

ORIGINAL ARTICLE

Title Page

Nerve stretch injury induced pain pattern and changes in sensory ganglia in a clinically relevant model of limb-lengthening in rabbits

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Short title: Pain pattern and changes in DRGs in a limb-lengthening model

Summary

We used a model of tibial lengthening in rabbits to study the postoperative pain pattern during limb-lengthening and morphological changes in the dorsal root ganglia (DRG), including alteration of substance P (SP) expression. Four groups of animals (naïve; OG: osteotomised only group; SDG/FDG: slow/fast distraction groups, with 1mm/3mm lengthening a day, respectively) were used. Signs of increasing postoperative pain were detected until the 10th postoperative day in OG/SDG/FDG, then they decreased in OG but remained higher in SDG/FDG until the distraction finished, suggesting that the pain response is based mainly on surgical trauma until the 10th day, while the lengthening extended its duration and increased its intensity. The only morphological change observed in the DRGs was the presence of large vacuoles in some large neurons of OG/SDG/FDG. Cell size analysis of the S1 DRGs showed no cell loss in any of the three groups; a significant increase in the number of SP-positive large DRG cells in the OG; and a significant decrease in the number of SP-immunoreactive small DRG neurons in the SDG/FDG. Faster and larger distraction resulted in more severe signs of pain sensation, and further reduced the number of SP-positive small cells, compared to slow distraction.

Key words: limb-lengthening, substance P, DRG, neuropathic pain, postoperative pain, rabbit

Introduction

Congenital anomalies and acquired limb length discrepancies cause several physical and mental problems in children suffering from these deformities. Graded limb-lengthening by callus distraction (callotaxis) is a surgical procedure widely used to correct tubular bone deformities. Although the functional improvement in children and changes of their life quality are dramatic after the surgery, “its cost in terms of pain” may be high (Young *et al.* 1994). While postoperative pain following other orthopaedic surgical procedures decreases rapidly, continued pain throughout the entire lengthening process, followed by a decreased pain level after the distraction process, is observed in patients undergoing limb-lengthening (Young *et al.* 1994).

Nerve injury is one of the most serious complications of limb-lengthening (Galardi *et al.* 1990, Nogueira *et al.* 2003, Velazquez *et al.* 1993) leading to nerve palsy and chronic neuropathic pain (Antoci *et al.* 2006, Green 1991, Young *et al.* 1994). The severe pain is one of the most common causes of interruption of the lengthening process. In this case the fixator has to be removed and the total regeneration of the bone lasting for several months must be waited for. Then further operations are necessary to replace the fixator and to remove it again when the planned extremity length is achieved.

Clinical studies have reported nerve injuries in 0.7-30.0% of the patients undergoing limb-lengthening procedures. Better understanding of the background of pain associated with limb-lengthening may result in more effective treatments which could save the children from many unnecessary operations and the risk of anesthesia. Although the limb-lengthening techniques attract wide attention because of the improvements provided (93% increase in bone segment length), only a few studies have been carried out focusing on the effects of the lengthening process on the peripheral and central nervous system.

Peripheral nerve injury generates behavioural signs of persistent pain, and initiates a cascade of events resulting in changes in peptide expression in dorsal root ganglion (DRG) cells and in the spinal dorsal horn. The tachykinin peptide SP is localized in small DRG cells which convey information from peripheral nociceptors (Kellstein *et al.* 1990, Lawson *et al.* 1997). SP is released from the primary afferents into the spinal dorsal horn following intense peripheral stimulation (Duggan *et al.* 1988, Duggan *et al.* 1995, Marvizon *et al.* 1997) and interacts with neurons expressing neurokinin-1 (NK1) receptor (Brown *et al.* 1995, Littlewood *et al.* 1995, Liu *et al.* 1994, Mantyh *et al.* 1995). It has been shown that SP is required to generate moderate to intense pain (Cao *et al.* 1998) and is also responsible for adaptive response to stress (De Felipe *et al.* 1998). The great majority of spinal lamina I projection neurons express NK1-receptor (Marshall *et al.* 1996, Todd *et al.* 2000) and it has been demonstrated that NK1-receptor-expressing cells in superficial dorsal horn are essential for central sensitization, and for establishing and maintaining inflammatory and neuropathic pain states (Khasabov *et al.* 2002, Mantyh and Hunt 2004, Mantyh *et al.* 1997, Nichols *et al.* 1999).

In this study, we developed a clinically relevant model of tibial lengthening in rabbits to detect and quantify the signs of postoperative pain during limb-lengthening and to study the morphological changes and alteration of SP expression in the DRG.

