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Characterization of the acute and persistent pain state present in K/BxN serum transfer arthritis

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Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune arthritis that affects approximately 1% of the population. Synovial inflammation cannot fully explain the level of pain reported by patients and facilitation of pain processing at the spinal level has been implicated. We characterized the K/BxN serum transfer arthritis model as a model of joint inflammation-induced pain and examine pharmacologic responsiveness and spinal glia activation. Mechanical allodynia developed congruently with joint swelling. Surprisingly, allodynia persisted after resolution of inflammation. At the peak of joint inflammation, (days 4–10) hypersensitivity was attenuated with i.p. etanercept, gabapentin, and ketorolac. Following resolution of synovial inflammation (day 19–28), only gabapentin relieved allodynia. The superficial dorsal horn of arthritic mice displayed increased staining of microglia at early and late time points, but astrocyte staining increased only during the inflammatory phase. ATF3, a marker of nerve injury, was significantly increased in the lumbar dorsal root ganglia during the late phase (day 28). Hence, serum transfer in the K/BxN serum transfer arthritis model produces a persistent pain state, where the allodynia during the inflammatory state is attenuated by TNF and prostaglandin inhibitors, and the pharmacology and histochemistry data suggest a transition from an inflammatory state to a state that resembles a neuropathic condition over time. Therefore, the K/BxN serum transfer model represents a multifaceted model for studies exploring pain mechanisms in conditions of joint inflammation and may serve as a platform for exploring novel treatment strategies for pain in human arthritic conditions.

K/BxN serum transfer arthritis produces persistent mechanical hypersensitivity despite resolution of clinical signs with evidence of transition from an inflammatory to neuropathic pain state.

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Keywords

arthritis; allodynia; astrocytes; microglia; inflammation; hyperalgesia

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease hallmarked by synovial inflammation and matrix destruction. Therapy reduces clinical signs of arthritis, yet joint pain often persists [52]. Common rodent models used to study inflammation-induced pain include intra-plantar and intraarticular injections of inflammatory agents. Although useful, these models often do not fully address the impact of autoimmune mechanisms, such as B and T cell responses, nor do they allow assessment of chronic inflammation. From this aspect, the K/BxN serum transfer model may be useful as a model of arthritis-induced pain.

K/BxN mice spontaneously produce autoantibodies against the ubiquitous protein glucose-6-phosphate isomerase (GPI), which is present in the joints, with resultant immune complex mediated development of inflammatory arthritis [33,39]. Serum from these mice can be used to induce transient inflammatory arthritis in a wide range of mouse strains [42]. This broad transfer applicability distinguishes the K/BxN serum transfer model from many other common models RA, such as collagen induced arthritis (CIA) which requires a specific genetic background [9,25]. The K/BxN serum transfer model has a predictable onset of clinical signs of arthritis as recipient mice receive the same quantity of autoantibodies at the time of injection, and this model is not dependent upon breaking T cell tolerance. The clinical profile has a severe inflammatory phase that reliably resolves as the autoantibodies are cleared and not replenished by autologous cells.

While well established in the RA field, the K/BxN serum transfer arthritis model has not been characterized as an experimental model of inflammatory pain. In humans and animal models, persistent inflammation in the joint is associated with ongoing pain and a hypersensitivity to innocuous stimuli [34,49]. As ligaments, fibrous capsule, meniscus, periosteum and the synovial layer are innervated by sensory A β , A δ - and C-fibers noxious sensation can be evoked from these structures [49]. The increased pain sensitivity is thought to reflect changes in the innervated joint, peripheral nerve, dorsal root ganglia (DRG) as well as in the spinal cord neurons, leading to altered processing of nociceptive transduction [24,31,49]. Noteworthy, an increased expression of activating transcription factor 3 (ATF3) [60] has been reported in DRGs following chronic joint inflammation, indicating that long-term inflammation may lead to changes resembling a nerve injury state [3,24,30]. In addition, activation of microglia and astrocytes has been implicated in spinal nociceptive processing, both in models of inflammation and nerve injury-induced pain (reviewed in [40]). Pharmacologic interventions reducing glia activation reduce pain behavior, supporting a role for these cells in pain signaling [4,23,46,55,63].

The aim of this study was to characterize the K/BxN serum transfer model as a model of arthritis-induced pain. Hence we examined i) the temporal profile of pain behaviors, ii) the antinociceptive effect of three conventional analgesics, iii) spinal microglia and astrocyte activation as well as iv) expression of the nerve-injury marker ATF3 protein in the DRG.

