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MECHANICAL ALLODYNIA AND SPINAL UP-REGULATION OF $P2X_4$ RECEPTOR IN EXPERIMENTAL AUTOIMMUNE NEURITIS RATS

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Abstract—Experimental autoimmune neuritis (EAN) is the animal model of acute inflammatory demyelinating polyradiculoneuropathy (AIDP) that is the most common subtype of Guillain-Barre syndrome (GBS). While neuropathic pain is a common symptom of GBS, its underlying mechanisms remain elusive. Central sensitization, particularly spinal glia (microglia and astrocytes) activation, is important for the initiation and maintenance of neuropathic pain. $P2X_4$ receptor ($P2X_4R$) is an ATP-gated ion channel and its spinal up-regulation has been found to be crucial for the development of neuropathic pain following peripheral nerve injury. The initiation of mechanical allodynia in rat EAN was observed at day 9 before the onset of neurological signs. Maximal level of mechanical allodynia was observed from days 17–19 and then a slow recovery, long after the cessation of typical neurological signs of EAN, until day 37 was observed. Expression of $P2X_4R$ in lumbar spinal cords was studied by immunohistochemistry. $P2X_4R$ immunoreactivity (IR) was mainly seen in gray matter, particularly in the dorsal horn. Accumulation of $P2X_4R^+$ cells in the lumbar dorsal horn was observed at day 9, reached the maximal level at day 17 and remained elevated until day 37 after immunization. Furthermore, a negative correlation between the density of $P2X_4R^+$ cells in the lumbar dorsal horn with mean hind-paw withdrawal threshold in EAN rats was seen, indicating that $P2X_4R$ might contribute to EAN mechanical allodynia. Double staining revealed that almost all $P2X_4R^+$ cells co-expressed CD68, a marker for reactive microglia, but not the astrocyte marker, glial fibrillary acidic protein (GFAP). Our data demonstrate that EAN induces mechanical allodynia and $P2X_4R$ expression in spinal microglia, suggesting that EAN is a good animal model for neuropathic pain in polyneuropathy and spinal microglia activation might participate in EAN-induced neuropathic pain. **© 2008 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: experimental autoimmune neuritis, neuropathic pain, $P2X_4$ receptor, microglia.

Guillain-Barre syndrome (GBS) is a human acute inflammatory demyelinating neuropathy caused by an autoimmune attack on the peripheral nervous system, and characterized by motor disorders as well as variable sensory disturbances (Hughes and Cornblath, 2005). The acute inflammatory demyelinating polyradiculoneuropathy (AIDP) is the most common subtype of GBS that is the world's leading cause of acute autoimmune neuromuscular paralysis. Neuropathic pain, which is caused by lesion or inflammation of the nervous system (peripheral and/or CNS), is a common symptom of GBS, occurring in 55–85% of cases and requires aggressive treatment (Pentland and Donald, 1994; Moulin et al., 1997). Despite the fact that a high percentage of patients with GBS experience intense pain, the symptom is often overlooked (Howarth, 2000), animal models were lacking and the underlying mechanisms remained unresolved. Experimental autoimmune neuritis (EAN) is a T cell–mediated acute demyelinating disease of the peripheral nervous system, mirrors many of the clinical, electrophysiological and immunological aspects of human demyelinating polyneuropathy and therefore serves as the animal model of the AIDP subtype of GBS (Hahn, 1996). Recently, thermal hyperalgesia and mechanical allodynia have been successfully observed in a newly established EAN pain model, which facilitates the investigation of mechanisms underlying autoimmune neuropathies, like GBS (Moalem-Taylor et al., 2007).

Neuropathic pain can be due to peripheral and/or central sensitization. And accumulated data reveal that spinal glia (microglia and astrocytes) are essential for the creation and maintenance of neuropathic pain. Spinal glia are activated in response to a diversity of stimuli, ranging from peripheral inflammation to CNS injury. Reactive spinal glia express varieties of receptors and secrete inflammatory mediators, such as interleukin (IL)-1ß, IL6, tumor necrosis factor-α and neurotrophin, which act either directly on dorsal horn neurons that transmit pain (nociceptive neurons) or indirectly on primary afferents, leading to increased sensitivity of the nociceptive neurons. Spinal glia activation is necessary and sufficient to induce neuropathic pain. Inhibition of spinal glia can effectively attenuate or even block the development of neuropathic pain in many animal models (see reviews Ji and Strichartz, 2004; McMahon et al., 2005; Tsuda et al., 2005).

