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Assessment and analysis of mechanical allodynia-like behvior induced by spared nerve injury (SNI) in the mouse

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La première description dans une publication médicale des douleurs neuropathiques remonte à 1872, le Dr S.W. Mitchell les résumant ainsi [...]" la causalgie est la plus terrible des tortures qu'une lésion nerveuse puisse entraîner "[...]. Par définition, la douleur neuropathique est une douleur chronique faisant suite à une lésion ou dysfonction du système nerveux. Malgré les progrès faits dans la compréhension de ce syndrome, le détail des mécanismes impliqués nous échappe encore et son traitement reste insuffisant car moins de 50% des patients sont soulagés par les thérapies actuelles.

Différents modèles expérimentaux ont été élaborés chez l'animal de laboratoire, en particulier des modèles de lésion de nerfs périphériques chez le rat, permettant des investigations tant moléculaires que fonctionnelles des mécanismes impliqués dans le développement des ces douleurs. En revanche, peu de modèles existent chez la souris, alors que cet animal, grâce à la transgénèse, est très fréquemment utilisé pour l'approche fonctionnelle ciblée sur un gène.

Dans l'étude présentée ici, nous avons évalué chez la souris C57BL/6 l'adaptation d'un modèle neuropathique, proposé une nouvelle modalité de mesure de la sensibilité douloureuse adaptée à la souris et défini une méthode d'analyse performante des résultats. Ce modèle, dit de lésion avec épargne nerveuse (spared nerve injury, SNI), consiste en la lésion de deux des trois branches du nerf sciatique, soit les nerfs peronier commun et tibial. La troisième branche, le nerf sural est laissé intact et c'est dans le territoire cutané de ce dernier que la sensibilité douloureuse à des stimulations mécaniques est enregistrée. Des filaments calibrés de force croissante sont appliqués sur la surface de la patte impliquée et la fréquence relative de retrait de la patte a été modélisée mathématiquement et analysée par un modèle statistique intégrant tous les paramètres de l'expérience (mixed-effects model). Des variantes chirurgicales lésant séquentiellement les trois branches du nerf sciatique ainsi que la réponse en fonction du sexe de l'animal ont également été évaluées.

La lésion SNI entraîne une hypersensibilité mécanique marquée comparativement aux souris avec chirurgie contrôle ; cet effet est constant entre les animaux et persiste durant les quatre semaines de l'étude. De subtiles différences entre les variables, y compris une divergence de sensibilité mécanique entre les sexes, ont été démontrées. La nécessité de léser le nerf tibial pour le développement des symptômes a également été documentée par notre méthode d'évaluation et d'analyse.

En conclusion, nous avons validé le modèle SNI chez la souris par l'apparition d'un symptôme reproductible et apparenté à l'allodynie mécanique décrite par les patients souffrant de douleurs neuropathiques. Nous avons développé des méthodes d'enregistrement et d'analyse de la sensibilité douloureuse sensibles qui permettent la mise en évidence de facteurs intrinsèques et extrinsèques de variation de la réponse. Le modèle SNI utilisé chez des souris génétiquement modifiées, de par sa précision et reproductibilité, pourra permettre la discrimination de facteurs génétiques et épigénétiques contribuant au développement et à la persistance de douleurs neuropathiques.

Assessment and analysis of mechanical allodynia-like behavior induced by spared nerve injury (SNI) in the mouse.

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Introduction

Neuropathic pain was described by the physician S.W. Mitchell in 1872 as "the most terrible of all tortures which a nerve wound may inflict" (Mitchell 1872). Despite progress in our understanding of this syndrome, the mechanistic details underlying neuropathic pain remain elusive and its treatment is still sub-optimal (Woolf and Decosterd 1999;Dworkin et al. 2003).

In order to tease out neuropathic pain-related mechanisms, rat experimental models have been developed. Partial or complete peripheral nerve transections (Bennett and Xie 1988;Seltzer et al. 1990;Kim and Chung 1992;Decosterd and Woolf 2000), spinal cord injuries (Hao et al. 1992) as well as toxic and inflammatory models of neuritis (Courteix et al. 1993;Eliav et al. 1999;Polomano et al. 2001) all lead to pain hypersensitivity and mimic to some extent pain symptoms such as allodynia and hyperalgesia that are encountered in patients suffering from neuropathic pain (Jensen et al. 2001).

