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Evoked hypoalgesia is accompanied by tonic pain and immune cell infiltration in the dorsal root ganglia at late stages of diabetic neuropathy in mice

Nitin Agarwal, Johanna Helmstädter, Daniel R Rojas, Kiran K Bali, Vijayan Gangadharan, and Rohini Kuner

Abstract

Diabetic peripheral neuropathy is a major debilitating late complication of diabetes, which significantly reduces the quality of life in patients. Diabetic peripheral neuropathy is associated with a wide spectrum of sensory abnormalities, where in loss of sensation or hypoalgesia to applied external stimuli is paradoxically accompanied by debilitating tonic spontaneous pain. In numerous studies on animal models of diabetic peripheral neuropathy, behavioural measurements have been largely confined to analysis of evoked withdrawal to mechanical and thermal stimuli applied to dermatomes, whereas spontaneous, on-going pain has not been widely studied. In the Streptozotocin model of type 1 diabetes, we employed the Conditioned Place Preference test to assess tonic pain. Our results indicate that both phases, that is, early evoked hypersensitivity (i.e. 5–7 weeks post-Streptozotocin) as well as late stage hypoalgesia (i.e. 17–20 weeks post-Streptozotocin) are accompanied by significant tonic pain in mice with diabetic peripheral neuropathy. We also report on the temporal relation between on-going pain and neuropathological changes in the dorsal root ganglia of mice with diabetic peripheral neuropathy up to 6 months post-Streptozotocin. Neither early hypersensitivity nor late hypoalgesia were associated with markers of cellular stress in the dorsal root ganglia. Whereas significant neutrophil infiltration was observed in the dorsal root ganglia over both early and late stages post-Streptozotocin, T-cell infiltration in the dorsal root ganglia was prominent at late stages post-Streptozotocin. Thus, longitudinal analyses reveal that similar to patients with chronic diabetic peripheral neuropathy, mice show tonic pain despite sensory loss after several months in the Streptozotocin model, which is accompanied by neuroimmune interactions in the dorsal root ganglia.

Keywords

Neuropathic pain, diabetes, DRG, immune cell

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Introduction

Diabetic peripheral neuropathy (DPN) is one of the most frequent late complications of diabetes, which severely limits the quality of life in patients.[1] Pain in the extremities and hypersensitivity to innocuous stimuli, such as tactile allodynia, are frequent in patients with DPN. About 30% of diabetic patients develop neuropathic pain.[2] Peripheral nerve complications seen in diabetic patients are irreversible and can neither be prevented nor reverted by lowering blood glucose levels; at best, it may serve to delay the progression of DPN.[3] Therefore, there is a critical need to understand the manifestations of DPN and their underlying mechanisms in order to develop new medications.

One of the major challenges in understanding and treating DPN is that it comprises a wide spectrum of painful and non-painful symptoms, which come together in various combinations and differing profiles in patients. Thus, a wide range of symptoms ranging from tingling, burning spontaneous pain and temperature hypersensitivity may be combined with hypoalgesia

Institute of Pharmacology, Heidelberg University, Germany

Corresponding Author:
Rohini Kuner, Institute of Pharmacology, Heidelberg University, Im Neuenheimer Feld 366, D-69120 Heidelberg, Germany.
Email: rohini.kuner@pharma.uni-heidelberg.de

loss of pain perception and numbness.[4] How hypoalgesia or numbness can be paradoxically accompanied by tonic, on-going pain in diabetic patients remains unknown.

Over the recent years, studies in animal models have started to yield important insights into mechanisms of pain in DPN. The most well-characterized model for type 1 diabetes is based upon injection of Streptozotocin (STZ) in rodents, which leads to progressive destruction of pancreatic beta cells, insulin deficiency and hyperglycemia over several weeks.[5–7] Despite its limitations, it is a widely applied model for type 1 diabetes. It is known that STZ-treated rodents demonstrate hyperalgesia to nociceptive stimuli over the early period with varying onsets depending upon the STZ doses and progressively show hypoalgesia and lack of sensation over several months post-STZ.[8] An increasing number of studies have addressed molecular mediators of nociceptive hypersensitivity over early period's post-STZ.[9,10] However, behavioural measurements have been largely confined to analysis of evoked withdrawal to applied mechanical and thermal stimuli. In contrast, spontaneous, on-going pain, which constitutes the debilitating component of diabetic neuropathic pain in human patients[4] has not been adequately studied and modelled in rodent's models of DPN so far.

In diverse models of chronic pain, conditioned place preference (CPP) to a chamber that was conditioned (i.e. paired) with pain relief via an analgesic drug has been employed to assess tonic pain.[11,12] Here, we undertook experiments in the STZ model of type 1 diabetes in mice to address analysis of on-going pain at early and late stages of DPN. Concurrent behavioural measurements of evoked behaviours were undertaken to test the temporal relationship between evoked pain and on-going pain in DPN. Our results indicate that both phases of early evoked hypersensitivity as well as later stage hypoalgesia and numbness to stimuli are accompanied by significant tonic pain in mice with DPN. We also systematically tested the temporal relation between tonic pain, sensory abnormalities, loss of peripheral afferents, cellular stress and immune cell infiltration in sensory ganglia.

Materials and methods

Animal experiments

All experiments were performed on C57Bl6/j male mice. Animals were purchased from Janvier labs, Europe. All animals were housed in individually ventilated cages with stable environment maintained at $22 \pm 1^{\circ}C$ with a 12/12-h light–dark cycle. All experimental procedures were approved by Animal Care and Ethics Committee (Regierungspräsidium), Karlsruhe, Germany, and we made all attempts to follow the ARRIVE guidelines. For each time point, four to six animals from each group were involved. Mice were randomized before the experiment and all experimental were blinded to the identity of the mice they were analysing. All tests were performed in an appropriate room with controlled light and sound conditions between 09.00 and 16:00 h.