2. Materials and methods**2.1 Animals**

All animal experiments were carried out according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego (under the Guide

for Care and Use of Laboratory Animals, National Institutes of Health publication 85–23, Bethesda, MD, USA) and the local Ethical Committee for animal experiments in Sweden (Stockholms Norra Djurförsöksetiska Nämnd).

Mice were housed up to four per standard cage at room temperature, maintained on a 12-h light/dark cycle (light on at 07:00 h). Testing was performed during the light cycle. Food and water were available *ad libitum*. C57BL/6 mice (male, 25–30 g) were purchased from Harlan (Indianapolis, IN). KRN T cell receptor (TCR) transgenic mice were a gift from Drs. D. Mathis and C. Benoist (Harvard Medical School, Boston, Massachusetts, USA) and Institut de Génétique et de Biologie Moléculaire et Cellulaire (Strasbourg, France), and were maintained on a C57BL/6 background (K/B). Arthritic mice were obtained by crossing K/B with NOD/Lt (N) animals (K/BxN). NOD/Lt mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA).

2.2 Serum transfer and arthritis scoring

Blood from arthritic adult K/BxN mice was collected, centrifuged at 10,000 rpm for 10 min and the sera pooled. Groups of six to nine recipient C57BL/6 mice received 100 μ l sera by 1 sera by intraperitoneal (i.p.) injection on days 0 and 2 (total volume 200 μ l). Control animals were injected with pooled sera from naïve C57BL/6 mice. Clinical arthritis scores were evaluated using a scale of 0–4 as described previously [8]. Briefly, one point was given if swelling was observed for i) any or all of the digits ii) the knuckles, iii) the dorsal aspect of the paw and/or iv) the ankle. Hence, a higher number represents a greater disease progression, with a maximum score per paw of 4 and 16 per mouse. Ankle thickness was measured with a caliper in mm and compared to baseline thickness.

2.3 Drugs and drug delivery

Groups of K/BxN serum treated arthritic mice (n=6) received a single i.p. injection of gabapentin (100mg/kg; Pfizer), ketorolac (7.5mg/kg; Sigma), or etanercept (5 mg/kg; Amgen) during the period of acute inflammatory arthritis (day 4–10) or during the period of persistent allodynia (day 19–23) and were measured for changes in tactile allodynia for four hours following treatment. Gabapentin, ketorolac, or vehicle were delivered randomized to the mice on day 4 and tactile allodynia monitored for four hours; subsequently drugs were allowed to wash out for 48 hours before the same procedure was repeated on day 6. Following a second drug washout period of 3 days, etanercept or vehicle were injected on day 10. A second cohort of arthritic mice was used to test the drugs at the post-inflammation stage following a similar protocol of testing on days 19, 21, and 23. All drugs were dissolved in normal saline, which was used as vehicle control. Etanercept [24], gabapentin [15,57], and ketorolac [13] dosages were based on previous studies.

2.4 Behavioral tests

Animals were subjected to two behavioral tests, the thermal paw threshold test and the von Frey mechanical threshold test. To assess the thermally evoked paw withdrawal response, a Hargreaves-type testing device was used [12] (UARDG, Department of Anesthesiology, University of California, San Diego, 92103-0818). The device consists of a glass surface (maintained at 30 °C) on which the mice are placed individually in Plexiglas cubicles. The thermal nociceptive stimulus originates from a focused projection bulb positioned below the glass surface. A timer is activated by the light source, and latency was defined as the time required for the paw to show a brisk withdrawal as detected by photodiode motion sensors that stopped the timer and terminated the stimulus.

Mechanical sensitivity was assessed using von Frey hairs with hair values ranging from 2.44 to 4.31 (0.03g to 2.00g) and the up–down method [6] was applied as previously described. The

50% probability withdrawal threshold (force of the von Frey hair to which an animal reacts to 50% of the presentations) was recorded. Given the systemic nature of this model mechanical and thermal sensitivity values for both paws were measured and averaged in order to attain a single data point per day of measurement.

The data were also presented as the hyperalgesic index, which represents the area under the time effect curve after drug delivery in which the percentage change from baseline (e.g. arthritic state) threshold is plotted against time. The resulting value has the units percentage change $\times$ time. The formula for calculating the percentage change is $100 \times (\text{baseline threshold} - \text{post-drug threshold}) / (\text{baseline threshold})$, where threshold was expressed in grams. Decreasing values indicate analgesic efficacy. The experimenter was blinded to drug treatments during all behavioral testing.

