunoreactivity was detected in the CN of sham-operated rats, suggesting that the presence of p-ERK in the CN is specific to the injured state.

**Fig. 1.** Phosphorylation of extracellular signal-regulated kinase (ERK) in the cuneate nucleus (CN) after chronic constriction injury (CCI). (A) The phosphorylated ERK (p-ERK) immunoreactivity in the ipsilateral CN (arrow) was more intense than that in the contralateral side (arrowhead) 7 days after CCI. (B) In the ipsilateral CN of sham-operated rats, there was little p-ERK immunoreactivity. (C–I) A temporal change of p-ERK immunoreactivity was observed in the ipsilateral CN at 5 h, 1 day, 3 days, 5 days, 7 days, 14 days, and 21 days after CCI. The p-ERK immunoreactivity was most prominent at day 7 after nerve injury. (J) Quantification of p-ERK expression in the CN of sham-operated and CCI rats using Western blot analysis. Compared with sham-operated rats, the p-ERK levels in the ipsilateral CN were significantly increased in CCI rats as early as 1 day after nerve injury. The ERK was used as a loading control. n = 6 rats at each time point. *P < 0.05 compared with sham-operated rats. (K) Dose-response effects of PD98059 on nerve injury-induced mechanical hypersensitivity. PD98059 was administered 1 day after CCI. Microinjection of PD98059 into the CN attenuated mechanical hypersensitivity in a dose-dependent manner. The black bold horizontal line indicates the period of drug infusion. n = 6 rats per group, except for sham-operated group (n = 5). *P < 0.05 for comparisons between each dose of PD98059 and vehicle. Error bars represent mean ± SD. Scale bar = 500 μm (A) and 100 μm (B–I). BL = basal level.
nearly no OX-42 immunoreactivity were observed in the contralateral CN of CCI rats (figure not shown).

**Impact of Allopregnanolone Treatment on CN ERK Phosphorylation, Glial Activation, Behavioral Testing, and Proinflammatory Cytokine Expression in CCI Rats**

Little p-ERK, GFAP, and OX-42 immunoreactivity was present in the ipsilateral CN of sham-operated rats (fig. 3A–C). In vehicle-treated CCI rats, intense p-ERK, GFAP, and OX-42 immunoreactivity was observed in the ipsilateral CN 7 days after surgery (fig. 3D–F). The p-ERK, GFAP, and OX-42 immunoreactivity was markedly decreased in the ipsilateral CN of CCI rats treated with 20 mg/kg allopregnanolone (fig. 3G–I). Western blot analysis showed a significant decrease in p-ERK, GFAP, and OX-42 levels in the CN of rats treated with different doses of allopregnanolone compared to vehicle-treated CCI rats (fig. 3J).

Mechanical withdrawal threshold was significantly lower in both drug-naive and vehicle-treated CCI rats compared to sham-operated rats (fig. 4A). In CCI rats, the mechanical withdrawal threshold was dose dependently increased by allopregnanolone administration, whereas allopregnanolone had no significant effect on rotarod motor performance at any of the tested doses (see figure, Supplemental Digital Content 1, http://links.lww.com/ALN/B318, which depicts the results of the rotarod test). The mechanical withdrawal threshold was higher in sham-operated rats administered allopregnanolone than in sham-operated vehicle-treated rats, but the difference did not reach statistical significance (fig. 4B).

Levels of the proinflammatory cytokines, TNF-α, IL-1β, and IL-6, were markedly higher in the ipsilateral CN of both drug-naive and vehicle-treated CCI rats compared to sham-operated control rats (fig. 4C–E) and were diminished in a dose-dependent manner by allopregnanolone.

**Effects of Intra-CN Administration of GABA$_{A}$ or GABA$_{B}$ Receptor Antagonist Plus Allopregnanolone on ERK Phosphorylation, Glial Activation, and Behavioral Testing in CCI Rats**

GABA$_{A}$ receptor immunoreactivity was detected in the ipsilateral CN of both sham-operated and CCI rats at 7 days postsurgery (see figure, Supplemental Digital Content 2, http://links.lww.com/ALN/B319, which shows GABA$_{A}$ receptor immunoreactivity in the CN). Quantitative analysis revealed a significantly greater number of GABA$_{A}$ receptor immunoreactive cells in the CN of CCI rats than in sham-operated rats (see figure, Supplemental Digital Content 2, http://links.lww.com/ALN/B319).