Streptozotocin model for type 1 diabetes

We employed the model of Streptozotocin (STZ)-induced type 1 diabetes in all our experiments, in which systemic delivery of STZ leads to selective destruction of pancreatic islet β-cells resulting in insulin deficiency and hyperglycemia.[6] We employed a regimen involving multiple administrations of low-dose STZ in mice.[13] Diabetes was induced in 8-weeks-old C57Bl6j mice of both sexes by intraperitoneal (i.p) injections of STZ (60 mg/kg in citrate buffer) over on five consecutive days. Citrate buffer was alone injected in mice as the control group. Blood glucose levels were measured using a glucometer (Accu-Chek Aviva, Roche Diagnostics) regularly in all STZ-injected mice throughout the experiment. Animals with glucose levels > 300 mg/dl were considered to be diabetic. Mice were analysed over a period of 5 weeks to 20 weeks post-STZ.

Behavioural analyses

All behavioural measurements were done in awake, unrestrained, age-matched mice of both sexes. Prior to measurements, all experimental groups of animals were habituated in experimental setup for 3 days in two separate sessions each day. The experimenter was fully blinded to the identity of the mice in the groups being tested. Von Frey measurement was done to measure mechanical sensitivity. Mice were placed on elevated wire grid and von Frey filaments exerting a force range from 0.07 to 2.0 g were tested on the plantar hindpaw. Paw withdrawal response were tested for five applications of each fibre type. We calculated 60% response frequency as 'thresholds', as described previously,[14] at basal and different time points after STZ injection. Thermal sensitivity was measured by recording paw withdrawal latency on application of infrared heat source (Ugo Basile Inc.) on the plantar hindpaw.[15] The IR intensity was set at 50 for all time points of measurement in all mice. A cut-off of 20 seconds was set to avoid burning of tissue. Heat applications were performed at intervals of 5 min.

Conditional place preference measurement

Conditional place preference (CPP) test was performed as described in details previously.[11,16] On day 1, mice were acclimatized to the setup for 20 minutes. On day 2, pre-conditioning for 20 min in morning was done to

reveal any pre-existing preference for one chamber of the setup. On days 3 and 4, the mice were conditioned for 50 min with vehicle (saline) injection paired with the preferred chamber in the morning and with injection of pregabalin (30 mg/kg, i.p) paired with the non-preferred compartment using distinct olfactory, visual and tactile cues for recognition of either chamber in the afternoon. On day 5, each mouse was put again in the arena for 20 min in a drug-free state (post-conditioning). The time spent on either side of the chamber for a total period of 20 min is measured and the increase in time spent in the drug-paired chamber directly reflects pain relief in diabetic mice.

Immunohistochemistry and cell counts

Mice were subjected to cardiac perfusion of 4% paraformaldehyde (PFA) under pentobarbital anesthesia at basal stage or at 8 weeks or 19 weeks after STZ or vehicle injection. The spinal column was extracted and post-fixed for 24 h in 4% PFA. The dorsal root ganglia (DRG) were dissected and 16-μm cryo-sections were cut and collected on Poly-L lysine–coated glass slides. For immunofluorescence analysis, the sections were permeabilized in 0.5% phosphate-buffered saline with Tween 20, washed and blocked with 7% horse serum. The sections were incubated overnight with Rat anti-CD3 (1:100, BD Pharmingen) at 4°C. Next day sections were washed and incubated with Alexa-594 conjugated secondary antibody. The sections were washed and mounted in Mowiol (Sigma). Images were obtained using a laser-scanning spectral confocal microscope (Leica TCS SP8 AOBS, Bensheim, Germany).

For 3,3′-diaminobenzidine tetra hydrochloride (DAB) staining, DRG-tissue sections were incubated in 1% hydrogen peroxide in phosphate-buffered saline: methanol (1:1). The sections were washed, permeablilized and blocked in horse serum. The sections were incubated with Biotin Rat anti-Gr1 (1:500, BD Pharmingen) antibody overnight at 4°C. Next day, sections were washed and incubated in an avidin–biotin complex solution (Vectastain Elite ABC Kit), washed and then stained with DAB solution (DAB substrate kit for peroxidase, Vector laboratories, Burlingame, CA, USA). Staining reaction was terminated by washing with water. Sections were mounted in Mowiol (Sigma). Bright-field coloured images were captured at Nikon imaging centre, Heidelberg. The quantification of positive-stained cells was done by counting immunopositive cells in successive L3–L4 DRG sections in blinded manner. Fifteen sections per DRG were analysed per condition.The average of number of cells/sections were then calculated.

Statistical analyses

All data were calculated and are presented as mean ± standard error of the mean. One-way or two-way analysis of variance (ANOVA) for repeated measures or random measures was employed as appropriate, and post-hoc Bonferroni test for multiple comparisons was performed to determine statistical significant differences. Changes with $p \leq 0.05$ were considered to be significant.