Recent studies in rat models of neuropathic pain highlight the contribution of non-injured neurons to neuropathic pain. Electrophysiological, molecular, and cellular changes identified in non-injured neurons include ectopic activity in C-fibers, activation of p38 intracellular signaling pathways, differential expression of sensory-specific voltage-gated sodium channels, augmentation of the transient receptor potential channel TRPV1, increased expression of BDNF, and activation of Remak Schwann cells (Wu et al. 2001;Fukuoka et al. 2001;Hudson et al. 2001;Decosterd et al. 2002;Gold et al. 2003;Obata et al. 2004;Murinson et al. 2005). Any or all of these may contribute to the development and maintenance of neuropathic pain.

In particular, differential investigation of injured and non-injured neurons is facilitated by the use of the spinal nerve ligation model (Kim and Chung 1992) and the spared nerve injury (SNI) model (Decosterd and Woolf 2000). SNI consists of sparing the sural nerve, when two other terminal branches of the sciatic nerve are injured (common peroneal and tibial nerves). Intense, reproducible and long-lasting mechanical-allodynia like behavior is measurable in the non-injured sural nerve skin territory (Decosterd and Woolf 2000;El Khoury et al. 2002;Broom et al. 2004;Decosterd et al. 2004;Zhao et al. 2004;Howard et al. 2005). The SNI model offers the advantage of a distinct anatomical distribution with an absence of co-mingling of injured and non-injured nerve fibers distal to the lesion such as the injured and non-injured nerves and territories can be readily identified and manipulated for further analysis (behavioral assessment, retrograde tracing, specific nerve treatment or recording) (Decosterd et al. 2002).

Although progress in generating genetically manipulated rats has been sustained by the recent publication of the rat genome sequence, the main accessible species with conventional or conditional gene targeting remains the mouse, at least for now (Abbott 2004). Genetically modified animals offer the possibility of correlating biochemical factors with functional changes (Woolf et al. 1998), but few models of peripheral neuropathic pain have been validated in mice (Malmberg and Basbaum 1998;Hao et al. 2000;Mansikka et al. 2000;Shields et al. 2003;Mansikka et al. 2004). Our aim in the present study was to investigate and validate whether SNI in mice induces signs of pain hypersensitivity. In C57BL/6 mice we tested the effect of SNI and two different surgical variants of SNI (tibial nerve injury alone or common

peroneal and sural nerve injury) on mechanical withdrawal response in the spared nerve territories. In addition, we investigated whether we can ameliorate the sensitivity of mechanical behavioral assessment by recording and analyzing relative frequency of paw withdrawal as a function of force applied. We demonstrated that injuries to the peripheral branches of the sciatic nerve induced robust mechanical-allodynia like behavior in the spared sural nerve skin territory when tibial and common peroneal nerves (original SNI model) or tibial nerve alone (SNI variant model) are injured. In addition, we document subtle changes responsible for variability by refining sensory assessment and improved methods of data analysis.

Methods

Animals and surgery

All experiments were approved by the Committee on Animal Experimentation of the Canton de Vaud, Lausanne, Switzerland, in accordance with Swiss Federal law on animal care and the guidelines of the International Association for the Study of Pain (IASP) (Zimmermann 1983). Eight week-old C57BL/6 male and female mice (Charles River, l'Abresle, France) were housed in the same room, one to a cage, at constant temperature (21±2°C) and a 12/12 dark/light cycle. A total of 62 mice were included. No other animals were housed in that room. Mice had ad libitum access to water and food.

An adaptation of SNI surgery was performed under 1.5-2.5% isoflurane (Abott AG, Baar, ZG, Switzerland) general anesthesia (Decosterd and Woolf 2000;Suter et al. 2003). The left hindlimb was immobilized in a lateral position and slightly elevated. Incision was made at mid-thigh level using the femur as a landmark (Figure 1A) and a section was made through the *biceps femoris* in the direction of point of origin of the vascular structure (Figure 1B). At that stage, the surgery continued with the help of a stereomicroscope (Wild AG, Heerbrugg, Switzerland). The three peripheral branches (sural, common peroneal and tibial nerves) of the sciatic nerve were exposed without stretching nerve structures (Figure 1C). In a slight modification of the original SNI procedure in rats, both tibial and common peroneal were ligated and transected together (ligated and transected one by one during rat procedure). A micro-surgical forceps with curved tips was delicately placed below the tibial and common peroneal nerves to slide the thread (6.0 silk instead of 5.0 in rats, Ethicon, Johnson & Johnson Intl, Brussels, Belgium) around the nerves (Figure 1D). A tight