With intra-CN microinjection of vehicle, there was intense p-ERK, GFAP, and OX-42 immunoreactivity in the ipsilateral CN 7 days after median nerve CCI (figure not shown), while...
Fig. 3. Effects of allopregnanolone treatment on extracellular signal-regulated kinase (ERK) phosphorylation and glial fibrillary acidic protein (GFAP) and OX-42 expressions in chronic constriction injury (CCI) rats. (A–C) Photomicrographs show phosphorylated ERK (p-ERK), GFAP, and OX-42 immunoreactivity in the ipsilateral cuneate nucleus (CN) of sham-operated rats. (D–I) Photomicrographs demonstrate p-ERK, GFAP, and OX-42 immunoreactivity in the ipsilateral CN 7 days after CCI in rats receiving vehicle or allopregnanolone at a dose of 20 mg/kg. (J) Western blot analysis show the expression of p-ERK, GFAP, and OX-42 in the CN 7 days after CCI in rats treated with allopregnanolone. The p-ERK, GFAP, and OX-42 levels in the CN were significantly decreased in CCI rats given different doses of allopregnanolone compared with those treated with vehicle (*, #, +, P < 0.05 compared with CCI plus vehicle group). The ERK and β-actin were used as a loading control. Error bars represent mean ± SD. n = 6 rats per group. Scale bar = 100 μm.
Immunoexpression levels were markedly decreased by intra-CN microinjection of allopregnanolone (fig. 5A–C). Comicroinjection of bicuculline with allopregnanolone mitigated the suppressive effects of allopregnanolone on p-ERK, GFAP, and OX-42 immunoreactivity in the CN of CCI rats (fig. 5D–F), while comicroinjection of phaclofen plus allopregnanolone did not alter p-ERK, GFAP, and OX-42 immunoreactivity in the CN compared to allopregnanolone microinjection alone (fig. 5G–I).

Western blot analysis revealed significantly lower p-ERK, GFAP, and OX-42 levels in the CN of allopregnanolone-treated CCI rats compared to vehicle-treated CCI rats (fig. 5J). The p-ERK, GFAP, and OX-42 levels were significantly increased in the ipsilateral CN of CCI rats microinjected with bicuculline plus allopregnanolone compared to allopregnanolone alone, while expression levels of p-ERK, GFAP, and OX-42 were similar between CCI rats receiving microinjection of allopregnanolone or phaclofen plus allopregnanolone.

Intra-CN microinjection of allopregnanolone, but not vehicle, reversed the decreased mechanical withdrawal threshold in CCI rats (fig. 6). However, comicroinjection of bicuculline with allopregnanolone abolished this effect, while there was no difference in the mechanical withdrawal threshold between CCI rats microinjected with phaclofen plus allopregnanolone versus allopregnanolone alone.

Influence of Medroxyprogesterone Treatment on CN ERK Phosphorylation, Gliarial Activation, Behavioral Testing, Proinflammatory Cytokine Expression, and Endogenous Allopregnanolone Production in CCI Rats

Little p-ERK, GFAP, and OX-42 immunoreactivity was observed in the CN of sham-operated rats treated with vehicle (fig. 7A–C); however, there was intense p-ERK, GFAP, and OX-42 immunoreactivity in the CN of sham-operated rats treated with 15 or 20 mg/kg medroxyprogesterone compared to vehicle treatment (fig. 7D–I). Western blot analysis showed significant increases in p-ERK and OX-42 in the CN of sham-operated rats receiving 20 mg/kg medroxyprogesterone compared to those receiving vehicle (fig. 7J). A significantly higher GFAP level was observed in the CN of sham-operated rats treated with 15 or 20 mg/kg medroxyprogesterone than in vehicle-treated sham-operated rats (fig. 7J). In CCI rats treated with 15 or 20 mg/kg medroxyprogesterone, significant increases in the p-ERK, GFAP, and OX-42 levels were found in the CN, compared to vehicle-treated CCI rats.