Recently, up-regulation of $P2X_4$ receptor ($P2X_4R$) on spinal microglia was demonstrated and was related to pain hypersensitivity following peripheral nerve injury (Tsuda et al., 2003). Deactivation of spinal $P2X_4R$ reverses tactile allodynia induced by peripheral injury, indicating the important roles of spinal $P2X_4R$ in neuropathic pain (Tsuda et al., 2003). $P2X_4R$ was first cloned from rat brain and is a subtype of the P2X receptor family (Bo et al., 1995). In the nervous system, $P2X_4Rs$ are involved in synaptic

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Abbreviations: AIDP, acute inflammatory demyelinating polyradiculoneuropathy; CFA, complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis; EAN, experimental autoimmune neuritis; GBS, Guillain-Barre syndrome; GFAP, glial fibrillary acidic protein; HPF, high-power field; HWT, hind-paw withdrawal threshold; IL, interleukin; IR, immunoreactivity; PBS, phosphate-buffered saline; $P2X_4R$, $P2X_4$ receptor; SCI, spinal cord injury.

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transmission in central and peripheral neurons and have excitatory effects in response to binding of extracellular ATP (North, 2002). $P2X_4R$ displays higher expression in spinal cord microglia after peripheral nerve injury and is believed to induce pain facilitation (Tsuda et al., 2003). Increased $P2X_4R$ expression in spinal microglia was also observed after spinal cord injury (SCI) (Schwab et al., 2005) and formalin-induced inflammatory pain in our group (Guo et al., 2005).

Thus, in the present investigation we studied the time course of mechanical allodynia, which is often related with central sensitization (Woolf and Salter, 2000), in the newly established EAN pain model and investigated the time course and cellular distribution of $P2X_4R$ in lumbar spinal cords of EAN rats.

EXPERIMENTAL PROCEDURES

Animal treatment

Male Lewis rats (8–10 weeks, 170–200 g, Charles River, Sulzfeld, Germany) were housed under a 12-h light/dark cycle and free access to food and water. All animal procedures were in accordance with a protocol approved by the University of Tuebingen Institutional Animal Care and Use Committee and the local Administration District Official Committee. All experiments conformed to international guidelines on the ethical use of animals and all efforts were made to minimize the number of animals and their suffering.

One symptom of EAN rats is ascending paraparesis/paralysis that can impact their ability to produce withdrawal responses. Since severity of EAN neurological scores and pathology correlates with the antigen dose used for immunization (Hahn et al., 1988) we used a reduced amount of P2 antigen to induce EAN to avoid complete paralysis. Further immunizations were done at the base of the tails instead of hind paws to circumvent interference of local inflammation with hind-paw withdrawal responses. Briefly, for EAN induction, rats were immunized by s.c. injection at the base of tail with 80 μL of an inoculum containing 80 μg of synthetic neuritogenic P2 peptide of peripheral myelin-amino acids 57–81, which were synthesized by GeneScript Corporation, Scotch Plains, NJ, USA, under ether anesthesia. The peptide was dissolved in phosphate-buffered saline (PBS) (2 mg/mL) and then emulsified with an equal volume of complete Freund's adjuvant (CFA) containing 2 mg/mL mycobacterium tuberculosis to get a final concentration of 1 mg/mL. CFA control rats were immunized with 80 μL inoculum that was prepared by emulsifying with equal volume of PBS and CFA containing 2 mg/mL mycobacterium tuberculosis.

EAN clinical scores of CFA control and EAN rats were evaluated every second day as follows: 0=normal, 1=reduced tonus of tail, 2=limp tail, impaired righting, 3=absent righting, 4=gait ataxia, 5=mild paresis of the hind limbs, 6=moderate paraparesis, 7=severe paraparesis or paraplegia of the hind limbs, 8=tetraparesis, 9=moribund, 10=death. Body weight of the rat was measured every second day as well.