ligation of both nerves (Figure 1E) was performed and a 1-2 mm section (2-4 mm in rats) of the two nerves was removed. The sural nerve was carefully preserved (Figure 1F) by avoiding any nerve stretch or nerve contact with surgical tools. Muscle and skin were closed in two distinct layers with silk 6.0 suture and surgical micro clips respectively. Two variants of SNI injury of the sciatic nerve branches were performed using the same surgical techniques but different combinations of nerve transections: $SNIv_{(t)}$, in which the common peroneal and sural nerves are sectioned, leaving the tibial nerve (t) intact (Figure 1G and H); and $SNIv_{(s,cp)}$, in which only the tibial nerve is injured, leaving the sural (s) and common peroneal (cp) nerves intact (Figure 1I and J). In both cases, the access used is several mm lower than for the original SNI procedure, allowing an easier separation of nerve branches. In both SNI variants, injury was produced by ligating the nerves tightly and removing a 2 mm nerve portion, just as in the original SNI procedure. The sham procedure consisted of the same surgery without ligation and transection of the nerves; instead, a 3 mm long thread of 6.0 silk was placed longitudinally at the level of the trifurcation. Thirteen animals were dedicated in a preliminary phase for optimization of the surgical procedures. After training, the total time required for the whole procedure was 15 minutes or less.

Behavior

Testing procedures started with one week of acclimatization of the animals to the testing room environment, with handling reduced to a minimum (Wilson and Mogil 2001). For another week, mice were habituated to the testing material. The experimenter placed each mouse on an elevated platform with a 20 mm soft wire

mesh floor, in a transparent plexiglas box (10x10x13 cm) for a fifteen-minute session every two days. During the last session, mice were familiarized with the application of von Frey monofilaments under the paw.

Mice were randomly assigned to sham and SNI groups and procedure coded. Recordings of mechanical sensitivity were then performed before and after surgery. Two sets of baseline measurements were taken, baseline A at 4 days before surgery and baseline B at 2 days before surgery. Although the investigator was blinded to the treatment applied, both gender and paw position on the floor after SNI were potentially recognizable during testing.

The plantar side of the paw ipsilateral or contralateral to the surgery was stimulated with calibrated von Frey monofilaments (Stoelting Co, Wood Dale, IL) (movie 1, Table I). Depending on the surgery done, only sural or both sural and tibial nerve territories were observed for the investigation of mechanical sensitivity (Figure 1K). Monofilaments were perpendicularly applied to the glabrous skin with sufficient force to cause filament bending. Ten stimuli were made with each of a series of Von Frey hairs comprised of the first eleven monofilaments supplied by the manufacturer (0.008g, 0.02g, 0.04g, 0.070g, 0.16g, 0.40g, 0.60g, 1.0g, 1.4g, 2.0g, 4.0g).

Withdrawal threshold: Testing started with filament 0.008g and positive response was determined by paw withdrawal occurring twice in the ten applications. In the case of negative responses, the next stiffer monofilament was applied. The monofilament that first evoked a positive response was designated the threshold (in grams) and no further monofilaments were applied (Tal and Bennett 1994;Decosterd and Woolf 2000).

Relative frequency of paw withdrawal: We also assessed mechanical sensitivity using another method similar described in rats and mice (Song et al. 1999;Mansikka et al. 2000). We measured the number of positive withdrawal responses for each monofilament of the series. The eleven monofilaments were tested in ascending order to determine the relative frequency of paw withdrawal. The withdrawal threshold as described above was also recorded during the procedure for comparison. Behavioral setting and procedures are described and illustrated in movie 1 and Table I.

Data analysis

We modeled mechanical threshold response and response frequency (expressed in mean ± SEM) for the overall effect of the treatment using a two-way analysis of variance (ANOVA), with time treated as repeated measure, followed by a *post hoc* Dunnett's test or *t*-test associated with Bonferroni correction when appropriate. Logarithmic transformed values were used for the statistical analysis, enabling ANOVA tests. A p value < 0.05 was considered significant. Analyses were performed using the JMP software version 5.01 (SAS institute Inc., Cary, NC).