Results

We employed a lose-dose protocol for the STZ model which comprises i.p. injections of 60 mg/kg body weight of STZ for five to six times with 24-h intervals between injections, employing injections of vehicle (Citrate buffer) as a control. Using this protocol, we achieved levels of blood glucose between 380 and 480 mg/dl starting from 2 weeks in STZ-injected mice, which were acutely controlled by administration of insulin, as necessary.[17] Importantly, unlike regimens involving single applications of high-dose STZ, this regimen of multiple injections of low dose STZ does not lead to toxicity in DRG neurons over acute time frames.[18,19] Moreover, the time frame chosen in our analyses (between 5 and 17 weeks post-STZ) is temporally separated from any potential toxic effects. As described previously,[20] we observed hypersensitivity to thermal and mechanical stimuli over the period between 5 to 7 weeks post-STZ treatment. STZ-treated mice showed a drop in the response threshold and an increase in the frequency of withdrawal responses to plantar application of von Frey mechanical stimulation at noxious intensities as well as non-noxious intensities (allodynia) as compared to sham-treated mice (Figure 1(a), left panel shows withdrawal threshold and right panel shows typical response rates to innocuous and noxious intensities of mechanical stimulation). Similarly, in Hargreaves test, the time required to withdraw the paw from radiant heat applied to the hindpaw surface was significantly decreased (thermal hyperalgesia) in STZ-treated mice (Figure 1(b)). STZ-induced mechanical as well as thermal hypersensitivity lessened in magnitude post 8 weeks and mice began to progressively develop hypoalgesia, which was significant at 17 weeks in most mice (not shown) and significant at 19 weeks post-STZ over the entire cohort (Figure 1(a) and (b)).

In diabetic patients, pregabalin is used to treat pain associated with DPN.[21] In rodent models of neuropathic pain, including DPN, pregabalin has been reported to attenuate nociceptive hypersensitivity. We therefore confirmed the effects of pregabalin on nociceptive hypersensitivity in our experimental setting, which also helped us to select the dose for the subsequent CPP analyses. A single i.p. injection of pregabalin at 30 mg/kg dose

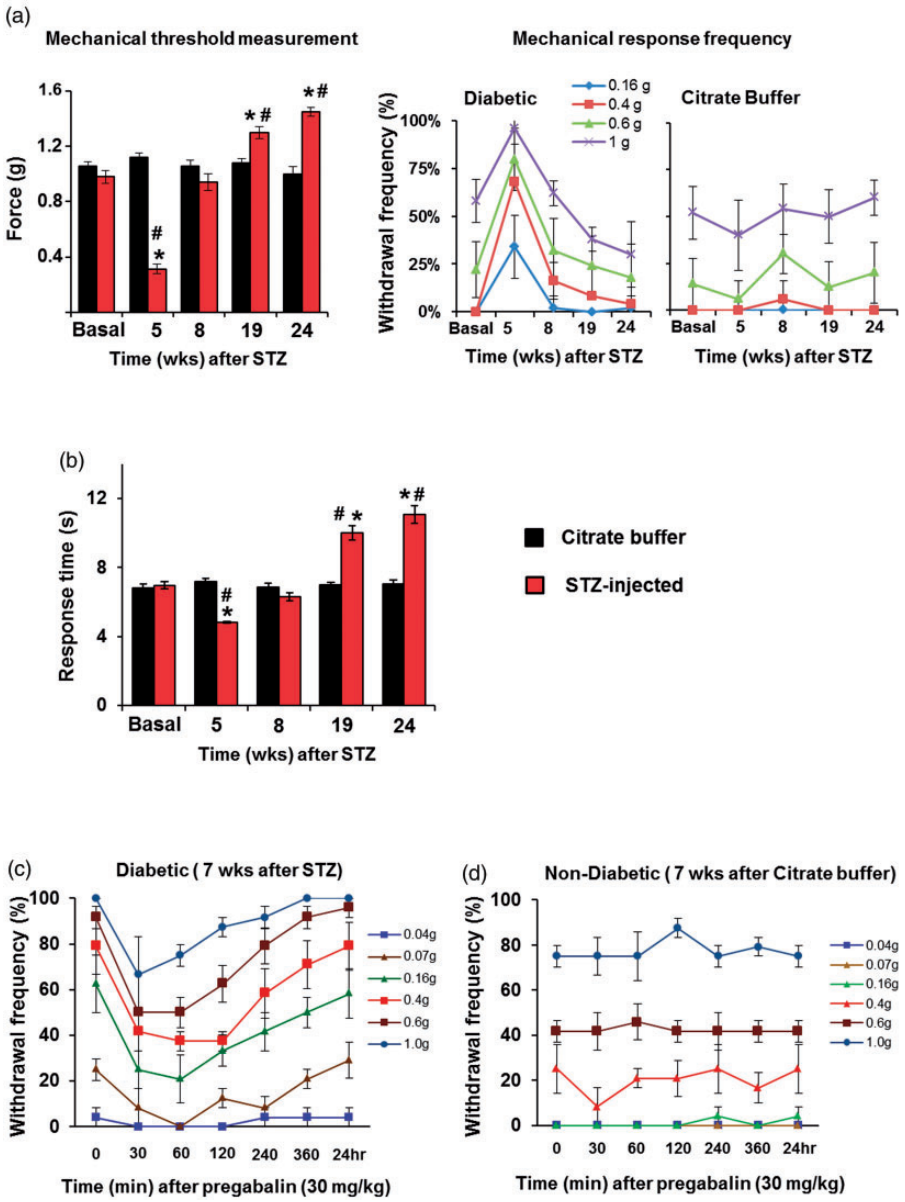

Figure 1. Biphasic change in sensitivity to mechanical and heat stimuli applied to the plantar paw surface over 24 weeks in mice with STZ-induced diabetes or control mice (citrate buffer-injected). (a) The left panel shows the summary of response threshold (defined as von Frey force eliciting response frequency of at least 60% response) ($n = 10$ mice/group) and the right panel shows examples of response frequency to particular von Frey filaments of innocuous and noxious strengths. (b) Latency to infrared heat in Hargreaves test ($n = 10$ mice/group). (c) and (d) Effect of a single intraperitoneal injection of pregabalin (30 mg/kg) on evoked hypersensitivity to mechanical von Frey stimuli in STZ-treated mice (c) or basal sensitivity in control mice (d). All data points represent mean ± SEM. In all panels, $^*p < 0.05$; Two-way ANOVA post-hoc Bonferroni for multiple comparisons.
*: as compared to basal; #: as compared to control group; ANOVA: analysis of variance; SEM: standard error of the mean; STZ: Streptozotocin.