Mechanical allodynia measurement

Mechanical allodynia was assessed by measuring rat hind-paw withdrawal threshold (HWT) using a mechanical plantar test apparatus (Ugo Basile, Milan, Italy), which is an automated von Frey–type system, in EAN and CFA control rats (six rats per group). The HWT was tested at a fixed time, between 9:00 and 12:00 AM, at every second day postimmunization. One week before measurement rats received training sessions every day. Tested rats were put in testing room that was kept silent and well controlled during testing for at least 10 min to habituate to environments. Thereafter, rats were placed in a Perspex enclosure (Ugo Basile) over a mesh floor and allowed to acclimate for 15 min. The mechanical force, which went from 0 to 50 g over a 15 s period, was exerted on the middle of the hind paw using a fine metal filament. Force was recorded when the withdrawal reflex could be observed. Left and right hind paw was measured five times each with a 2-min interval between stimuli and the mean value was calculated. Results for the left and right hind paws were combined. The baselines of HWT were obtained 1 day before immunization. According to the baseline, the rats were stratified into groups so that there were no significant differences of HWT mean values among groups.

Immunohistochemistry

To evaluate time course of $P2X_4R$ expression in lumbar spinal cords, rats were killed at days 0, 5, 9, 13, 16, 17, 27, or 37 (n=3 for each group) after immunization. Rats were deeply anesthetized with ether and perfused intracardially with 4 °C 4% paraformaldehyde in PBS. Spinal cords were quickly removed and postfixed in 4% paraformaldehyde overnight at 4 °C. Spinal cords were cut, divided into 8-mm segments, embedded in paraffin, serially sectioned (3 μm) and mounted on silane-covered slides.

After dewaxing, cross-sections of spinal cords were boiled (in a 600 W microwave oven) for 15 min in citrate buffer (2.1 g sodium citrate/L, pH 6). Endogenous peroxidase was inhibited with 1% H_2O_2 in methanol for 15 min. Slices were incubated with 10% normal pig serum (Biochrom, Berlin, Germany) to block nonspecific binding of immunoglobulins and then with a previously reported rabbit polyclonal antibodies: $P2X_4R$ (1:200; Alomone Laboratories, Jerusalem, Israel) against the $P2X_4R$ peptide (KKYKYVEDYEQGLSGEMNQ). Antibody binding to tissue sections was visualized with a biotinylated swine anti-rabbit (DAKO, Hamburg, Germany) IgG $F(ab)_2$ antibody fragment. Subsequently sections were incubated with a streptavidin–avidin–biotin complex (DAKO), followed by development with diaminobenzidine (DAB) substrate (Fluka, Neu-Ulm, Germany). Finally, sections were counterstained with Maier's hemalum. Specificity of our applied $P2X_4R$ antibody has been described in our previous paper (Guo et al., 2005).

Double staining

In double labeling experiments, sections were pre-treated as described above but without counterstaining with Maier's hemalum. Then they were once more irradiated in a microwave for 15 min in citrate buffer and were incubated with 10% normal pig serum (Biochrom). Subsequently, sections were incubated with the appropriate second primary antibodies for 1 h at room temperature. Consecutively, visualization was achieved by adding secondary antibody in TBS-BSA for 30 min and then alkaline phosphatase-conjugated avidin complex diluted 1:100 in Tris–BSA for another 30 min. Finally immunostaining was developed with Fast Blue BB salt chromogen–substrate solution, but by omission of counterstaining with hemalum. Following monoclonal antibodies were applied: mouse monoclonal antibodies against ED-1 (1:100; Serotec, Oxford, UK) to detect microglia and glial fibrillary acidic protein (GFAP) (1:50; BD Bioscience, Germany) for astrocytes.

Evaluation and statistical analysis

After immunostaining, $P2X_4R$ expression in lumbar spinal cords of different time points was examined by light microscopy. As $P2X_4R$ expression was mainly located to dorsal horns, numbers of $P2X_4R^+$ cells in the left and right dorsal horns were further semiquantified using the following method by two investigators unaware of the experimental grouping. For counting, sections were

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