Longitudinal data such as these are widely encountered in other domains of the biological sciences, and specialized models and associated statistical procedures have been developed to handle them (Crowder and Hand 1990;Pinheiro and Bates 2002;Diggle et al. 2002). The main potential advantage of such procedures over the ANOVA methods outlined above is an integrated treatment of the data, which takes into account the different sources of variation – between time, individuals, surgery, paws and sexes – resulting in more appropriate assessment of uncertainty, and thus in tests and confidence intervals that are better adapted to the experimental set-up. In the present case, graphs of the raw data on the relative frequency of paw

withdrawal showed that the individual curves for each combination of animal, day, and paw or nerve territory are similar to each other, and they might be well-described by a common type of curve, though with parameters varying between individual series. This suggested modeling the proportion of paw withdrawals out of ten applications of the filament as a function of log-force using a mixed-effects model (Venables and Ripley 2002;Pinheiro and Bates 2002), the details of which are given in the Appendix. The analysis can allow for two types of variation: systematic variation due to factors such as experimental treatment, sex, and time; and random variation among experimental subjects and among the occasions on which the subjects are tested. These two types of variation are respectively called fixed and random effects. The key parameters of the model we chose to fit are an intercept parameter α representing the overall sensitivity of an animal to stimulus, and a slope parameter β representing how sharply its reactions change when the stimulus is changed. Two animals with the same β but different values of α would show the same range of responses but for different levels of stimulus; in other terms, the animal with higher α intercept parameter responds to a lower intensity stimulus. Two animals with the same α but different values of β would have the same response to an average stimulus, but the reactions of the animal with higher β slope parameter change more rapidly as the stimulus varies over a given range. The fitting of this model was performed using the software packages S-PLUS 6.1 (Insightful Corporation, Seattle, WA) and R 1.9.1 (R Development Core Team, Vienna, Austria, 2004).

Results

Weight gain was identical in treated and sham group and no signs of severe discomfort (Hawkins 2002) were observed. Although close observation revealed abnormal hindlimb/paw movements, the animals were able to perform their usual activities in the cage, including walking, standing up on hindlimbs and climbing (movie 2, Table II). While most animals did not exhibit autotomy, we observed nail gnawing in a few operated mice, without subsequent bleeding or soft tissue injury, corresponding to degree 1 on a scale of autotomy behaviors (Wall et al. 1979).

Mechanical allodynia-like behavior after SNI

Following SNI, mice developed mechanical allodynia-like behavior as represented by the decrease in withdrawal threshold 3 days after the injury ($n = 9$, male) (Figure 2). The effect was stable from the first to the fourth week of the study period and the difference between SNI and sham-operated group ($n = 6$, male) was statistically significant ($p < 0.001$). There was no significant effect of either SNI or sham surgery on the withdrawal threshold for the contralateral paw ($p > 0.05$).

Comparison of mechanical stimulus-evoked response following SNI in male and female mice

In both male and female mice, SNI induced a dramatic drop in withdrawal threshold following mechanical stimuli to the ipsilateral paw (Figure 3; $p < 0.001$, $n = 6$ in each sex) and no change in mechanical threshold was observed for the contralateral paw ($p > 0.05$). The analysis of variance did not reveal any significant difference in response between genders ($p > 0.5$). However, a large proportion of the animals reached the maximal possible effect using the lightest monofilament possible (0.008), creating a false ceiling effect following the SNI procedure. We therefore investigated

whether determining the number of withdrawal responses for each filament yields a more useful analysis of these data.