2.5 Histology

Whole knee joint sections were stained with hematoxylin and eosin (H&E) (HistoTox, Boulder, Colorado, USA). Histopathological evaluation was performed as previously described for inflammation and erosion [8].

2.6 Immunohistochemistry

At specified time points, mice were deeply anesthetized with euthasol and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde. The lumbar spinal cord and L4–6 DRGs were removed, post-fixed and cryoprotected in sucrose. The DRGs were cut in transverse sections (10 μm) and mounted on glass slides. Lumbar sections (L4–L6) of the spinal cord were cut as free floating sections (30 μm). Tissue sections were incubated with anti-GFAP antibody (1:1000, Sigma), anti-Iba1 antibody (1:500, Abcam), anti-NeuN:biotinylated (1:1000, Chemicon) or anti-ATF3 (1:1000, Santa Cruz). Binding sites were visualized with secondary antibodies conjugated with fluoro-Alexa-488 and Alexa-555 or streptavidin conjugated fluoro-Alexa-555 where appropriate (1:5000, Molecular Probes, Eugene, OR). An avidin/ biotin blocking kit (Vector Labs) was utilized as appropriate before biotinylated NeuN. Images were captured by Leica TCS SP5 confocal imaging system and quantified using Image-Pro Plus v.5.1 software.

Reactivity of glia is characterized by both an increase in the number of cells, and in the conformational extension of these cells (rounding of the cell bodies and thickening of processes) leading to an increase in labeling with increasing glia reactivity. Microglia (Iba1) and astrocyte (GFAP) staining was quantified by measuring the total integrated intensity of pixels divided by the total number of pixels in a standardized area of the dorsal horn. The investigator was blinded to experimental conditions during the quantification. Staining intensity was examined in lamina I-II of the superficial dorsal horn or of a standardized box in lamina V of the deeper dorsal horn region with 3 slices (separated by at least 180 μm) examined per animal and 3 animals per m) examined per animal and 3 animals per experimental condition. Only pixels above a preset background threshold were included. An increase in the integrated intensity / pixels for Iba1 and GFAP staining was interpreted to signify microglia and astrocyte reactivity, respectively. All data is presented as % change from the corresponding control group. Statistics were performed on raw data values.

ATF3 staining was assessed by counting the number of cells with ATF3 positive nuclei over the total number of NeuN positive cells with visible nuclei. L4 and L5 DRGs were group mounted for individual animals onto slides. Each slide contained 2–4 DRGs (L4–L5). All slides were separated by at least 60 μm . Three to five slides per animal (n= m. Three to five slides per animal (n=3–4) were counted.

2.7 Quantitative Real-time PCR (RT-PCR)

The mRNA from flash frozen lumbar spinal cord lumbar was extracted using Trizol (Invitrogen) according to the manufacturer's protocol. Complementary DNA was prepared and quantitative real-time PCR performed with TaqMan Gene Expression Assays (both according to the manufacturer's instructions, Applied Biosystems) to determine relative mRNA levels, using the GeneAmp 7500 Fast Sequence Detection system (Applied Biosystems). Pre-developed specific primers were used to detect glial fibrillary acidic protein (GFAP) (Assay ID Mm00546086_m1), CD11b (Assay ID Mm00434455_m1), and HPRT1 (Assay ID Mm00446968_m1) (Applied Biosystems). Relative abundance was calculated by comparing delta-CT values [45] and the data were then normalized to HPRT1 gene expression to obtain relative concentration and presented as relative gene expression.

2.8 Statistics

Data are given as mean $\pm$ standard error. For comparison of change of pain behavior, a two-way ANOVA for repeated measures with Bonferroni *post hoc* tests was used. For comparison of microglia, and astrocyte changes a one-way ANOVA with Bonferroni *post hoc* test was used. ATF3 and mRNA changes were analyzed by a student's t-test compared to control values. Drug treatment data were also presented as a hyperalgesic index, a derived value that defines the magnitude of arthritis induced sensitization by quantifying the area under the curve compared to baseline values.