(selected based on previous studies[22,23]) significantly attenuated the diabetes-associated increase in frequency of paw withdrawal to von Frey hairs in the non-noxious to noxious range (0.04 g–1 g) for at least 2 h (and even up to 6 h in case of some filaments) (Figure 1(c)). Pregabalin did not alter mechanical sensitivity in non-diabetic control mice (Figure 1(d)).

It should be noted there is variability in the onset of both early hypersensitivity as well as late hypoalgesia in the STZ, since these changes occur secondary to fluctuations in increase in blood glucose levels, which vary in onset and magnitude across mice post-STZ. In subsequent analyses, we chose specific time windows to study phenomena associated with deviations in

nociceptive sensitivity post-STZ. In our hands, early hypersensitivity peaked somewhere between 5 and 7 weeks post-STZ across mice. Late hypoalgesia commenced in some mice at 14 weeks, but it became widespread across the cohort and reaches significant values around 17 to 19 weeks. These time points were chosen as windows of analysis.

As a measure of on-going pain,[11,12] we then tested the ability of systemically applied pregabalin to induce CPP. We first chose a time point of 17 weeks post-STZ, when mice demonstrate mechanical hypoalgesia. Sham-treated or STZ-treated mice received i.p. injections of saline or pregabalin and the time spent in the saline- or pregabalin-paired chambers before and after drug- or saline-conditioning was measured. Non-diabetic (sham-treated) mice did not show any significant difference in the time spent in the pregabalin-paired chamber pre- and post-conditioning (Figure 2(a)). Prior to the conditioning phase, STZ-treated mice also did not show appreciable differences in time spent in the two chambers (Figure 2(a)). Post-conditioning, diabetic mice showed a significant increase in the pregabalin-paired chamber, indicating a preference for pregabalin treatment (Figure 2(a)). This was reflected as a significant difference between preference for saline or pregabalin in diabetic mice, but not in sham-treated non-diabetic mice (Figure 2(b)). Thus, at a time period associated with evoked hyposensitivity to mechanical stimuli, diabetic mice showed CPP to an analgesic drug, indicating on-going pain.

We also tested STZ- or sham-treated mice over 5 to 7 weeks, a time period when mice show hypersensitivity in terms of evoked responses to nociceptive stimuli. Also at 7 weeks post-treatment, diabetic mice, but not non-diabetic mice, showed a significant preference for pregabalin- over saline-paired chamber (Figure 2(c)),