The relative frequency of paw withdrawal is shown in Figure 4. The frequency of withdrawal in response to low force filaments increased after SNI in both males and females and there was a statistically significant effect of paw (ipsilateral versus contralateral side) on relative response frequency due to the SNI procedure ($p < 0.001$). Mechanical allodynia-like behavior in the ipsilateral paw was first detected on the fourth post-operative day and persisted for the duration of the experiment. We observed a trend towards dissociation of response curves between genders at some time points, but this analysis did not detect any significant difference between males and females ($p > 0.05$). We then analyzed the data shown in Figure 4 using a more comprehensive mixed-effects analysis that integrates multiple factors including nerve injury, paw, gender, and time. Force-response curves for each individual at each time point are integrated to provide greater sensitivity (see methods; mathematical details are given in the Appendix). The two key parameters of this model are the α intercept, representing the overall sensitivity of an animal to the stimulus, and the slope parameter β, representing how sharply the animal's responses change when the stimulus intensity changes. With this analysis, the values of the two baseline recording sessions were not significantly different from each other ($p = 0.8$, Table III), suggesting that the testing method is reproducible. Analysis with the mixed-effects model confirmed a strong effect of SNI on response to mechanical stimuli to the ipsilateral paw (Figure 5, Table III) as seen by a marked shift of the response curve (change in the intercept α) beginning on the fourth day after the SNI procedure ($p<0.0001$). The response profiles of the operated paw were shifted toward smaller monofilament log-force values (f), corresponding to mechanical allodynia-like

behavior after SNI. Contralateral paws show no significant change ($p > 0.05$). Similar changes in intercept were seen for both female and male groups, but the baseline intercept values differ slightly, but significantly, between genders, with females slightly less sensitive than males ($p = 0.01$). This difference persisted over time. The effect of the surgery was the same for males and females, with no significant interaction between sex and the other variables that is day and paw ($p > 0.05$). Although significant, the baseline gender effect was tiny compared to the SNI-induced time-dependent difference: the day-paw interaction is nearly ten times larger than the gender effect on baseline responses. To investigate this further we conducted a randomization test on the data but found no significant difference ($p > 0.05$); the groups in this study are not large enough to reliably detect such a small difference.

There was no difference in the slope parameter, β, between the first and second baseline assessments. However, four days after SNI, β was significantly lower and remained lower for the duration of the experiment ($p < 0.0001$, Figure 4B, Table III). The same pattern of change was observed for both genders and for both paws: changes in β do not depend on paw or on sex. There is, however, a significant time-dependence that affects both paws equally.

Comparison of mechanical stimulus-evoked response following peripheral nerve injury in the SNI model and SNI variants

The relative frequency of paw withdrawal was observed for three weeks in SNI, $SNIv_{(t)}$, $SNIv_{(s,cp)}$ and sham groups (n = 6, 6, 6 and 4 male mice respectively). Responses in both sural and tibial nerve territories in the paw ipsilateral to the surgery were recorded and analyzed in order to identify changes in mechanical

behavior after SNI. Contralateral paws show no significant change ($p > 0.05$). Similar changes in intercept were seen for both female and male groups, but the baseline intercept values differ slightly, but significantly, between genders, with females slightly less sensitive than males ($p = 0.01$). This difference persisted over time. The effect of the surgery was the same for males and females, with no significant interaction between sex and the other variables that is day and paw ($p > 0.05$). Although significant, the baseline gender effect was tiny compared to the SNI-induced time-dependent difference: the day-paw interaction is nearly ten times larger than the gender effect on baseline responses. To investigate this further we conducted a randomization test on the data but found no significant difference ($p > 0.05$); the groups in this study are not large enough to reliably detect such a small difference.

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sensitivity in the respective spared nerve territories. The intercept parameters were extracted from every individual force-response curve (see Appendix) and Figure 6 shows how tibial nerve intercept depend on the sural nerve intercept parameters obtained from the four different groups. In all groups, baseline values for the tibial and sural intercepts for the same animal were very similar, implying identical mechanical sensitivity in both territories. After surgery, the SNI and $SNIv_{(s,cp)}$ groups and the sham and $SNIv_{(t)}$ groups behave strikingly differently: for the first two groups, SNI and $SNIv_{(s,cp)}$, the sural intercepts have larger values than the tibial intercepts for the same animal, implying mechanical hypersensitivity in the sural nerve territory. For the other two groups, the contrary appeared: after sham and $SNIv_{(t)}$ procedure, the tibial intercepts tend to be larger than the sural intercepts (although the shift generally appeared to be smaller), implying increased sensitivity in the tibial nerve territory. Within the SNI, a paired t-test confirmed the significance of this difference between the two territories on all post-surgery days ($p<0.01$), similarly confirmed it in the $SNIv_{(s,cp)}$ group two and three weeks after surgery ($p<0.01$), marginally confirmed it in the case of the $SNIv_{(t)}$ group only three weeks after surgery, but showed no significant difference of the means in any other case ($p>0.05$). The mixed-effects model (methods and mathematical details are given in Appendix) strongly confirmed the presence of mechanical hypersensitivity in the sural nerve territory after the SNI and $SNIv_{(s,cp)}$ procedures on every post-surgery day tested (Table IV, which shows the significant terms of the model). The variation of the