3. Results

3.1 Characterization of arthritic pain behavior and clinical signs

Mice were injected on days 0 and 2 with 100 μ l pooled K/BxN sera. As previously reported, 1 pooled K/BxN sera. As reviously reported, within 24 hours following serum transfer, mice developed significant clinical signs of arthritis [33]. These signs, including redness and swelling, were significantly increased over days 1–12, peaking at day 6, $p < 0.05$ – 0.001 (Figure 1A). Ankle joints diameter was also significantly increased compared to baseline levels on days 3–9, $p < 0.05$ – 0.001 (Figure 1B). Significant tactile allodynia was present in arthritic animals on days 2–28, excluding day 12, compared to control sera treated animals, $p < 0.01$ – 0.001 (Figure 1C). Animals reached maximum severity of tactile allodynia at day 4, which surprisingly remained robustly stable excluding day 12 through the end of the study at day 28. A mild thermal hypoalgesia was initially present in these arthritic mice compared to control sera treated mice from days 4–6, $p < 0.001$, before returning to baseline. Arthritic animals showed no other signs of thermal sensitivity after day 6 (Figure 1D).

Histopathologic changes in the knee joints were examined using H&E staining. Joint sections from mice injected with control sera harvested day 6 (Figure 2A) and day 28 (Figure 2B) showed no evidence of infiltrating inflammatory cells or alterations in the bone or cartilage architecture. In comparison, knee joint sections from K/BxN sera treated arthritic mice showed inflammatory cell infiltration (Figure 2C; as indicated by the arrow) at day 6, which paralleled visible ankle joint swelling measurements. Although the clinical swelling in the paw and ankle and accompanying microscopic inflammatory cell infiltrate visualized in the knee joint were resolved by day 28, joint sections from the mice that received K/BxN sera displayed persistent bony erosions (Figure 2D) at this time point.

3.2 Characterization of microglia and astrocyte spinal cord changes in acute and chronic phases

Persistent allodynia, especially during chronic pain can be linked to changes in the spinal cord [17,65]. To investigate this, lumbar spinal cord gene and protein expression levels of markers

for activated astrocytes (GFAP) and microglia (Cd11b or Iba1) were measured during peak inflammation (day 6) and during the persistent allodynia, without inflammation, late phase (day 28). Changes in mRNA transcription levels of CD11b and GFAP were quantified by real time RT-PCR. CD11b mRNA levels were increased in day 6 arthritic mice (6.67 ± 0.7 vs. 3.04 ± 0.54 relative expression units (REU) in arthritic vs. control mice, respectively, $p < 0.01$) and in day 28 arthritic mice (7.93 ± 1.6 vs. 3.55 ± 0.8 REU in control mice, $p < 0.05$), Figure 3A. GFAP mRNA levels were increased in day 6 arthritic mice (6.87 ± 0.7 vs. 2.9 ± 0.4 REU in control mice, $p < 0.001$) and day 28 arthritic mice (9.75 ± 2.0 vs. 3.58 ± 0.9 REU in control mice, $p < 0.05$), Figure 3B.

GFAP and Iba1 protein expression levels were assessed using immunoreactivity. The average of control scores for each day of interest were set to 100% and changes in staining were reported as percent change compared to control values for the day of interest. Immunostaining of sections from L4–L6 with antibodies targeting microglia (Iba1) showed an increase in microglia staining in the superficial region of the dorsal horn (lamina 1 and 2) and in deeper dorsal horn areas (sample from lamina 5) at day 6 compared to control (Figure 4A and 4C). On day 6 superficial microglia dorsal horn staining is $539 \pm 114\%$ ($p < 0.001$) of control levels. Deeper dorsal staining is $381 \pm 28\%$ ($p < 0.05$) of control levels. On day 28 staining of microglia was still significantly elevated in the superficial dorsal horn region as compared to control animals (Figure 4A and 4D). On day 28, superficial microglia dorsal horn staining is $201 \pm 34\%$ ($p < 0.05$) of day 28 control levels. Microglia staining in the deeper dorsal horn region on day 28 is $142 \pm 26\%$ ($p > 0.05$) of control staining values.

The same spinal cord sections were also stained for the astrocyte marker (GFAP) and evaluated according to the same protocol as used for microglia staining. The integrated intensity of superficial dorsal horn staining was markedly enhanced on day 6 (Figure 4B and 4E) but did not remain elevated by day 28 as compared to control mice (Figure 4B and 4F). On day 6 superficial astrocyte dorsal horn staining is $170 \pm 16\%$ ($p < 0.05$) of control staining. No significant changes in deep dorsal horn staining were seen at either time point measured.