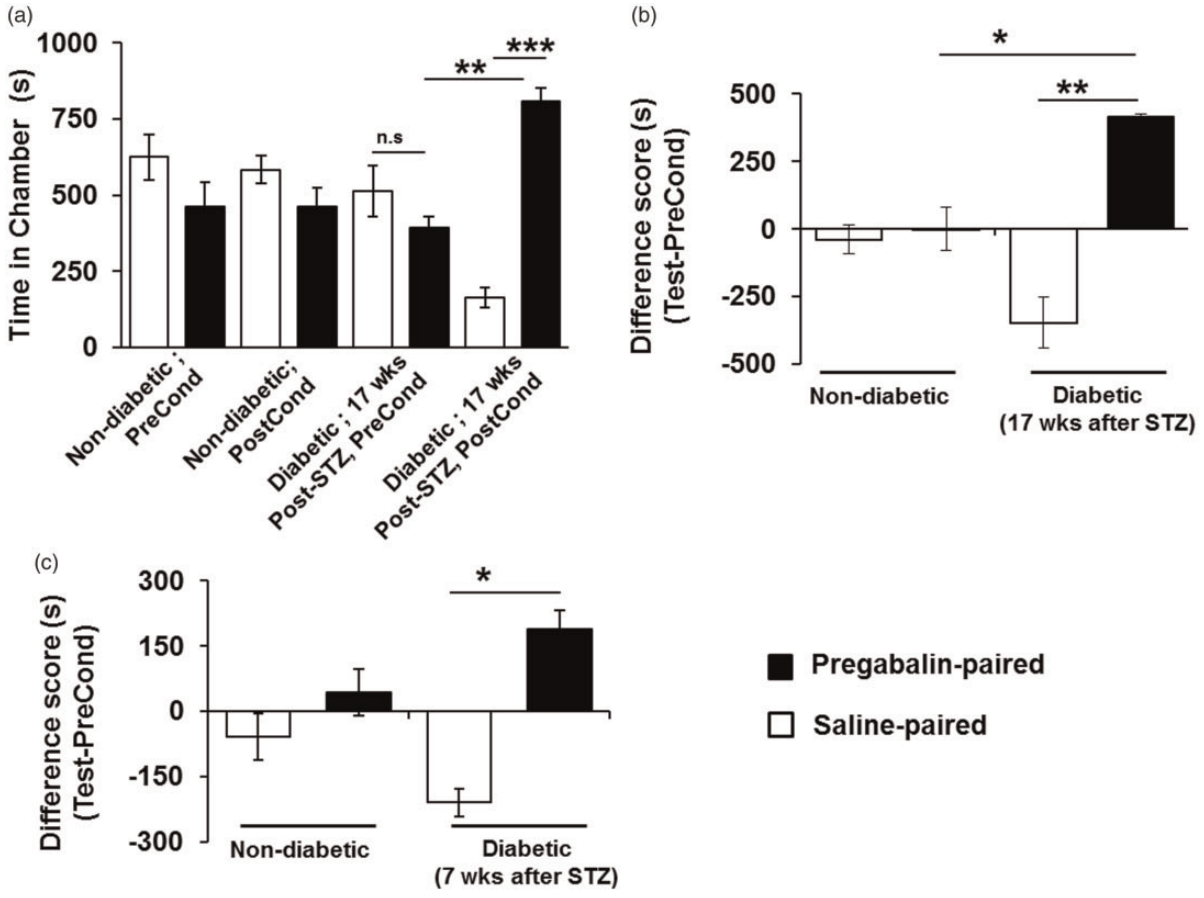

Figure 2. Conditioned place preference test with intraperitoneally injected pregabalin (30 mg/kg) in control mice or mice with diabetic neuropathy at 17 weeks post-STZ (a, b) or 7 weeks post-STZ (c). (a) Absolute time mice spent in the drug- or vehicle-paired chambers before (pre-conditioning) and after (post-conditioning)($n = 6$ mice/group). (b, C) Difference in time spent in drug- or saline-paired chamber before and after treatment with pregabalin or vehicle at 17 weeks (b) or 7 weeks (c) post-STZ or control treatment ($n = 6$ mice/group). All data points represent mean ± SEM. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ as indicated. Two-way ANOVA post-hoc Bonferroni test for multiple comparisons.

ANOVA: analysis of variance; SEM: standard error of the mean; STZ: Streptozotocin.

indicating that tonic pain is a feature that is established early on in the course of DPN. Thus, pregabalin was efficacious against nociceptive hypersensitivity as well as tonic pain in mice with DPN at early stages.

Evoked hyposensitivity to applied stimuli has been attributed to loss of intra-epidermal nerve fibre endings, particularly of nociceptors, at late stages post-STZ.[24] Our results on CPP with pregabalin at 17 weeks post-STZ suggested that mice demonstrate tonic pain despite hypoalgesia and loss of intra-epidermal nerve fibre endings, indicating that other mechanisms account for tonic pain. However, the mechanistic basis of tonic pain in chronic DPN is unknown. We therefore undertook neuropathological analyses on the DRG of STZ-injected and control mice, comparing DPN-induced changes at early and late stages post-STZ. ATF3 is a marker of cellular stress, which is prominently upregulated in injured DRG neurons upon peripheral nerve lesions.[25] However, in the context of the STZ model, neither early nor late stages of DPN were associated with marked expression and upregulation of ATF3 (see Figure 3(a) for typical examples and Figure 3(b) for negative staining control), not even at 24 weeks when sensory loss had set in in all STZ-treated mice; in contrast, ATF3