Methods

Animals. The experimental procedures were carried out on New Zealand white rabbits of either sex, weighting 2.5-3.0 kg. All animal experiments were performed in accordance with European Communities Council Directive of 24 November 1986 (86/609/ECC) and were approved by the Committee on Animal Experiments, Semmelweis University, Budapest, Hungary. All efforts were made to minimize the number of animals used and their suffering.

Limb-lengthening model. Insertion of an external skeletal fixator and osteotomy was performed under deep ketamine-medetomidine general anaesthesia (25 mg/kg and 0.5 mg/kg i.m., respectively) in 15 rabbits. The skin was incised on the medial aspect of the hindlimb and the tibia was exposed. An Orthofix-M 101 minifixator with four self-drilling cortical screws intended for metacarpal lengthening in humans was applied and a transverse tibial osteotomy was performed. Care was taken to avoid damaging nerves, muscles and other soft tissues. The cut ends of the tibia were compressed to support callus formation until the 7th postoperative day. The 15 rabbits undergoing the surgical procedure were divided into three groups (n=5 for each group): in the osteotomised group (OG) osteotomy was not followed by lengthening; in the slow and fast distraction groups (SDG and FDG) osteotomy was followed by distraction initiated at a rate of 1mm/day done once a day (for 14 days) and three times a day (for 8 days), respectively, until the bone had reached 120% and 130% of its original length (Fig. 1.). A further 5 naïve rabbits, which did not undergo any surgical procedure were used as controls. After 3-4 days following the end of the distraction period the animals were sacrificed.

During the experimental procedures, the rabbits were kept in separate standard cages (0.5 m² / animal) with food and water available *ad libitum*. The environment was maintained at 22 ± 2°C (60% humidity) with a 12h light-dark cycle.

X-ray analysis Three postoperative radiographs (the first taken immediately after the operation, the second at the 10th postoperative day and third at the end of the lengthening period) were made in standard dorsoplantar and mediolateral projections (50 kV, 8.0 mAs). The positions of the pins were checked on the first radiogram. The callus formation and the lengthening were analysed on the second and third X-ray picture respectively (Fig. 1).

Autopsy. Since nerve damage could occur intraoperatively due to physical injury by equipment, or in the distraction phase as a result of nerve impingement on a pin or wire, or entrapment at certain predisposed locations, the proximal tibiofibular junctions were examined immediately after perfusion fixation. The peroneal nerve was exposed at the level of the stifle and was followed to the mid part of the tibia. The position of the pins was examined, and search was made for signs of nerve entrapment.

Behavioural study. The measurements of the effects of the osteotomy as well as the lengthening processes were carried out from the 7th until the 18th (FDG) or 24th (SDG) days after the osteotomy by an Incapacitance Tester (SG1 3QD, Churchill Electronic Services, Ltd) capable of measuring the load on hindlimbs in grams. The incapacitance was defined as the difference between the loads exerted by hindlimbs expressed as a percentage of the total load exerted.

The statistical comparisons of the data were performed by one way ANOVA followed by Tukey-Kramer Multiple Comparisons Test.

Immunocytochemistry. All the rabbits used in the study were perfused with a fixative consisting of 4% (para)formaldehyde in 0.1M phosphate buffer (PB), pH adjusted to 7.4. First sacral (S1) ganglia were removed and postfixed overnight. Longitudinal 50µm sections were cut with a Vibratome and kept in serial order, treated in 50% ethanol to enhance antibody

penetration and then incubated for 72 hours in rat monoclonal SP antibody (AbD Serotec-Oxford Biotechnology; 1:200), and for 24 hours in donkey anti-rat IgG conjugated to Alexa-594 (Invitrogen-Molecular Probes, Budapest, Hungary; 1:500). Both the primary and secondary antibodies were diluted in phosphate buffered saline with 0.3% Triton X-100. Finally, sections were treated for 30 mins with the NeuroTrace blue fluorescent Nissl stain (Invitrogen-Molecular Probes; 1:200), then rinsed in PB, mounted in Vectashield (Vector Laboratories) and scanned on a Nikon Eclipse E800 microscope attached to a Bio-Rad Radiance 2100 Rainbow confocal laser scanning system.

Analysis Longitudinal sections of S1 DRGs were selected for cell size analysis by using every third section of each series. In each of these sections, overlapping fields of approximately $296 \times 296 \mu\text{m}^2$ covering the whole ganglion cross-section were scanned with a 40x lens using laser beams of 405nm and 543nm in λ -mode to reveal fluorescent Nissl staining and SP immunoreactivity. For each field, 20 optical sections were acquired at a z-separation of $1 \mu\text{m}$ in the middle of the full section thickness to avoid double counting of cells in different sections.