3.3 Mechanical hypersensitivity induced by arthritis during the peak inflammation phase is attenuated by anti-inflammatory and analgesic agents

To further characterize pain behavior in the K/BxN serum transfer model of arthritis we assessed reversal of allodynia during peak inflammation following injection of different classes of analgesic compounds. The average C57BL/6 control mechanical threshold is presented on these graphs as a dotted line at 1.52g (Figure 5). All drugs were given i.p. with saline as vehicle and the mice tested by application of von Frey filaments, using the up-down method, for four hours following drug delivery. Gabapentin, ketorolac, or vehicle were delivered randomized to the mice on day 4 and tactile allodynia monitored; subsequently drugs were allowed to wash out for 48 hours before the same procedure was repeated on day 6. Following a second drug washout period of 3 days, etanercept or vehicle were injected on day 10. To begin with, we assessed the anti-allodynic effect of ketorolac, a mixed cyclooxygenase (COX) inhibitor. Ketorolac treated animals (7.5mg/kg) had significantly increased tactile thresholds at 60, 120, and 180 minutes ($p < 0.01$, 0.01 and 0.05) after treatment (Figure 5A). The second drug investigated was gabapentin, originally approved for seizures, but also currently prescribed for neuropathic pain. Gabapentin treated animals (100mg/kg) had significantly increased tactile thresholds at 30, 60, and 120 minutes ($p < 0.05$, 0.001 and 0.001) after treatment (Figure 5B). Lastly, we tested the anti-allodynic effect of etanercept, a TNF decoy receptor fusion protein, which significantly reduced swelling, clinical signs, and tactile allodynia in CIA [24]. Etanercept treated animals (5mg/kg) had significantly increased tactile thresholds at 120 and 180 minutes ($p < 0.01$ and 0.001) after injection. Drug efficacy was also quantified by comparing the hyperalgesic index for animals receiving and not receiving treatment. Etanercept ($p < 0.05$),

gabapentin ($p<0.001$), and ketorolac ($p<0.01$) all display significant analgesic activity in comparison to their respective saline treated vehicle control arthritic animals according to this measure (Figure 5D).

3.4 Post inflammation arthritis induced mechanical hypersensitivity is attenuated by gabapentin but not by etanercept or ketorolac

Given the changes in joint inflammation, clinical signs, and spinal cord glial staining in mice between day 6 and day 28 we decided to investigate changes in analgesic responsiveness during this second time period. The average C57BL/6 control threshold is also presented on these graphs as a dotted line at 1.52g (Figure 6). All drugs were given i.p. in saline at identical dosages to day 6 and tested in the same fashion as during day 6. Etanercept treated animals (5mg/kg) had a very modest but significant increase in tactile threshold at 60 minutes ($p<0.01$) after injection (Figure 6A). Gabapentin treated animals (100mg/kg) had significantly increased tactile thresholds at 30, 60, and 120 minutes ($p<0.05$, 0.001 and 0.01) after treatment (Figure 6B). Ketorolac treated animals (7.5mg/kg) displayed no significant difference in tactile thresholds during the four hours following treatment (Figure 6C). Drug efficacy was also quantified by comparing the hyperalgesic index for animals receiving drug treatment and those receiving vehicle treatments. Only gabapentin ($p<0.05$) displayed a significant analgesic activity in comparison to saline treated vehicle controls according to this measure (Figure 6D).

3.5 Changes in ATF3 in the DRG during inflammation and post inflammation

Given the efficacy of gabapentin on pain behavior in the K/BxN serum transfer arthritis, DRGs from 6 and 28 day control and arthritic mice were immunostained for ATF3, a marker of nerve injury, and NeuN. DRGs were scored such that values represent the number of ATF3 positive stained cells out of the total number of neuronal cells containing a visible nucleus. Control animals from day 6 and day 28 had a mean ATF3/NeuN ratio of 0.004 ± 0.0005 and 0.05 ± 0.03 , respectively (Figure 7A, D). Day 6 arthritic animals (Figure 7B, C) had a mean ATF3/NeuN ratio of 0.14 ± 0.09 ($p>0.05$ vs control) and day 28 arthritic animals (Figure 7E) had a mean ATF3 staining ratio of 0.23 ± 0.06 ($p<0.05$ vs control). ATF3 expression in DRGs from day 6 arthritis animals was variable. Hence, representative images from low ATF3 (Figure 7B) and high ATF3 expressing mice are depicted in Figure 7C. The staining is quantified in Figure 7F.