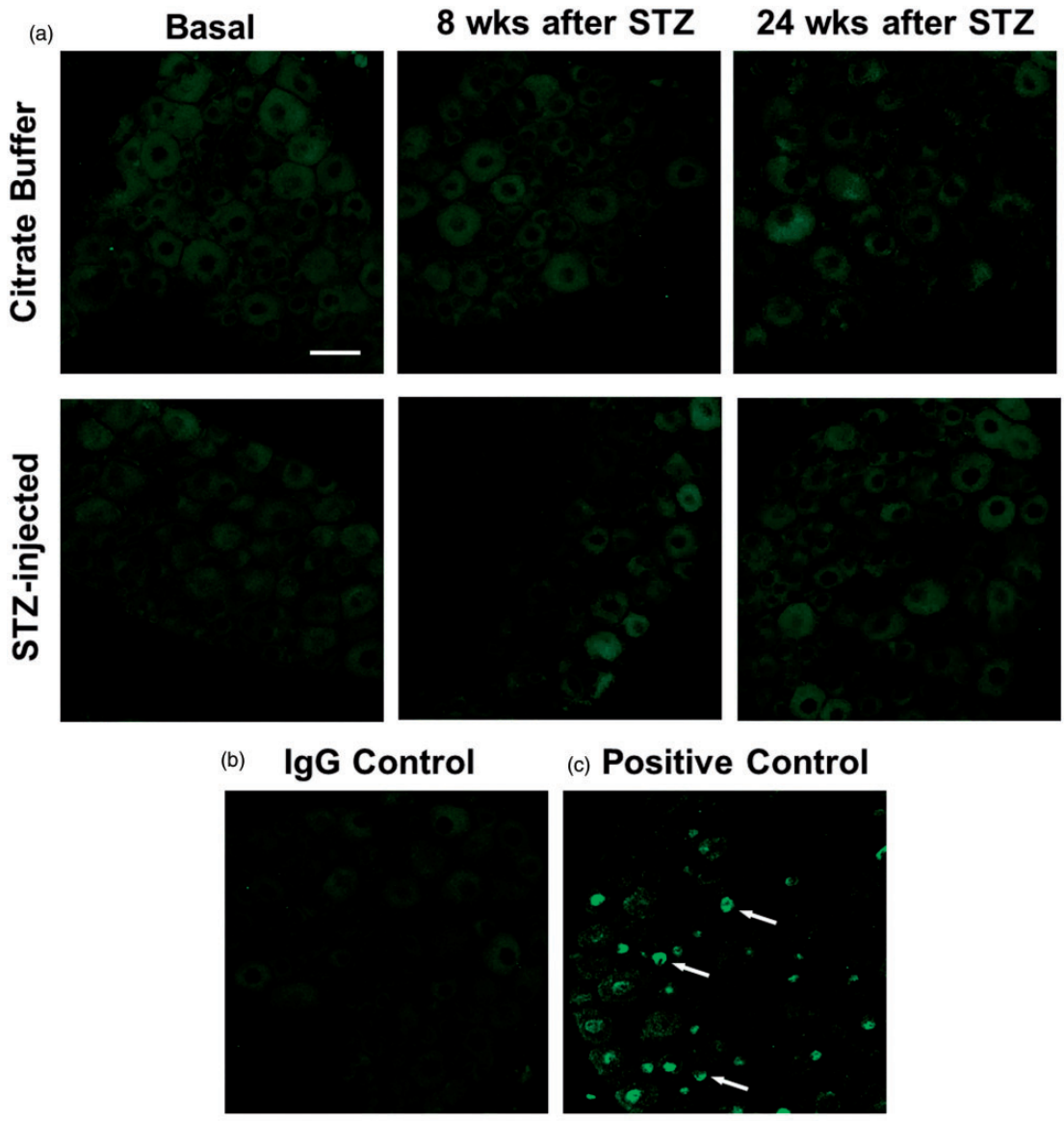

Figure 3. Immunohistochemical analysis of expression of ATF3 in dorsal root ganglia sections of mice at basal, 8 and 24 weeks post-STZ injection or control injection. Negative controls lacking primary antibody and positive controls from mice with spared nerve injury are also shown. Arrows indicate positive staining. Scale bars represent 50 μm.
STZ: Streptozotocin.

expression was prominently observed in the DRGs of mice with peripheral nerve lesions (spared nerve injury), which were included as positive controls (Figure 3(c)).

Human biopsies of patients with DPN and neuropathic pain have revealed significant neural infiltration of immune cells[26,27] and recent studies in animal models indicate that immune cells also invade DRGs and the spinal parenchyma in several models of neuropathic pain. We then compared numbers of T-cells and macrophages infiltrating the DRG in STZ-treated mice at early and late stages corresponding to evoked nociceptive hypersensitivity and hyposensitivity, respectively. To identify T-cells, we performed immunohistochemical staining against CD3 on lumbar DRGs of diabetic and non-diabetic control mice (typical examples are shown in Figure 4(a) to (c); a negative control for antibody staining is shown in Figure 4(d); arrows indicate CD3-positive or Gr-1 positive immune cells in Figure 5). There was a significant increase in the numbers of T-cells infiltrating the DRGs in mice post-STZ treatment as compared to basal only at late stages post-STZ