NeuroLucida for Confocal 4.34 software (MicroBrightField, Williston, VT, USA) was used to measure the long (LA) and short (SA) axes of the neuron cross-sections. Only cells that had a visible nucleolus within the $20 \mu\text{m}$ thick stack of optical sections were analysed. This method led to the counting of 1369 ± 389 neurons per DRG. Since the shape of the majority of neurons in transverse sections through the ganglion was elliptical, the cross-sectional areas were calculated using the formula: $\text{area} = \pi(\text{LA})(\text{SA})/4$ (Farel and McIlwain 2000, Weissner *et al.* 2006).

Due to the fact that the fluorescent Nissl method and the SP immunoreaction resulted a slightly different pattern of staining within individual neurons, the LA and SA of a selected neuron differed slightly, depending on whether SP immunoreactivity or Nissl staining was

used to make the measurements. To standardize the measurement, all SP-immunoreactive cells were measured both ways and a correction factor was calculated and applied for those cells which were not SP-positive: $\text{area} = \pi(1.07 \text{ LA})(1.07 \text{ SA})/4$. For statistical analysis, proportions of the SP-positive neurons in different groups were compared by z-test ("Difference between two proportions-test"). The level of significance used was $p < 0.05$. Statistical analysis was done using SAS statistical software package (SAS/STAT, Software Release 9.1.3., SAS Institute Inc., NC, USA).

Results

Animals. All rabbits recovered well after surgery. During the study there was no observable prostration, lack of appetite and body weight loss of the rabbits. No signs of infection or inflammation were observed in any of the animals. Furthermore, neither malposition of the pins nor malunion was found during the distraction period. In addition, neither impingement nor entrapment of the nerve was observed in any rabbits.

Behavioral study. Measures of weight-bearing on the two hindlimbs in each group (naïve; OG: osteotomised only group; SDG/FDG: slow/fast distraction groups, with 1mm/3mm lengthening a day, respectively) are shown in Fig. 2. All operated animals preferentially bore weight on the unoperated hindlimb.

The differences in the incapitance of the naïve and the operated groups were significant throughout the investigation period. The incapitance gradually increased till the 10th postoperative day (3rd day of elongation) in all 3 operated groups and started to decrease slightly in the OG from that time point. **Statistically significant differences in the incapitance between the OG and lengthened groups were recorded between the 15th and 21st postoperative days and disappeared again in the SDG as distraction was terminated but a moderate decrease of incapitance was observable also in the FDG.** Application of the 3mm/day elongation produced an increased incapitance during the study.

SP expression. The pattern of immunostaining for SP (Fig. 3.) and the proportion of SP-positive neurons in naïve rabbit DRGs were very similar to those reported in rats (Weissner *et al.* 2006). SP was expressed in $26.6\% \pm 2.9$ of the total number of counted cells in S1 DRGs. Detailed size analysis of DRG neurons in rabbits has not been carried out previously. The cross-sectional area of 6 707 cells in the 5 DRGs was calculated using the measured longest

and smallest diameter of the individual neurons according to the description of (Weissner *et al.* 2006). The size distribution was skewed, and appeared bimodal when plotted on log scale (Fig. 4.), as reported for rat (Lawson 1979, Weissner *et al.* 2006). The two peaks of the histogram represented two overlapping cell populations by size. The SP-positive neurons seemed to be associated with the first peak. Fitting the two size distributions by maximum likelihood placed the intersection at $992.3 \mu\text{m}^2$ in naïve rabbits, which divided the neurons into two groups: small cells ($\leq 992 \mu\text{m}^2$) and the large cells ($\geq 992 \mu\text{m}^2$) in the ganglion. In naïve rabbits $19.7\% \pm 3.5$ and $6.9\% \pm 2.8$ of the SP-immunoreactive DRG neurons belonged to the small and large cell groups, respectively.