4. Discussion

Several monoarticular arthritis models are commonly used as pain models of “inflammatory arthritis” including intra-articular injection of inflammatory agents such as carrageenan or CFA [2,32]. These models are very useful in studies of local acute inflammation and the short-term impact of such inflammation on the nervous system. Given the chronic inflammation present in RA we sought to define the effects of a longer-lasting inflammation, using the K/BxN serum transfer arthritis model, upon nociceptive processing. The current report is the first to examine this model as a tool for evaluating pain behavior and understanding changes in nociceptive processing during arthritic disease progression. Mice injected with K/BxN serum display robust mechanical allodynia. The onset correlates with an increase in paw and ankle swelling and the number of affected joints. Surprisingly, the mechanical hypersensitivity did not return to baseline even two weeks after loss of visible and histologic signs of synovitis. An increase in spinal microglia (Iba1) and astrocyte (GFAP) staining was observed, with the most prominent increase at day 6, during peak inflammation. During this stage of K/BxN serum transfer mediated arthritis, i.p. bolus injection of etanercept, ketorolac, and gabapentin had anti-allodynic effects. At day 28, microglia staining remained elevated, while astrocyte staining was not significantly different from control. Interestingly, arthritic mice at the post inflammation stage showed no response to the previously effective dose of etanercept or ketorolac, but an anti-allodynic effect was observed following bolus injection of gabapentin.

Recently, the CIA model, the most widely used model of chronic murine arthritis, was validated as a pain model [24]. CIA mice display maximal clinical scores 7–10 days post arthritis induction, with correlating tactile allodynia. A robust tactile allodynia is characteristic of both models, however the thermal hypersensitivity reported in the CIA model was not detected in the K/BxN serum transfer model. Rather, a transient mild hypoalgesia was observed corresponding to the period of peak inflammation. There is a clear correlation between development of arthritis and mechanical hypersensitivity in animal models and RA patients [11,19]. However, thermal hypersensitivity in RA patients is unclear. Leffler and colleagues reported no differences in thermal sensitivity over the affected joint in RA patients of short (< 1 year) duration, longer duration (>5 years) or in control groups [34]. In contrast, Edwards et al. reported that RA patients have reduced thermal thresholds [14]. Differential control of thermal and mechanical hypersensitivity have been observed in other animal models of pain [18,35,37,43,56,58]. Further studies are warranted to expand our understanding of the mechanisms that lead to differential sensory processing.

Consistent mechanical allodynia occurs following induction of K/BxN serum transfer arthritis. Based on our observations that i) etanercept, a TNF blocker not crossing the blood-brain barrier, diminishes allodynia, ii) ATF3 expression is induced in the DRGs and iii) spinal glia are activated, hypersensitivity is likely driven by both peripheral sensitization of the joint innervating nerves and central sensitization. During joint inflammation low threshold A δ fibers have increased output in response to noxious and non-noxious stimuli while high threshold A δ and C fibers have reduced mechanical thresholds (reviewed in [49]). ATF3, a member of the ATF/CREB transcription factors family, is induced in a variety of tissues exposed to stress [20]. Although its function is not clearly elucidated, ATF3 is commonly used as a marker of nerve injury [21,60,61]. This study reports clinical signs of inflammation lasting approximately 2 weeks which appears to be sufficient to induce signs of nerve damage. In the CIA model, ATF3 was induced in DRGs 14 days after arthritis onset [24]. Of note, ATF3-induction has been observed in DRGs of horses with laminitis in hoof [30] and in rats with monosodium iodoacetate-induced osteoarthritis [27], indicating that other long-term painful conditions, which are not directly associated with nerve injury but rather chronic inflammation and joint damage, respectively, may also transition to a painful state with a neuropathic trait. Hence, it is possible that long-term inflammation or joint damage leads to a state that exhibits some features in common with neuropathic pain. However, further studies to examine the underlying mechanisms for such transition are necessary, and at current state we cannot exclude the possibility that the persistent pain is driven by continuous nociceptive input from the damaged joint.

Spinal sensitization is a complex event linked to diverse mechanisms including repeated activation of glutamate NMDA receptors (reviewed in [5,22]), tachykinin NK1 receptor activation (reviewed in [5]), spino-bulbo-spinal loop activity [62] and glial activation (reviewed in [40],[59]). Spinal microglia and astrocyte activation during central sensitization has been reported in variety of nociceptive states including peripheral inflammation [54], osteosarcoma [50], HIV-1 gp120 induced pain [41], and spinal nerve ligation induced neuropathic pain [10,17]. The role of microglia and astrocytes in central sensitization has attracted significant interest as interventions directed at blocking the action of these cells are anti-nociceptive in many pain models (reviewed in [26,40,53]). K/BxN serum transfer arthritis increased spinal astrocyte and microglia associated mRNA levels and immunoreactivity, which we interpreted to indicate activation. However, we did not functionally assess the consequences of glia blockade. Common between other chronic pain models such as the paw incision and L5 nerve transection model and our investigation are early microglia reactivity. Increases in astrocyte immunoreactivity are present in all models, but they display varying temporal patterns [46,47].