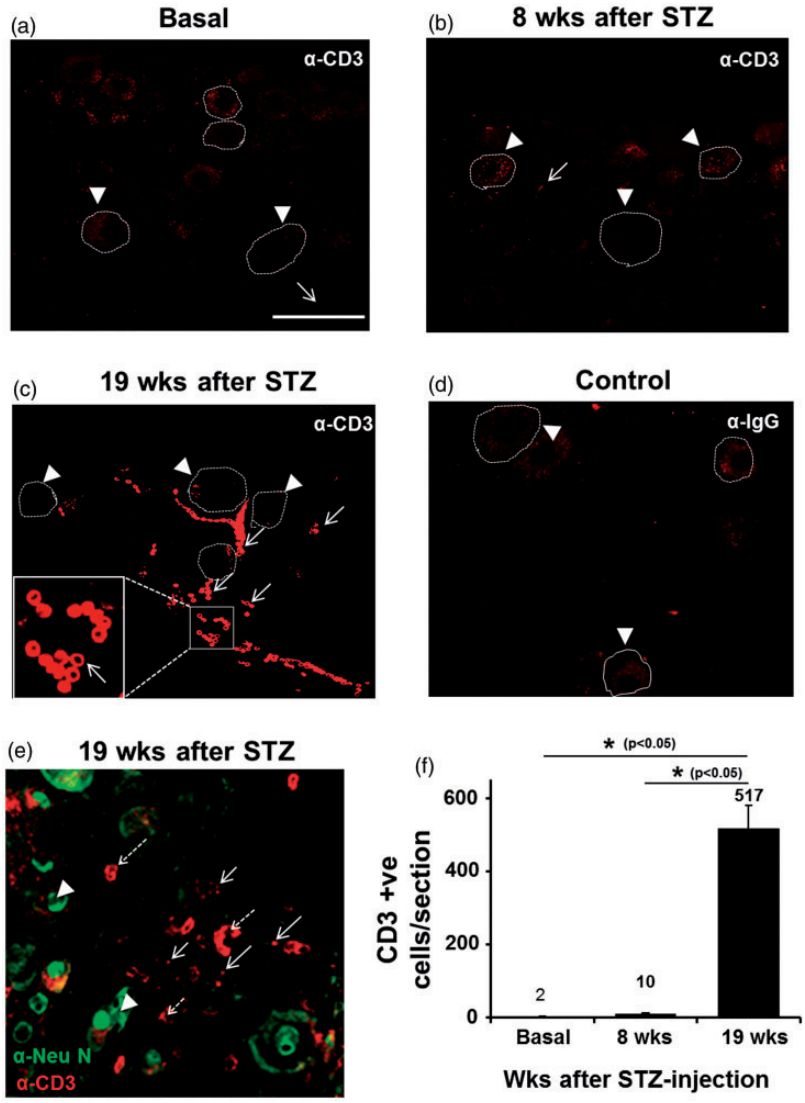

Figure 4. Immunofluorescence analysis of CD3-immunoreactive T-cells infiltrating DRG of mice in the basal state or at 8, 19 or 24 weeks after STZ injection or control injection. (a–c). Typical examples of infiltrating T-cells. Arrowheads represent the soma of DRG neurons whereas arrows represent T-cells. (d) Negative staining control lacking primary antibody. (e) Double immunostaining of CD3 (red) and NeuN (green) immnuoreactive in DRG section of 19 weeks post-STZ injected mice. Arrows represent individual CD3-positive cells whereas dotted arrow represents clumped CD3-positive immune cells. Arrow heads represent NeuN-labelled DRG neurons. (f) Quantification of CD3-immunoreactive T-cells in DRG sections ($n = 15$ sections). All data points represent mean $\pm$ SEM. *$p < 0.05$, ANOVA followed by post-hoc Tukey's test. Scale bars represent 50 μm.
ANOVA: analysis of variance; DRG: dorsal root ganglia; SEM: standard error of the mean; STZ: Strepozotocin.

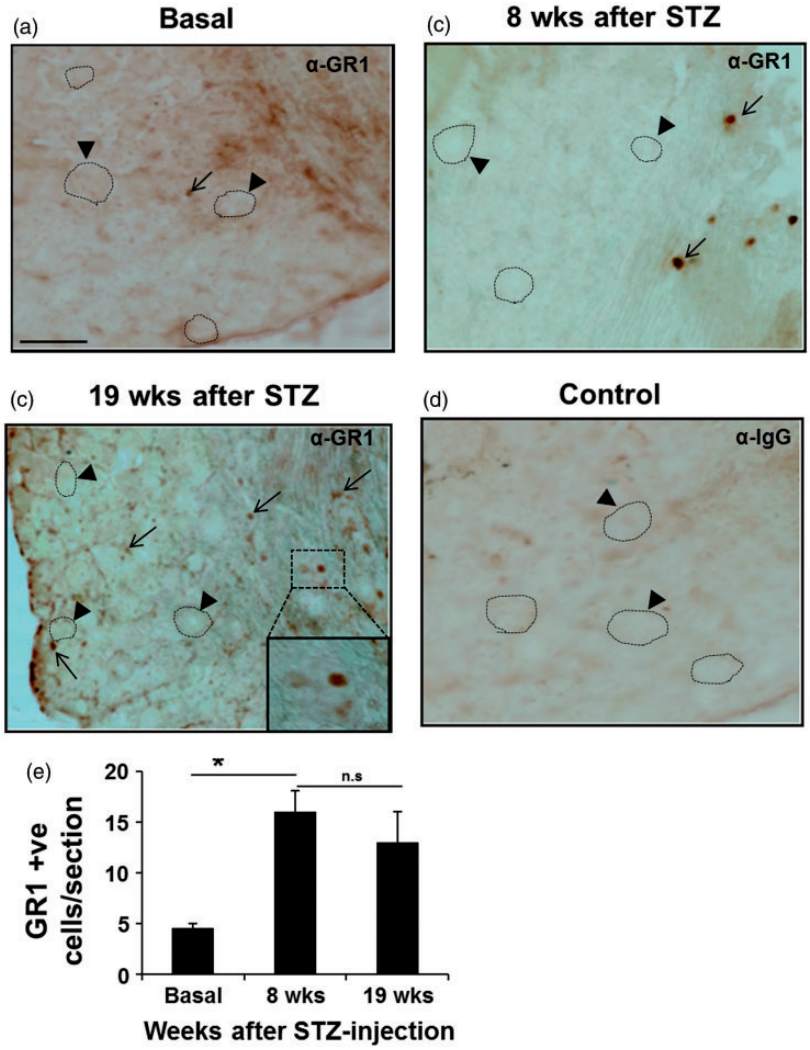

Figure 5. Immunofluorescence analysis of Gr1-immunoreactive neutrophils infiltrating DRG of mice in the basal state or at 8, 19 or 24 weeks after STZ injection or control injection. (a–c). Typical examples of infiltrating neutrophils. Arrowheads represent the soma of DRG neurons whereas arrows represent neutrophils. (d) Negative staining control lacking primary antibody. (e) Quantification of Gr1-immunoreactive neutrophils in DRG sections ($n = 15$–30 sections). All data points represent mean ± SEM. *$p < 0.05$, ANOVA followed by post-hoc Tukey's test. Scale bars represent 50 μm.
ANOVA: analysis of variance; DRG: dorsal root ganglia; SEM: standard error of the mean; STZ: Streptozotocin.

(arrows in Figure 4(c); double immunohistochemistry with anti-NeuN as a neuronal marker is shown in Figure 4(e) and quantification shown in Figure 4(f)).

To label neutrophils invading the DRG, we performed immunohistochemistry against the pan neutrophil marker, Gr1. Significant neutrophil infiltration was observed over both early and late stages post-STZ (arrows in Figure 5(b) and (c), quantification in Figure 5(e); negative staining control in Figure 5(d)). Thus, tonic pain and nociceptive hypersensitivity is concurrent with neutrophil invasion in the DRG over early phase of DPN. In chronic DPN, sensory loss and tonic pain are accompanied by infiltration of T-cells and neutrophils in the DRG.

