The Journal of Pain, Vol 7, No 5 (May), 2006: pp 337-345
Available online at www.sciencedirect.com

Covariance Among Age, Spinal p38 MAP Kinase Activation and Allodynia

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Abstract: This study examined effects of age (young rats, approximately 35 days, vs mature rats, approximately 75-110 days) on spinal nerve ligation (SNL)–induced tactile allodynia and phosphorylation of p38 (as measured by phospho-p38 MAP kinase [P-p38]) in dorsal root ganglia and spinal cord. Effects of SNL combined with spinal nerve transection also were assessed. Mature rats displayed milder SNL-induced allodynia than young rats. Addition of spinal nerve transection distal to the ligation in older animals resulted in an allodynia comparable to that seen in young animals. In DRG, both groups displayed early (5 h) and late (10 days) peaks in P-p38 following surgery as compared to naïve rats. Tight nerve ligation plus transection had no additional effect on P-p38 levels in DRG. In spinal cord, young rats had increased levels of P-p38 from 5 h to 3 days after SNL. Phosphorylated p38 levels then decreased, with a second peak at 10 days. In contrast, spinal cord from mature rats showed less early p38 phosphorylation, although they also displayed a late 10-day peak. Addition of a transection to the ligation produced restoration of the early peak along with intensification of allodynia. Alterations of spinal P-p38 at early time points thus seem to covary with intensity of tactile allodynia.

***Perspective:** Age and modifications to spinal nerve ligation, a common model of neuropathic pain, influence spinal p38 phosphorylation and allodynia. Early levels of spinal P-p38 seem to covary with allodynia intensity. This may mean that small variations of an injury could affect the therapeutic window of a p38 antagonist.*

***Key words:** Age, nerve transection, MAP kinase, pain, neuropathic pain model.*

Spinal nerve ligation (SNL) is one of the more commonly used animal models of neuropathic pain. As originally described,[10] either the L5 or both the L5 and the L6 spinal nerves are tightly ligated. Although both surgical preparations show a profound mechanical allodynia, the preparation with 2 ligated nerves is reported to produce a slightly greater and longer-lasting effect.[10] The magnitude and duration of the allodynia has been reported to be age dependent,[3] with young rats (defined as 40-50 days old, body weight less than 150 g) achieving the highest degree of allodynia and mature or old rats (4 months to 1 yr old, body weight over 380 g) developing less mechanical allodynia, no cold allodynia, and only a small degree of cold hyperalgesia with a longer onset time. Lack of mechanical allodynia with SNL has been reported in some strains of rat,[12] and major genetic differences in the probability and magnitude of SNL-evoked allodynia have been demonstrated in the mouse.[15] Perhaps in response to decreased allodynia in adults or to varying strains, the literature includes references to a modified SNL in the rat.[25] In these animals, the original procedure is supplemented by a transection just distal to the tight ligation. This modification is said to enhance the degree of allodynia. Thus far, these comparative data are anecdotal and have not apparently been published.

Our lab recently demonstrated in young Sprague-Daw-

Received December 8, 2004; Revised November 29, 2005; Accepted December 22, 2005.

Supported by National Institutes of Health Grant NS NS41580 (L.S.S.) and the Bavaria California Technology Center.

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1526-5900/$32.00

doi:10.1016/j.jpain.2005.12.007

ley rats that there is a time-dependent increase in phosphorylated p38 mitogen-activated protein kinase (MAPK) (P-p38) in both dorsal root ganglia (DRG) and lumbar spinal cord following SNL lesions.[18] The purpose of the present study was to determine: 1) whether age or modification of the SNL procedure affected the magnitude of the allodynia; 2) whether age covaried with expression of P-p38 in either the DRG or the spinal cord and/or with magnitude of allodynia; and 3) if addition of nerve transection changed P-p38 expression in mature rats. Some of these results have been presented previously in abstract form.[19]

Materials and Methods

Subjects and Procedures

Male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were used in procedures approved by the Animal Care and Use Committee of the University of California, San Diego. Rats were housed in groups of 2-3 with food and water available ad libitum, on a 12:12 h light-dark cycle. Animals were used for longitudinal behavioral experiments, or, if intended for Western blots, tissue was harvested at the designated time points. Animals in the behavioral experiment included young rats (100-130 g, approximately 33-38 days old) and mature rats (350-390 g, approximately 75-110 days old). Young rats received an SNL, and the mature rats received either a sham operation, SNL, or modified SNL (SNL+). Western blots were performed on L5-6 DRGs and on the ipsilateral lumbar (L4-6) spinal cord in young and mature rats with either sham surgery or SNL lesions. In addition, tissue from mature rats with modified SNL was harvested at 2 time points after surgery: 5 hours and 10 days.