In OG animals, $28.0\% \pm 1.0$ of the neurons were SP-positive which did not differ significantly from the naive group ($p=0.2903$). In the lengthened groups, although the total number of cells did not change, the proportion that were SP-immunoreactive decreased significantly ($18.0\% \pm 1.8$ and $15.9\% \pm 2.5$ in SDG and FDG, respectively) compared to both the naive (naive vs. SDG: $p<0.01$; naive vs. FDG: $p<0.01$) and the OG (OG vs. SDG: $p<0.01$; OG vs. FDG: $p<0.01$). Furthermore, the ratio of the SP-positive small and large cells also altered in the OG and the lengthened groups. The proportion of SP-positive small cells (SDG: $11.5\% \pm 0.9$, FDG: $7.9\% \pm 1.9$) decreased significantly (control vs. SDG: $p<0.01$, control vs. FDG: $p<0.01$), while the proportion of stained large cells (SDG: $6.7\% \pm 1.5$, FDG: $7.9\% \pm 2.4$) did not change significantly (control vs. SDG: $p=0.81$; control vs. FDG: $p=0.21$) in the lengthened groups. In addition, the faster distraction yielded a further significant decrease in the number of SP immunostained small cells in the FDG compared to the SDG (SDG vs. FDG: $p<0.01$). Interestingly, in the OG, the number of the SP-immunoreactive small neurons ($17.1\% \pm 2.7$) decreased only slightly ($p=0.11$), whereas the number of the stained large cells ($11.1\% \pm 3.5$) increased significantly ($p<0.01$) compared to the control group (small neurons: $19.7\% \pm 3.5$, large neurons: $6.9\% \pm 2.8$). Proportions of SP-containing cells in different groups are summarized on Fig. 5.

Morphological changes in DRG cells. At the light microscopic level, the location and distribution of the supporting (satellite) cells did not show any abnormality either in osteotomised and the lengthened animals following the surgery. The general morphological appearance of the small ganglion cells was normal in these groups. The only obvious difference was found in some large cells. The nucleus of certain large neurons became eccentric and huge vacuoles appeared in the cytoplasm (Fig. 6.). Among the counted neurons, 3-4 vacuolated large cells in OG, 13-15 in SDG and 13-17 in FDG were found per DRG.

Vacuolated large DRG neurons expressing SP were not found in any operated groups.

Discussion

Behavioral study. Animal models of neuropathic pain have been established in rats and mice, however, the limb-lengthening procedure would not be easy to carry out in these species because of the size of their limbs. Since precise insertion of the fixator was essential to avoid surgical complications, a longer bone was required and, hence, the rabbit tibia was selected. However, quantifying signs of pain in rabbits is complex. The most commonly used behavioural tests (e.g. hot-plate test, von Frey filaments) developed for rodents are difficult to carry out because rabbit hindpaw is hairy. Consequently, these tests have not been standardised on rabbits, and no baseline data is available.

In this study we measured the load on hindlimbs in grams then applied the definition of the incapacitance. This measurement clearly showed the time course and the extent of the changes in the weight bearing capacity of the operated limbs. The decreased weight bearing capacity suggested tenderness of the involved limbs. Since no signs of infection or inflammation were detected during the study, it suggested that the increased tenderness of the operated legs resulted from the surgical and lengthening procedures.

Model of limb-lengthening. The pain measurements in our study demonstrated an increasing postoperative pain response till the 10th postoperative day both in the OG and the lengthened groups, which then decreased gradually in the OG animals, but lasted for a further 2 weeks in the lengthened groups. These results suggest that the pain response was based mainly on the surgical trauma until the 15th day. Since the autopsy did not show any signs of nerve impingement or entrapment, the long-lasting pain response which started to decrease when the lengthening process completed in the lengthened groups was presumably maintained by the nerve stretch during the distraction period. Furthermore the more rapid lengthening (by 3mm/day) of the affected limb led to more severe signs of pain. The time course and pattern

of postoperative pain described in this study are in very good agreement with clinical reports taken from humans following limb lengthening (Young *et al.* 1994).

Cell size distribution. Detailed size analysis of S1 DRG neurons was carried out in rabbits as demonstrated in rats by Weissner *et al.* (2006). Fig. 3. shows that the “large” cell population in the rabbit S1 DRG is much bigger (relative to the “small” population) than is the case for the rat L5 ganglion. Furthermore the SP staining is limited to small neurons in normal rats, whereas SP is also expressed in a small population of large DRG cells in naïve rabbits.

Vacuolar changes in the DRGs. Vacuolar changes in large DRG neurons in both the OG and the lengthened groups, described in this work, were also reported previously in rats after unilateral transection of sciatic nerve (Groves *et al.* 1997). It was demonstrated that these neurons were viable and existed for a long period of time following axotomy. Administration of neurotrophin-3 markedly reduced the incidence of the vacuolated cells in the DRGs in this type of injury. It was concluded that vacuolation of certain type of cells represented a specific type of pathological response to nerve injury (Groves *et al.* 1997).