Spinal glial changes are presumed to be an effect of the peripheral inflammation and resulting nociceptive input. Serum transfer could, however, have a direct effect upon the spinal cord. This is unlikely, given unaltered tactile thresholds in control mice following injection of naïve mouse serum. Furthermore, the GPI autoantibody has been identified as the pathogenic factor during K/BxN serum transfer arthritis. Positron emission tomography has tracked anti-GPI antibodies following serum transfer, and describes preferential localization to the paws, ankles and knees but not to the vertebral joints [64]. Though a direct spinal action of the serum cannot be completely eliminated, spinal glial activation is more likely the result of sustained peripheral inflammation and associated nociceptive input.

The clinical signs of arthritis during K/BxN transfer arthritis are reduced by COX and IL-1b inhibitors [7,8,28]. Here, we assessed the anti-allodynic effect as opposed to reduction of clinical signs for three classes of drugs. We tested a non-selective COX-1/COX-2 inhibitor (ketorolac), a TNF receptor fusion protein (etanercept), and synthetic gabapentin. All three compounds reduced mechanical hypersensitivity during the inflammatory phase. Surprisingly, only i.p. gabapentin robustly reversed the post inflammation mechanical hypersensitivity. Gabapentin and pregabalin are approved for neuropathic pain treatment with efficacy against diabetic neuropathy [48] and post herpetic neuralgia [51]. These compounds bind to the 2 subunit of voltage sensitive calcium channels and *in vitro* acutely alter neurotransmitter release including glutamate, noradrenaline, GABA, substance P and CGRP [16,38]. Though not commonly prescribed for pain indications besides neuropathic pain, gabapentin also attenuates inflammation-induced pain in rats [36]. Hence, the observation that only gabapentin was effective in the post-inflammatory phase strongly suggests that the mechanisms that drive the allodynia in the two phases are different, it does not prove that the post-inflammatory allodynia has a neuropathic trait.

TNF-blockers such as etanercept improve clinical signs and slow disease progression in RA. Beyond being an important inflammatory factor, TNF has a direct neuronal action [29,44]. Boettger et al demonstrated that intra-articular etanercept injection reduced joint afferents' responses to rotation of the inflamed joint within 30 minutes after injection, without gross changes in inflammation or alteration of the normal joint response to rotation [1]. Accordingly, we observed an anti-allodynic effect during the inflammation phase within 1 hour following bolus i.p. injection of etanercept, indicating a peripheral anti-nociceptive effect of TNF-neutralization. Current data suggests that COX and TNF play a minor role in pain processing during the post-inflammation phase, or that a single injection is not sufficient to reverse established allodynia. The anti-allodynic effect of a single dose of gabapentin has potential therapeutic implications given that the most common agents for RA therapy include TNF blockers and NSAIDs, which address different mechanisms. Further investigation of gabapentin and pain relief in RA patients would be of great interest.

In summary, K/BxN serum transfer arthritis induces tactile allodynia during the inflammatory phase with an expected sensitivity to systemic NSAIDs and etanercept. Of interest, however, is the extension of the allodynic state beyond the period of clinical inflammatory arthritis, with a time dependent shift towards characteristics indicative of a neuropathic state, including loss of sensitivity to NSAIDs, a continued sensitivity to gabapentin, and the appearance of ATF3. At the spinal level this model demonstrated continued microglial activity, but not astrocytic activation. We believe that the K/BxN serum transfer arthritis model will be of importance in pain studies as it can be applied to different strains of mice. Characterization of this model describes a pain state transition, which is recapitulated in this model unlike other models, and might also occur in RA despite adequate therapy. In conclusion, the K/BxN serum transfer model represents a multifaceted model for studies exploring pain mechanisms in conditions of joint inflammation and may serve as a platform for exploring novel treatment strategies for pain in human arthritic conditions.

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Figure 1.