Surgery

All surgical procedures were performed under isoflurane anesthesia (5% for induction and 2% for maintenance) in 50% O_2. The SNL was performed as described previously.[10] A midline incision was used to expose the left L6 transverse process. This process was removed from animals in all surgical groups and the L5 and L6 spinal nerves isolated. In the SNL and SNL+ groups the spinal nerves were tightly ligated with 6-0 silk. The SNL+ animals then had both spinal nerves transected just distal to the ligature during the same surgical procedure.

Evaluation of Allodynia

Rats were placed in individual Plexiglas compartments (26 × 11 × 20 cm) with wire mesh bottoms. Following a 30-min acclimation period, mechanical allodynia was assessed using von Frey filaments and the Dixon up-down method as described by Chaplan et al.[1] Briefly, calibrated filaments (Stoelting, Wood Dale, IL.) with buckling forces between .41 and 15.2 g were applied perpendicularly to the mid-paw plantar surface until the filament was slightly bent (L4 dermatome) and held there for 4-6 s. Stimuli were separated by several seconds or until the animal was calm with both hindpaws placed on the grid. Testing always began with the 2.0-g filament. The 50% probability withdrawal threshold was determined. Tests were performed on animals before surgery and on days 1, 4, 8, 11, 14, and 17 after surgery. While it was obviously not possible to blind the person performing the behavioral testing to the age (size) group of the rat, she was unaware of the surgical procedure (sham, SNL, or SNL+) performed on the mature rats. All testing was done between 9 am and 1 pm.

Western Blots

At 5 hours or 1, 3, 5, or 10 days after surgery, animals were quickly anesthetized with isoflurane (5%). After decapitation, the spinal cord was hydroextruded with iced saline, and the ipsilateral lumbar enlargement and L5-6 DRG were collected. Naïve animals were treated similarly. Tissue was immediately placed in iced 50 mmol/L Tris buffer [pH 8.0, 0.5% Triton X-100, 150 mmol/L NaCl, 1 mmol/L EDTA, and phosphatase inhibitor cocktail 1 and 2 (Sigma 1:100)] and sonicated. The protein concentration of each supernatant was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL) and 50 μg of protein was loaded in each well. Prior to loading, samples were denatured and subjected to NuPAGE 4%-12% Bis-Tris gel electrophoresis and then electrophoretically transfered to nitrocellulose membranes (Osmonics, Minnetonka, MN). Membranes were blocked for 1 h at room temperature in 5% low-fat milk in PBS containing 0.1% Tween 20 and then incubated with antibodies overnight at 4°C. After washing, the antibody protein complexes were probed with secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature and detected with chemoluminescent reagents (Supersignal, Pierce Biotechnology, Rockford, IL.). The nitrocellulose membranes were stripped with the Re-Blot Western blot recycling kit (Chemicon, Temecula, CA) and reblotted with different antibodies. The antibodies used detected P-p38 (1:1000), total p38 (1:500; Cell Signalling Technology, Beverly, MA), and β-actin (1:5000; Sigma, St Louis, MO). Immunopositive bands were quantified using Image Quant software (Amersham Biosciences, Piscataway NJ). Data for P-p38 and total p38 were normalized against β-actin expression in the corresponding sample and compared to, and expressed as a percentage of, levels detected in DRG/spinal cord from naïve animals of the same age that were run on the same gel.

Statistical Analysis

To determine differences from baseline, percentage differences in allodynia for different age or surgical groups were calculated by comparing areas under the curve. Unpaired *t* tests were used to compare individual time points of the behavioral data as well as the Western blot data. *P* values of $\leq .05$ were considered to be statistically significant. All data are presented as mean ± SEM.