SP expression and pain. It is well known that peripheral nerve injury not only generates behavioural signs of persistent pain but results in changes in peptide expression in dorsal root ganglion (DRG) cells and in the spinal dorsal horn. Calcitonin gene-related peptide (CGRP) and SP are down-regulated while other peptides such as vasoactive intestinal polypeptide (VIP) and galanin (GAL) are upregulated, while neuropeptide Y (NPY) is expressed *de novo* (Baranowski *et al.* 1993, Henken *et al.* 1990, Hokfelt *et al.* 1994, Jessell *et al.* 1979, Nielsch *et al.* 1987, Noguchi *et al.* 1993, Noguchi *et al.* 1990, Noguchi *et al.* 1990, Noguchi *et al.* 1989, Villar *et al.* 1989, Wakisaka *et al.* 1992). Since the antibodies from different hosts and

sources against CGRP, VIP, GAL and NPY, routinely used in our laboratory, did not work on rabbit tissue, the only change we could study was the SP expression.

Although it has been demonstrated that SP is down regulated in small but up regulated in large DRG cells in different models of nerve injury (Baranowski *et al.* 1993, Bisby and Keen 1986, Honore *et al.* 2000, Kobayashi *et al.* 2004, Ma and Bisby 1998, Swamydas *et al.* 2004, Tessler *et al.* 1985, Villar *et al.* 1989, Weissner *et al.* 2006), the functional meaning of these changes is still unknown.

It has been reported that alterations in peptide expression appear 3 days after nerve injury and last much longer than 2 weeks (Kajander and Xu 1995, Malmberg and Basbaum 1998, Munglani *et al.* 1996, Sommer and Myers 1995). This suggests that if osteotomy and the lengthening processes affected the SP expression, this should have been detected at the time point at which animals were sacrificed in our experiments (18 days in FDG and 24 days in OG and SDG following osteotomy).

It has been proposed that in a distal tibial fracture model, SP signalling contributes to the vascular and nociceptive changes (pain and allodynia) (Guo *et al.* 2004). In our limb-lengthening model, although all animals were osteotomised in the operated groups, the changes in SP expression were completely different in the OG and in the lengthened groups, suggesting that the osteotomy induced postoperative pain might not be related to the alteration of SP expression. Possible explanations for the discrepancy between the findings in these two studies include species differences (rat vs. rabbit), and the fact that cast immobilization was used by Guo *et al.* (2004). A further difference between the two studies was that distal limb oedema was observed in the fracture model, but was not seen in our study.

Mechanical allodynia (pain in response to non-noxious mechanical stimuli) is one of the most characteristic features of chronic pain, including inflammatory, neuropathic and postoperative pain syndromes. Allodynia appears in patients undergoing limb-lengthening (Pap *et al.*, unpublished observation). Several lines of evidence suggested that *de novo*

expression of SP in A β cutaneous afferents (Noguchi *et al.* 1994, Weissner *et al.* 2006) may contribute to allodynia after certain types of nerve injuries (Weissner *et al.* 2006). However, other studies failed to detect SP release in the spinal cord following electrical stimulation of A β afferents belonging to the chronically transected sciatic nerve (Allen *et al.* 1999, Malcangio *et al.* 2000). Furthermore, Hughes *et al.* (2007) did not observe SP-immunoreactivity in the central terminals of A β afferents in the dorsal horn either in chronic constriction injury or in spinal nerve ligation models. One of the most notable results of our study is that although osteotomy was carried out, vacuolisation was seen in the large DRG neurons and the pain pattern was very similar till the 10th day in all 3 operated groups, significant increase in the number of SP positive large cells appeared only in the OG. This data does not support the contribution of A β afferents to the pain behaviour in our model.

Kozlova *et al.* (1986) have pointed out a significant growth-promoting effect of SP for sympathetic ganglia and spinal cord culture and highlighted the functional significance of SP in nerve tissue development and regeneration. Since the osteotomy was not followed by further intervention in the OG in our study, the decreasing signs of pain from the 15th day suggested an ongoing regeneration in the nervous system. Accordingly, *de novo* expression of SP in large sensory neurons may reflect the regeneration of the injured cells in the DRG or the nervous system. This finding is in a good agreement with the previous suggestion, that after nerve injury the primary role of the sensory neurons changes from neurotransmission to cell survival and regeneration (Hokfelt *et al.* 1994).