Mechanical withdrawal threshold was significantly lower in sham-operated rats given medroxyprogesterone at doses of 15 or 20 mg/kg than in vehicle-treated sham-operated rats (fig. 8A). The mechanical withdrawal threshold was also...
Fig. 5. Effects of intra-cuneate nucleus (CN) microinjection of γ-aminobutyric acid type A (GABA_a) or type B (GABA_b) receptor antagonist plus allopregnanolone on extracellular signal-regulated kinase (ERK) phosphorylation and glial fibrillary acidic protein (GFAP) and OX-42 expressions in chronic constriction injury (CCI) rats. (A–C) Little, if any, phosphorylated ERK (p-ERK), GFAP, and OX-42 immunoreactivity was seen in the ipsilateral CN of CCI rats after intra-CN microinjection of allopregnanolone; however, there was intense p-ERK, GFAP, and OX-42 immunoreactivity in the CN of CCI rats microinjected with bicuculline plus allopregnanolone (D–I). (G–I) Upon microinjection with phaclofen plus allopregnanolone into CN of CCI rats, p-ERK, GFAP, and OX-42 immunoreactivity was scarcely observed in the CN. (J) Quantification of p-ERK, GFAP, and OX-42 expressions in the CN of CCI rats microinjected with GABA_a or GABA_b receptor antagonist plus allopregnanolone using Western blot analysis. Allopregnanolone-treated CCI rats displayed a significant decrease in p-ERK, GFAP, and OX-42 levels in the CN compared to vehicle-treated CCI rats (*, #, +P < 0.05 compared with CCI plus vehicle group). The p-ERK, GFAP, and OX-42 levels were significantly increased in the ipsilateral CN of CCI rats microinjected with bicuculline plus allopregnanolone as compared to those microinjected with allopregnanolone alone (**, ###, +++P < 0.05 compared with CCI plus allopregnanolone group). Similar expressions of the p-ERK, GFAP, and OX-42 were observed in CCI rats microinjected with allopregnanolone with and without phaclofen. The ERK and β-actin were used as a loading control. Error bars represent mean ± SD. n = 6 rats per group. Scale bar = 100 μm.
Fig. 6. Influence of intra-cuneate nucleus (CN) administration of γ-aminobutyric acid type A (GABA_A) or type B (GABA_B) receptor antagonist plus allopregnanolone on nerve injury-induced mechanical hypersensitivity. The chronic constriction injury (CCI) rats receiving microinjection of allopregnanolone had a significant increase in the mechanical withdrawal threshold compared with those receiving vehicle (*P < 0.05). Compared with allopregnanolone administration, concomitant delivery of bicuculline and allopregnanolone in CCI rats significantly decreased the mechanical withdrawal threshold (#P < 0.05). No significant difference in the mechanical withdrawal threshold was observed between CCI rats microinjected with phaclofen plus allopregnanolone and those receiving allopregnanolone alone. Error bars represent mean ± SD. n = 6 rats per group.

Discussion

This study demonstrates that CCI of the median nerve induced a significant increase in p-ERK immunoreactivity in the ipsilateral CN and led to the development of mechanical hypersensitivity. Mechanical hypersensitivity induced by CCI was attenuated in a dose-dependent manner by microinjection of the MEK inhibitor PD98059 into the CN. Double immunofluorescence labeling with cell-specific markers showed that p-ERK immunoreactive cells coexpressed GFAP and OX-42 immunoreactivity. Allopregnanolone treatment effectively reduced p-ERK, GFAP, and OX-42 immunoreactivity and the release of proinflammatory cytokines and successfully ameliorated nerve injury-induced mechanical hypersensitivity in CCI rats. Intra-CN coadministration of the GABA_A receptor antagonist bicuculline, but not the GABA_B receptor antagonist phaclofen, with allopregnanolone blocked these effects. In addition, medroxyprogesterone treatment resulted in a reduction in endogenous allopregnanolone levels and rendered rats more susceptible to nerve injury-induced neuropathy.