mean intercepts over time is presented in Figure 7. Mechanical hypersensitivity following SNI and $SNIv_{(s,cp)}$ procedures was clearly demonstrated by the sharp difference between the sham and the SNI and between the sham and the $SNIv_{(s,cp)}$ groups on the sural nerve territory 7, 14 and 21 days after surgery ($p<0.001$ for day 7, $p<0.0001$ for days 14 and 21, Table IV). Equally clear is the large jump from baseline values to post-injury values in these two groups ($p<0.001$ and $p<0.0001$). A peculiarity of the data was the high value of the intercepts in the sham group 7 days after surgery - the values for $SNIv_{(t)}$ group are significantly lower at that time point for the sural nerve territory. In the spared tibial nerve territory of the $SNIv_{(t)}$ group, mechanical sensitivity was not significantly different from the sham group at all time points.

None of the statistical methods applied showed significant changes in the slope parameter β, although there was a slightly larger dispersion of the slope values after surgery in all four groups.

Discussion

All SNI operated mice developed rapid, strong, and persistent hypersensitivity to mechanical stimuli, beginning three days after surgery and lasting throughout the four weeks of the study. Mechanical allodynia-like behavior was present in the SNI ipsilateral paw only, in the glabrous lateral plantar area of the paw that corresponds to the skin territory of the non-injured sural nerve. The increase in mechanical responsiveness in the skin territory of the non-injured nerve may be representative of extra-territorial pain encountered in the clinical picture of human neuropathic pain (Ochoa and Yarnitsky 1993;Baron 2000;Jensen et al. 2001).

Severing only the tibial nerve (the $SNIv_{(s,cp)}$ group in our study) leads to hypersensitivity in the sural nerve territory similar to that seen following SNI. In contrast, when the peroneal and sural nerves were severed (the $SNIv_{(t)}$ group in our study), the mechanical sensitivity recorded in the spared tibial nerve is not significantly different from the sham-operated group. Similar results have been described for these two SNI variant procedures in the rat (Lee et al. 2000;Robinson et al. 2003) and we suggest that pain hypersensitivity may be correlated to properties specific to the injured nerves or the spared nerve. Function, composition of efferent/afferent fibers (motor efferent axons, myelinated and non-myelinated affrerent axons, sympathetic axons) and number of fibers involved are the main variables between the three branches of the sciatic nerve (Schmalbruch 1986). The tibial nerve contains the largest proportion of motor and sensory fibers. It may be that the development of mechanical hypersensitivity depends upon the injury of a critical number of one of these subpopulations and that injury of the common peroneal nerve does not affect sufficient numbers. Reactions of cutaneous and

muscle afferent neurons to peripheral injury are selective and may also contribute differentially to the development of mechanical allodynia (Hu and McLachlan 2003). Although our findings in mice are consistent with previous reports in rat models, they are in contrast to a previous study which reported that SNI procedure did not induce mechanical allodynia-like behavior in the sural nerve skin territory in mice, although it was observed in the spared tibial nerve territory when the sural and common peroneal nerves were injured (Shields et al., 2003). We have no clear explanation for why these results differed from ours, however, divergences in surgical procedure, behavioral assessment, analysis of data, and strain of mice (European strain of C57BL/6 versus American strain) may account for this discrepancy (Chesler et al. 2002a;Chesler et al. 2002b).

In addition to detection of mechanical withdrawal threshold in our experimental groups, we applied a variant of an assessment method and measured the relative frequency of paw withdrawal for each force applied (Song et al. 1999;Mansikka et al. 2000;Mansikka et al. 2004). This integrates a force-response curve for each individual at each time point that gives much more information on the response to mechanical stimuli than does single measurement of the mechanical withdrawal threshold, avoiding the false ceiling effect encountered in our study (in a majority of SNI-injured mice the threshold lies at or below the smallest filament stimulus). Although this method increases duration of testing and number of stimulus applied, it is in a reasonable fashion that animals (and investigators) did not manifest signs of augmented stress. We perform a mixed-effects analysis of the force-response curve and we reveal two significant changes induced by SNI that were not detected by the more classical, but restricted, analysis of variance. Specialized mathematical models, including mixed-effects models, have been developed for parallel analysis of multiple