Previous studies have demonstrated that the anatomical and behavioural changes correlate in different models of nerve injury (Cameron *et al.* 1997, Malmberg and Basbaum 1998). An important finding of our study is that a significant decrease in the number of SP-immunoreactive small DRG neurons was detected solely in the lengthened groups. The increased and maintained pain response was in good correlation with the decreased SP expression in the small DRG cells in the lengthened groups. Faster and larger distraction

resulted in a more severe pain response and lowered further the number of SP positive small cells compared to slow distraction. These observations demonstrate that nerve stretch injury shares common features with other nerve injury models. At the same time, in contrast to other models, this model clearly shows the correlation between the rate of the injury and the changes in the SP expression and pain responses.

Future perspective. Since the gradual lengthening itself does not cause permanent injury, the model used in this work is suitable not only for studying the pathological changes but also the regeneration processes which may contribute to preventing or ameliorating the postoperative pain. As the pain pattern described in this model is in good agreement with human studies, the effects of new analgesics could also be tested.

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Figures

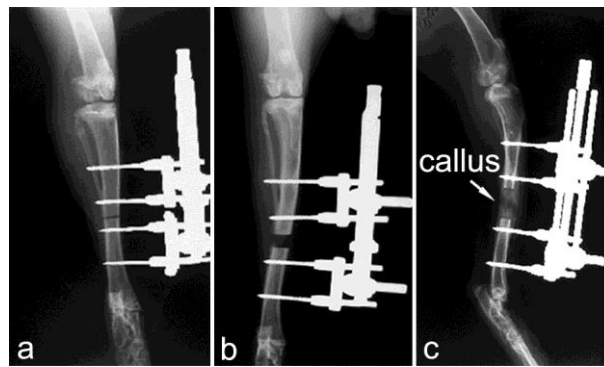


Figure 1. Postoperative radiographs of the rabbit tibia in standard dorsoplantar and mediolateral projections. Images taken a) immediately after the operation, b) at the 10th postoperative day, and c) at the end of the lengthening demonstrate the positions of the pins, the lengthening and the callus formation, respectively.

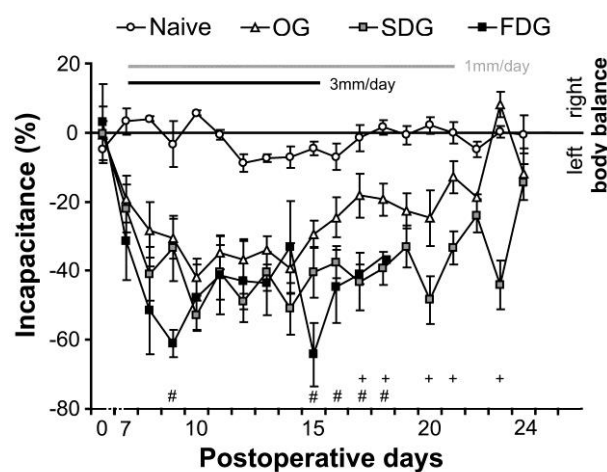


Figure 2. Postoperative pain measurements in rabbits. Time-course of changes in incapacitance calculated from the measurements of weight bearing capacity of the hindlimbs in naïve, osteotomised (OG), slow (SDG) and fast (FDG) distraction animals. Data indicate mean \pm SEM. **Statistical analysis: one-way ANOVA, Tukey-Kramer *post hoc* test, +: $p < 0.05$ OG vs. SDG, #: $p < 0.05$ OG vs. FDG.**