ERK activation in the central nervous system is a common phenomenon after peripheral nerve injury.3–5 In the current study, p-ERK immunoreactivity was significantly increased on the lesion side of the CN as early as 1 day after CCI and reached its peak on postinjury day 7. The temporal alterations of p-ERK immunoreactivity were consistent with the expression pattern in the lumbar spinal dorsal horn after peripheral nerve ligation.17 Ciruela et al.34 reported that activation of ERK after sciatic nerve injury may contribute to the development of mechanical hypersensitivity that was alleviated by inhibition of ERK signaling. We administered PD98059 1 day after median nerve CCI, a time when ERK was already activated, and rats displayed mechanical hypersensitivity. Microinjection of PD98059 into the CN ameliorated mechanical hypersensitivity in a dose-dependent manner. These findings indicate that median nerve injury-induced ERK activation in the CN modulated the development of mechanical hypersensitivity.

There is evidence that astroglial and microglial activation after peripheral nerve damage is accompanied by up-regulation of the intermediate filament protein, GFAP, and the surface antigen, complement receptor 3, respectively.45 We evaluated glial activation in the CN after median nerve CCI by immunohistochemistry using monoclonal antibodies specific to GFAP and OX-42 (which recognizes complement receptor 3). The current study found that p-ERK was strongly expressed in both GFAP and OX-42 immunoreactive cells in the CN of CCI rats. Phosphorylation of ERK leads to glial activation in the brain,46–48 but how ERK is activated in the CN after CCI remains poorly understood. It has been reported that glial ERK activation is regulated by intracellular calcium (Ca^{2+}) levels.49,50 Peripheral nerve injury induces the release of transmitters (e.g., glutamate and adenosine triphosphate) from primary afferents as a result of immediate injury-associated discharges and altered ectopic activity.32,51,52 This afferent activity may contribute to the activation of ERK in the CN, glutamate is one of the major neurotransmitters in primary afferent terminals.53,54 Pharmacologic studies showed that in cultured...
Fig. 7. Effects of medroxyprogesterone treatment on extracellular signal-regulated kinase (ERK) phosphorylation and glial fibrillary acidic protein (GFAP) and OX-42 expressions. (A–C) In sham-operated rats treated with vehicle, little phosphorylated ERK (p-ERK), GFAP, and OX-42 immunoreactivity was observed in the ipsilateral cuneate nucleus (CN). (D–I) There was intense p-ERK, GFAP, and OX-42 immunoreactivity in the ipsilateral CN 7 days after chronic constriction injury (CCI) in rats treated with vehicle or medroxyprogesterone at a dose of 20 mg/kg. (J) Western blot analysis shows p-ERK, GFAP, and OX-42 levels in the CN after medroxyprogesterone treatment. The sham-operated rats receiving 20 mg/kg, but not 15 mg/kg, medroxyprogesterone displayed a significantly higher p-ERK and OX-42 levels than those receiving vehicle (*, +P < 0.05 compared with the sham-operated plus vehicle group). There was a significantly higher GFAP level in sham-operated rats receiving either 15 or 20 mg/kg medroxyprogesterone than in those receiving vehicle (#P < 0.05). In CCI rats, significantly increased p-ERK, GFAP, and OX-42 levels were observed after medroxyprogesterone treatment at 15 or 20 mg/kg compared with vehicle administration (**, ##, ++P < 0.05 compared with CCI plus vehicle group). The ERK and β-actin were used as a loading control. Error bars represent mean ± SD. n = 6 rats per group. Scale bar = 100 μm.
Fig. 8. Effects of medroxyprogesterone treatment on the mechanical withdrawal threshold, proinflammatory cytokine expression, and endogenous allopregnanolone production. (A) Sham-operated rats receiving 15 or 20 mg/kg medroxyprogesterone displayed a significant decrease in mechanical withdrawal thresholds compared with those receiving vehicle. The mechanical withdrawal threshold was significantly decreased in chronic constriction injury (CCI) rats receiving 20 mg/kg medroxyprogesterone compared with those receiving vehicle. (B–D) The proinflammatory cytokine levels were increased in the cuneate nucleus (CN) of sham-operated rats receiving medroxyprogesterone compared with those receiving vehicle. The expression of proinflammatory cytokines in the ipsilateral CN on day 7 after CCI was more prominent in rats subjected to different doses of medroxyprogesterone than those given vehicle. (E) The allopregnanolone level was significantly increased in the ipsilateral CN of CCI rats receiving vehicle as compared with sham-operated rats receiving vehicle. A significant decrease in the level of allopregnanolone was observed in sham-operated rats treated with 20 mg/kg medroxyprogesterone compared to those treated with vehicle. The CCI rats treated with either 15 or 20 mg/kg medroxyprogesterone displayed a significantly lower allopregnanolone level in the CN when compared to those treated with vehicle. Error bars represent mean ± SD. n = 6 rats per group. *P < 0.05 between groups.
glia, glutamate activates N-methyl-D-aspartate (NMDA) receptors that depolarize the cell membrane and open voltage-gated Ca\(^{2+}\) channels, leading to a large Ca\(^{2+}\) influx and activation of the ERK signaling pathway. The Ca\(^{2+}\) channel blockers, gabapentin and pregabalin, are efficacious inhibitors of glial activation and treatment of neuropathic pain associated with diabetic neuropathy and CCI-induced allodynia.44,56 Accordingly, an increase in glial intracellular Ca\(^{2+}\) concentration may promote phosphorylation of ERK, which subsequently drives the activation of glia.