variables and/or covariates present in biological experiments and can be regarded as a modern reformulation and extension of analysis of variance models (Pinheiro and Bates 2002;Diggle et al. 2002). The principal advantage of using a mixed-effects model is in treating the error structure of the data appropriately, enabling an integrated analysis which can account for different sources of variation. There are several aspects to this: (1) the response of a mouse to the stimulus is summarized in terms of simple quantities (intercept and slope) which are computed using data from the entire stimulus-response curve. The resulting increase in precision means that it is possible to detect effects which are undetectable using a conventionally-estimated withdrawal threshold, which incorporates only a part of the data. (2) Such a model allows a clear distinction between variations due to variables of central interest (time, ipsi- and contralateral paws, gender, and treatment) and those which simply cloud the main issues (e.g. individual differences between mice of the same sex). Variations in the first category are treated as fixed effects, to which parameters are ascribed, and on which tests may be performed, and those of the second type are treated as random, because the mice can be regarded as taken from a population. (3) Such a model allows the separation of different levels of variation in the experiment: there is variation between mice, but also variation within the responses taken from a single mouse. This type of analysis is now widely used in many areas of the biological and behavioral sciences (Davidian and Giltian 1995;Pinheiro and Bates 2002).

We identify a gender-based difference in baseline withdrawal frequency. In our study, male and female animals both developed comparable signs of mechanical hypersensitivity, but started from different baselines. Gender-based differences in pain sensitivity and drug-induced analgesia have been reported both clinically and

experimentally, but clear documentation of these differences requires a better understanding of the factors that contribute to inter- and intra-experimental variability (Mogil et al. 2000;Craft 2003). We show here that gender is an additional source of variability in the response to innocuous mechanical stimuli and supports the usual recommendation that experiments may yield a clearer picture when conducted on animals of the same sex. In addition, we revealed a global decrease in the slope of the stimulus intensity-response curve in the male-female study. This change was consistent over time after SNI and was independent of the paw tested and the animal's gender. A decrease in the slope in this case implies that there is less of a difference between the minimal and maximal responses to the same range of stimuli in SNI-operated animals than in control conditions. Such a change in the slope was not present in the study comparing sham and SNI-variant surgeries; thus we suggest that unidentified environmental factors or investigator bias rather than the surgical procedure itself were responsible for these changes.

The mechanisms underlying the generation and maintenance of neuropathic pain are likely to be complex and to act with several different temporal profiles. Unknown or uncontrollable external factors that affect experimental models of neuropathic pain add another layer of complexity. It is therefore important to optimize behavioral assessment and experimental analysis to factor in as many potential variables as possible (Wilson et al. 2002;Chesler et al. 2002b;Francis et al. 2003).

In summary, we have shown that SNI in the mouse produces profound allodynia-like behavior, similar to that seen in the rat. In addition, we have demonstrated the reproducibility and sensitivity of a mixed-effects analysis of behavioral data from the

mouse SNI model. We identified a gender-based difference in baseline response to mechanical stimuli and a possible change in the dynamics of response to mechanical stimuli following SNI. This refined method of behavioral analysis should permit the identification of experimental variability factors as well as allow more detailed exploration of the mechanisms underlying neuropathic pain. Use of the SNI model with enhanced behavioral analysis in genetically altered mice should prove to be a powerful tool for evaluating the contribution of genetic and epigenetic factors to the development and maintenance of neuropathic pain.

Acknowledgements

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Appendix

In the mixed-effects analysis, we considered the score data and modeled the proportion r_i of paw withdrawals out of ten applications of the filament with log-force f_i (relative frequency of paw withdrawal). We assumed that the proportion of positive responses shows dependence on f_i of logistic form

$$r^m_{i,d,s,p} = 1 - \frac{1}{1+\exp(\mathcal{A}^m_{d,s,p} + \mathcal{B}^m_{d,s,p} f_i)} + \varepsilon^m_{i,d,s,p},$$