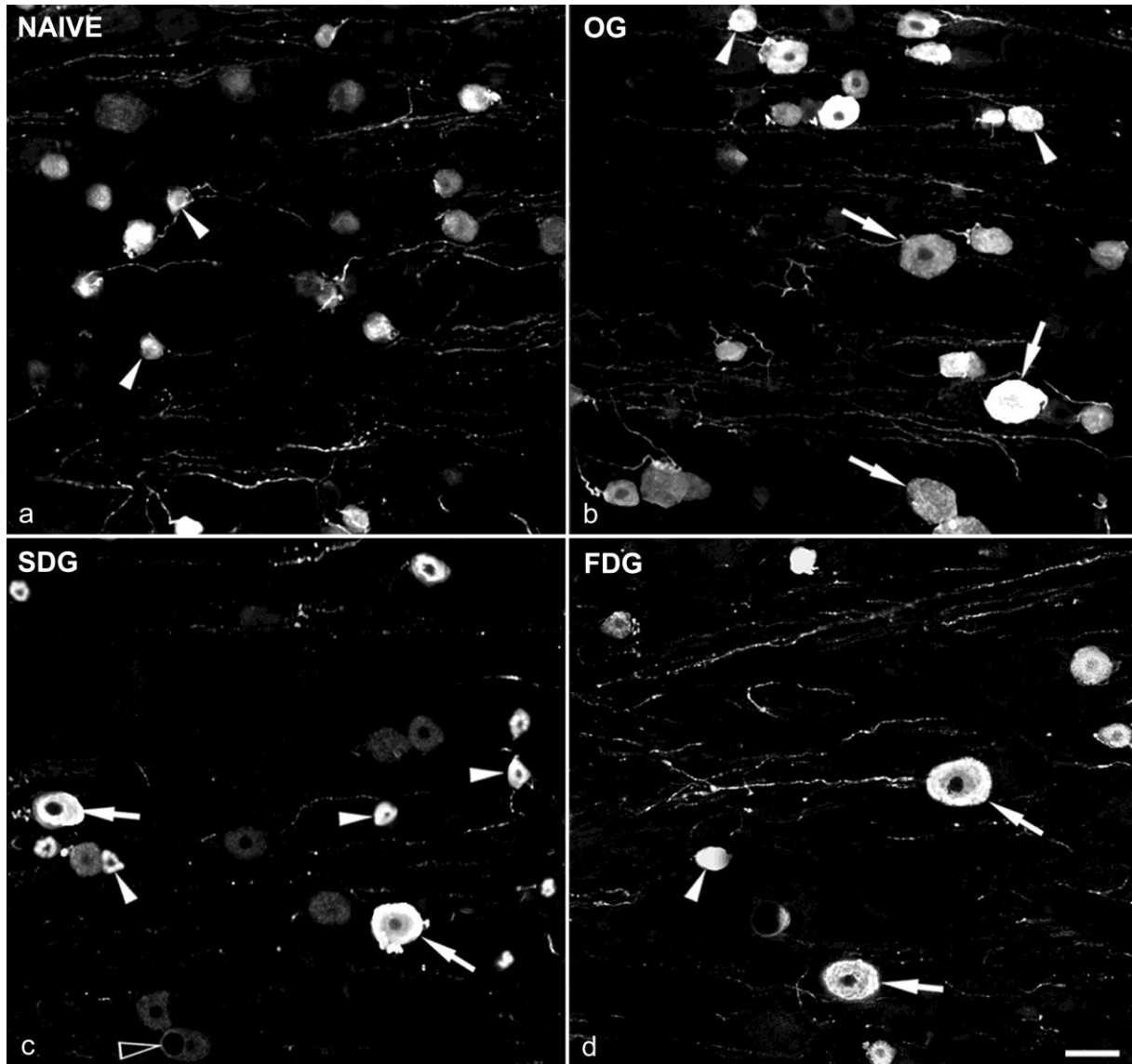


Figure 3. Confocal images showing SP immunoreactivity in S1 dorsal root ganglia. DRGs were obtained from a) naïve, b) osteotomised (OG), c) slow (SDG), and d) fast (FDG) distraction groups. Arrowheads: SP-positive small neurons, arrows: SP stained large DRG cells. Each picture shows a projection of 5 optical sections at 1 μm z-separation. Scale bar: 50 μm