Recently, Guan et al. increased our knowledge of the connection between peripheral nerve injury and microglial activation. They found that peripheral nerve injury induces de novo expression of colony-stimulating factor 1 (CSF1) in injured small- and large-diameter dorsal root ganglion neurons and that CSF1 is then transported to the spinal cord, where it interacts with the CSF1 receptor on microglia. That study also found that DAP12, a microglial transmembrane adapter protein, is critical for CSF1-induced mechanical hypersensitivity; moreover, the CSF1–CSF1 receptor–DAP12 pathway, a key signaling cascade in neuropathic pain, acts in parallel to the known adenosine triphosphate–P2X4 pathway.57 Given that the CN also receives synaptic input from large-diameter sensory neurons, we speculate that this novel pathway may also be involved in the alteration of CN microglial phenotype after median nerve injury. Nonetheless, more work is required in the near future.

In addition to glial activation, ERK phosphorylation also regulates the glial release of proinflammatory cytokines.46–48 Recent work demonstrated that activated glia produce signaling mediators, such as proinflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6), cyclooxygenase-2, and inducible nitric oxide synthase, and that these mediators appear to contribute to different features of pathologic pain.58,59 Electrophysiologic studies demonstrated that TNF-α enhances NMDA-induced currents and facilitates excitatory synaptic transmission in superficial dorsal horn neurons.60 IL-1β increases both the frequency and amplitude of spontaneous excitatory postsynaptic currents and reduces the frequency and amplitude of spontaneous inhibitory postsynaptic currents.61 IL-6 suppresses GABA- and glycine-induced currents and decreases the frequency of spontaneous inhibitory postsynaptic currents.62 These results indicate a pivotal role for proinflammatory cytokines in the development of neuropathic pain via modulation of synaptic plasticity and neuronal excitability with subsequent central sensitization.

The current study reports that oral administration of allopregnanolone significantly attenuated p-ERK, GFAP, and OX-42 immunoreactivity, as well as proinflammatory cytokine levels in the CN, and ameliorated nerve injury-induced mechanical hypersensitivity. The neurosteroid allopregnanolone is a positive allosteric modulator of the GABA\(_{A}\) receptor, an ionotropic receptor consisting of α1, β2, and γ2 subunits, with several allosteric binding sites.63 Pharmacologic studies demonstrated that allopregnanolone blocks the opening of voltage-gated Ca\(^{2+}\) channels by binding to the neurosteroid binding site on the GABA\(_{A}\) receptor.64 Many in vitro and in vivo observations have shown the presence of functionally active GABA\(_{A}\) receptors on the glia cell membrane.65–67 In addition, an in vitro electrophysiologic study revealed that allopregnanolone also depresses NMDA receptor activation and associated downstream responses by altering GABA\(_{A}\) receptor activation-evoked membrane potential changes.68 We show herein that intra-CN administration of a GABA\(_{A}\), but not a GABA\(_{B}\), receptor antagonist to CCI rats reversed the effects of allopregnanolone. Based on these observations, we suggest that allopregnanolone attenuates the release of proinflammatory cytokines from glia and the development of neuropathic pain via GABA\(_{A}\) receptor–mediated suppression of glial intracellular Ca\(^{2+}\) levels and ensuing inhibition of ERK phosphorylation.