Discussion

Clinically, DPN represents a perplexing mix of symptoms which paradoxically combine a loss of sensation at extremities (particularly feet) with burning, on-going pain.[28] However, rodent analyses on DPN have largely focused on hyperalgesia to thermal and mechanical stimuli early after the onset of diabetes. Late periods post-diabetes induction, in contrast, which largely correspond to chronic stages of highly painful DPN in patients, have been largely ignored in rodent models owing to the hypoalgesia that sets in progressively. Here we report that later stages post-diabetes induction, which are characterized by sensory loss, are paradoxically associated with tonic pain. We observed that this tonic pain does

not temporally correlate with cellular pathology in the somata DRG neurons, but rather with invasion of immune cells.

In order to promote translation of research insights, there is a large need in the pain field to align rodent models with clinically relevant forms of pain, mimicking the temporal and pathophysiological course of clinical disorders.[29] Therefore, it is important to thoroughly characterize behavioural outcomes in rodents, focusing not only on stimulus-dependent, evoked behaviours, but also behavioural measures of emotional components of pain and pain effect. In diabetic models in rodents, studies have largely addressed molecular mechanisms underlying thermal hyperalgesia, with a focus on ion-channels such as TRP channels, sodium channels, etc., with a focus on peripheral sensory neurons and afferents.[30,31] In contrast, there are very few pharmacological studies on tonic pain and pain affect in models of diabetic neuropathy. In the CPP test, a reinforcing or rewarding effect of pain relief is considered indicated by a relative increase in time spent in the area that had been paired with the pain-relieving treatment.[10] So far, the CPP test has been successfully employed to study tonic pain in a wide range of neuropathic and inflammatory pain disorders in rodents.[32] In the context of diabetes, delivery of a soluble epoxide hydroxylase inhibitor has been reported to induce CPP at early stages in a model of diabetes.[32] Razieh Samandari[33] studied the impact of diabetes on morphine-induced CPP at 7 days post-STZ and concluded that the rewarding properties of morphine increased at 7 days post-STZ. These data could also interpreted as an increase in tonic pain in STZ-treated mice at 7 days post-STZ.[33] Our data now indicate that stages of hypersensitivity, which develop at 5 to 7 weeks post-STZ, are marked by both tonic pain as well as hypersensitivity to heat and mechanical stimuli. Interestingly, electrophysiological recordings performed at 4 weeks post-STZ treatment in peripheral skin–nerve recordings have revealed an on-going discharge in diabetic, but not control, C-fibres as well as exaggerated sensitivity to nociceptive and non-nociceptive strengths of somatic stimuli.[34] These observations are highly consistent with the behavioural outcomes of tonic pain as well as hypersensitivity that we report here.

Another key requirement towards improved translation from mouse models to human disorders is to consider the temporal course of behavioural analyses and match chronic stages of pain disorders accordingly with rodent analyses in longitudinal studies.[29] Thus, given the chronic, progressive nature of pain in DPN, it is crucial to study chronic phases of diabetic pain in rodent models. However, pain-related studies in diabetic models are typically studied in days to a few weeks post-diabetes induction, and longitudinal, long-term studies are missing. In contrast, studies addressing the metabolic and cell death-related mechanisms of neuropathy do consider chronic stages of neuropathy, but do not address pain.[35,36] We therefore found it imperative to study sensory and affective components of pain in a long-term manner and observed that as diabetic mice progressively develop progressive hyposensitivity to external stimuli, they still maintain the component of tonic, on-going pain. This phenotype faithfully replicates the manifestations of DPN in the human condition and open the way for addressing mechanisms of tonic pain at late (chronic) stages of the disorder.

ATF3 is marker of cellular stress and injury, which is upregulated in injured neurons in models of nerve injury.[37] Here, we used it to test whether diabetic neuropathy involves a similar pattern of cellular stress and injury in DRG neurons. We observed that this is not the case, indicating that dysfunction of sensory neurons is different between conditions of metabolic dysfunction versus direct traumatic injury.

Neuro-immune interactions are a cardinal feature of not only inflammatory pain disorders, but also play a critical role in neuropathic pain.[38] Recent studies particularly implicate T-cells and neutrophils in regulating the excitability and function of peripheral and spinal neurons in chronic pain models of lesion-induced neuropathic pain.[14,39,40] In case of diabetes, because there is no focal damage in one particular avenue, it is even more likely that inflammation downstream of metabolic damage contributes to spontaneous pain. We therefore studied immune cell infiltration in a longitudinal analysis in conjunction with spontaneous pain in diabetic neuropathy.

We observed that in mice modelling type 1 diabetes, marked infiltration of Gr-1-positive immune cells occurs in the DRG parenchyma at stages associated with nociceptive hypersensitivity. The Gr-1-positive population comprises the Ly6C and Ly6G components and thus includes inflammatory monocytes/macrophages, neutrophils and eosinophils.[41] Here we observed that the number of infiltrating T-cells markedly exceeded the number of Gr-1-positive immune cells. Our observations here are consistent with our recent finding that pharmacological blockade of neutrophil elastase (leukocyte elastase), which is expressed in both neutrophils and T-cells,[14] significantly reduces the magnitude of nociceptive hypersensitivity at 5to 8 weeks post-STZ.[42] Importantly, we also report here that at chronic stages of DPN, where tonic pain is apparent despite hypoalgesia, a significant infiltration of neutrophils and T-cells is observed in the DRG. In nerve biopsies of patients with severe DPN, similar filtrations of T-cells and neutrophils have been reported.[27] Thus, the DPN mouse model reproduces important clinical pathophysiological features, thereby opening the way for mechanistically addressing the functional contributions of

neutrophil- and T-cell–derived mediators in tonic pain at chronic stages of DPN.

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Declaration of Conflicting Interests

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