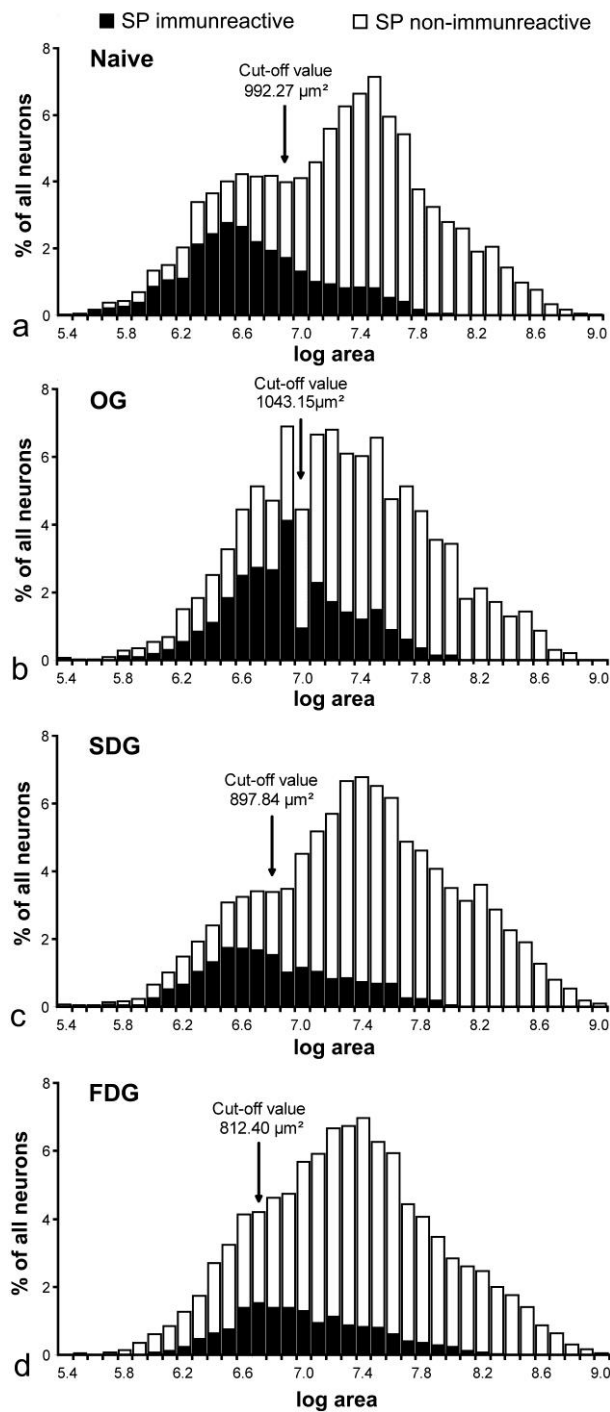


Figure 4. Size distribution of S1 dorsal root ganglion neurons in naïve (a), osteotomised (b; OG), slow (c; SDG) and fast (d; FDG) distraction animals. All measured neurons (27 376 cells of 20 S1 DRGs) were separated by the presence (shaded bars) or absence (open bars) of SP and were binned by area in 0.1 natural log increments as it was demonstrated in rats by Weissner et al. (2006).

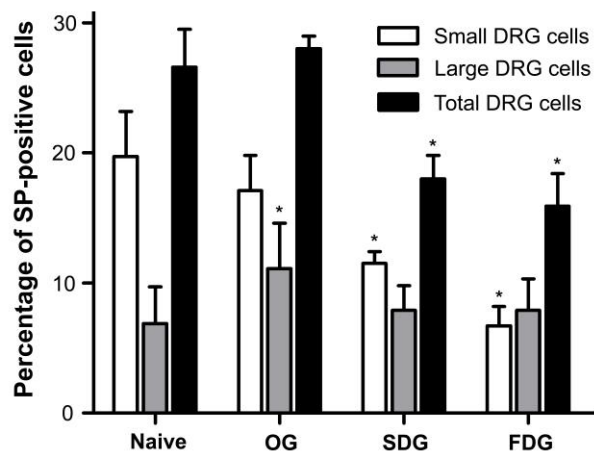


Figure 5. Proportions of SP-immunoreactive small and large DRG cells. Parallel decrease of percentage of small and total SP-immunopositive DRG cells was observed in both lengthened groups compared to the naïve and sham-operated animals. Significant increase of large DRG neurons was exclusively characteristic for OG rabbits. *: $p < 0.01$.

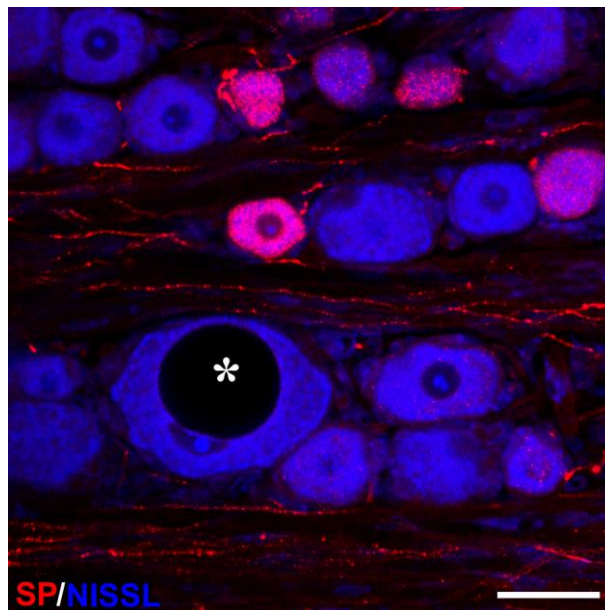


Figure 6. Double labelled confocal image of a S1 ganglion taken from an animal in the slow distraction group. Nissl-staining in blue and SP immunoreactivity in red. Note the large neuron (asterisk) with eccentric nucleus and huge vacuole in the cytoplasm. Image was obtained from a stack of 3 optical sections, z-spacing: $1\mu\text{m}$. Scalebar: $50\mu\text{m}$.