In the current study, we observed that production of allopregnanolone was increased in the ipsilateral CN of CCI rats, compared to sham-operated rats. It has been suggested that endogenous allopregnanolone in the nervous system may be derived not only from progesterone in the gonads, particularly in the ovary, but also from progesterone synthesized de novo from precursors such as cholesterol and pregnenolone in the central and peripheral nervous systems.22 The biosynthesis of allopregnanolone from progesterone requires the complementary activities of two key enzymes, a 5α-reductase that converts progesterone into dihydroprogesterone and a 3α-HSOR that produces allopregnanolone from dihydroprogesterone.22 Meyer et al. demonstrated that 5α-reductase and 3α-HSOR activities were markedly increased together with allopregnanolone levels in the rat spinal cord dorsal horn after sciatric nerve ligation. Furthermore, Qiu et al. found that 3α-HSOR is mainly coexpressed in neurons and microglia in the spinal dorsal horn after sciatric nerve CCI. In order to clarify the effects of endogenous allopregnanolone production in the CN on neuropathic pain development, we administered the 3α-HSOR inhibitor medroxyprogesterone into the CN of sham-operated and median nerve CCI rats. Medroxyprogesterone injection reduced endogenous allopregnanolone levels and led to further increases in p-ERK, GFAP, and OX-42 immunoreactivity and proinflammatory cytokine levels in the CN. Further, an even greater decline in mechanical withdrawal threshold was observed in CCI rats administered medroxyprogesterone. Taken together, these findings suggest that allopregnanolone exerts a physiologic analgesic effect as reduced allopregnanolone makes the rats more susceptible to nerve injury-induced neuropathy. However, the endogenous allopregnanolone biosynthesis appears insufficient to restore normal homeostasis after peripheral nerve injury; thus, exogenous supplementation may be an effective strategy for interfering with the development of the chronic pain state.

Neuropathic pain is a chronic disorder having multiple causes and affecting millions of patients. Effective alleviation and treatment of this often intractable pain remains an unmet medical need, and the development of therapeutic strategies that are devoid of adverse reactions and unwanted side effects is
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urgently required. The data presented herein show allodynia
effects of the neurosteroid allopregnanolone in an animal model
of neuropathic pain. In humans, it is also observed that serum
allopregnanolone levels were inversely associated with self-
reported low-back pain and chest pain,70 suggesting its activity
as an endogenous analgesic. Furthermore, apart from fatigue
and mild nausea, intravenous dosing of allopregnanolone did
not have any adverse effects in healthy women.71 In conclusion,
our study shows that allopregnanolone, which possesses a high
therapeutic potential and a good safety profile, may be an ideal
drug candidate against chronic neuropathic pain.

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Competing Interests
The authors declare no competing interests.

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For those who favor adequately oxygenating their anesthetized patients, 1936 was indeed a banner year. That was the year when neuropathologist Cyril Courville, M.D. (1900 to 1968, top left), published his landmark paper in the journal Medicine titled, “Asphyxia as a Consequence of Nitrous Oxide Anesthesia.” That same year, a future biomedical engineer and pioneer of pulse oximetry, Takuo Aoyagi (top right), was born in Niigata Prefecture, Japan. And finally, also in 1936, the New York Society of Anesthetists nationalized into the American Society of Anesthetists (ASA). Fifty years following that name change, the ASA (now rebranded “Anesthesiologists” and with its seal colorized, bottom center) would publish its “Standards for Basic Intra-operative Monitoring” with “pulse oximetry…encouraged.” Today, 80 years after the banner year of 1936, the ASA Charitable Foundation supports the Lifebox initiative to supply pulse oximeters worldwide for safer anesthesia in “under-resourced countries.” (Copyright © the American Society of Anesthesiologists’ Wood Library-Museum of Anesthesiology.)

courville, aoyagi, and ASA: 1936 Was a Banner Year!