Characterization of K/BxN arthritis pain behavior. Graphs display (A) arthritis clinical scores assessed for 28 days, demonstrating an increase in clinical signs of arthritis day 1–12, (B) ankle thickness measured with calipers showing a significant ankle swelling in arthritic animals day 3–9, (C) tactile thresholds (g) showing tactile allodynia day 2–28 (excluding d12) and (D) thermal thresholds (sec) demonstrating that arthritic animals displayed significant thermal hypoalgesia day 3–6, with no changes from baseline at any other time point. Each time point represents mean $\pm$ SEM (n=9 mice/group), * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$ by Bonferroni post test.

Figure 2. K/BxN serum transfer induced joint destruction. Mice were sacrificed on days 6 and 28 and the knee joints removed and prepared for histology, sectioned and stained with Hematoxylin and Eosin. Representative images are shown. There was a prominent inflammatory infiltrate on Day 6 in the mice that received K/BxN sera (white arrow) and residual bony erosions on Day 28 (black arrowheads). Original $\times 50$ magnification.

Figure 3. Microglia (CD11b) and astrocyte (GFAP) mRNA levels are elevated in the spinal cords of arthritic animals compared to control mice. Graphs show (A) CD11b and (B) GFAP mRNA relative expression units (REU) in the spinal cords of day 6 and day 28 arthritic compared to control serum treated animals. Each time point represents mean $\pm$ SEM (n=5–6 mice/group), * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$ by student's t-test.

Figure 4.

Microglia and astrocyte immunoreactivity are elevated in the lumbar regions of spinal cord following induction of serum transfer mediated arthritis. (A) Representative images for Iba1 immunoreactivity in arthritic and control mice on days 6 and 28 post induction of arthritis. Increased staining for Iba1 is present in the superficial dorsal horn (lamina I-II) and deep dorsal horn (lamina V) 6 days in the superficial dorsal horn day 28. (C, D) Graphs showing quantification of Iba1 signal intensity. (B) Representative images of GFAP immunoreactivity days 6 and 28 post serum injection. Increased GFAP staining was observed in the superficial dorsal horn (lamina I-II) day 6, but not day 28 or in the deep dorsal horn, after induction of arthritis. (E,F) Graph depicting quantification of GFAP signal intensity. Each time point

represents mean $\pm$ SEM (n=3 mice per group, 3–5 sections per mouse), *= p<0.05, and ***=p<0.001 by Bonferroni post test. Scale bars represent 50 μ m.

Figure 5.

Intraperitoneal injection of etanercept, gabapentin and ketorolac reverses tactile allodynia in the K/BxN serum transfer arthritis model. Graphs showing anti-allodynic effect of i.p. injection of (A) etanercept (5mg/kg), (B) gabapentin (100mg/kg) and (C) ketorolac (7.5mg/kg) at the peak of paw swelling when tested for changes in mechanical thresholds every 30 minutes for four hours. Saline was used as vehicle for all drugs. (D) Hyperalgesic index indicates a significant decrease in pain behavior for etanercept, gabapentin, and ketorolac. Dotted line indicates average control tactile threshold (1.52g). Each time point and bar represents mean $\pm$ SEM (n=6–7 mice/group for all groups), * = $p < 0.05$, ** = $P < 0.01$, and *** = $p < 0.001$ by Bonferroni post test.

Figure 6.

K/BxN serum transfer arthritis induced day 28 tactile allodynia is reduced by intraperitoneal injection of gabapentin, but not etanercept or ketorolac. Graphs depicting effect of i.p injection of vehicle and (A) etanercept (5mg/kg), (B) gabapentin (100mg/kg i.p.) and (c) ketorolac at one hour intervals for four hours. (D) Hyperalgesic index indicates a significant decrease in pain behavior for gabapentin, but not for etanercept or for ketorolac as compared to vehicle. Dotted line indicates average control tactile threshold (1.52g). Each time point represents mean $\pm$ SEM (n=6 mice/group), * = $p < 0.05$, ** = $P < 0.01$, and *** = $p < 0.001$ by Bonferroni post test.

Figure 7.

The number of ATF3 positive cells is increased in dorsal root ganglia following induction of K/BxN serum transfer arthritis. Representative images of (A) day 6 control mice and day 6 arthritis mice with (B) low and (C) high ATF3 immunoreactivity. ATF3 staining in 28 days (D) control and (E) arthritic mice showed that injection of K/BxN serum lead to a significant increase in number of ATF3 positive neurons day 28. (F) Graph showing the number of cells positive for ATF3 divided by the total number of NeuN positive nuclear cell bodies in the different groups. Each bar represents mean $\pm$ SEM (n=3–4/group, 3–5 sections per mouse) and $*=p<0.05$ by student's T-test. Scale bars represent 100μm.