where *m* denotes the mouse, *i=1,..,11* indexes the force applied by the filament, d, s, p are the indices for day (*d*), sex (*s*)and paw (*p*) (*d* = Baselines A and B at days -*4* and -*2* then days *4, 7,14, 21,* and *28* after SNI for the seven experimental days, *p* = *0* for contralateral and *1* for ipsilateral paw and *s* = *0* for female and *1* for male mice), and $\varepsilon^m_{d,s,p,i}$ are the within-group errors, assumed to follow a normal distribution. The logistic curve describes the common form of the response profiles for each animal, day and paw well, and by choosing an appropriate model for its parameters $\mathcal{A}^m_{d,s,p}$ and $\mathcal{B}^m_{d,s,p}$, we can obtain a good fit for each individual series of monofilament applications. $\mathcal{A}^m_{d,s,p}$ characterizes the location of the curve along the *f* axis and $\mathcal{B}^m_{d,s,p}$ represents its steepness. Both of these parameters were assumed to decompose into two terms: a deterministic part that depends linearly on the explanatory variables sex *s*, day *d*, paw *p* and their interaction terms (fixed effects $A_{d,s,p}$ and $B_{d,s,p}$) and a random component for each individual series of applications of eleven filaments (random effects $a^m_{d,p}$ and $b^m_{d,p}$), giving decompositions $\mathcal{A}^m_{d,s,p} = A_{d,s,p} + a^m_{d,p}$ and $\mathcal{B}^m_{d,s,p} = B_{d,s,p} + b^m_{d,p}$. The validity of the assumption of normal errors in the study of gender differences was checked using normal quantile-quantile (Q-Q) plots.

For all explanatory variables 0-1 coding was used with the baseline day, sex female and right (contralateral) paw as reference categories. Variable selection for the fixed effects was performed by backward elimination starting from a model containing all main effects and all possible interaction terms, with the decision to keep or drop a term based on likelihood ratio tests, Akaike's Information Criterion (AIC) and residual and random effects plots. Model-fitting was performed by maximum likelihood estimation. The fixed effects $A_{d,s,p}$ and $B_{d,s,p}$ were found to depend on the explanatory variables in the following way:

- For females, contralateral paw: $A_{d,0,0} = \alpha_0$ and $B_{d,0,0} = \beta_0$ for *d* = -4, -2, 4, 7, 14, 21, 28
- For males, contralateral paw: $A_{d,1,0} = \alpha_0 + \alpha_s$ and $B_{d,1,0} = \beta_0$ for *d* = -4, -2, 4, 7, 14, 21, 28
- For females, ipsilateral paw: $A_{BL\_A,0,1} = \alpha_0$, $B_{BL\_A,1,1} = \beta_0$ for *d* = Baseline A (day -4) and $A_{d,0,1} = \alpha_0 + \alpha_d$ and $B_{d,0,1} = \beta_0 + \beta_d$ for *d* = -2, 4, 7, 14, 21, 28 with values of α_d and β_d given in Table III;
- For males, ipsilateral paw: $A_{BL\_A,1,1} = \alpha_0 + \alpha_s$, $B_{BL\_A,1,1} = \beta_0 + \beta_s$ for *d* = Baseline A (day -4) and $A_{d,1,0} = \alpha_0 + \alpha_s + \alpha_d$, $B_{d,0,0} = \beta_0 + \beta_d$ for *d* = -2, 4, 7, 14, 21, 28 with values of α_d given in Table III.

A question in a statistical model is the validity of the assumptions concerning the within-group errors $\varepsilon^{m}_{d,s,p,i}$. We also attempted to fit a generalized linear mixed model with binomial errors, but this failed to converge. As departures from normality of the

errors were found to be small, the conclusions above are based on the fit of the normal error model, used as an approximation to the binomial.

The validity of the assumption of normal errors in the study of the different surgical procedures was not justified. Therefore, to compare the effects of the different surgical procedures we used an empirical logistic transformation of the responses (Cox 1970), that is

$$q^m_{i,d,g,t} = \log \frac{r^m_{i,d,g,t} + 0.5}{R - r^m_{i,d,g,t} + 0.5},$$

where the index g is for the surgery group, t represents nerve territory, *m* denotes the mouse, *i=1,...,11* indexes the force applied by the filament, and d is the day. $R = 10$ is the number of filament applications. This transformed response is a linear function of the log-force f_i of the monofilaments, and can be fitted by a linear mixed-effect model:

$$q^m_{i,d,g,t} = \mathcal{A}^m_{d,g,t} + \mathcal{B}^m_{d,g,t} f_i + \varepsilon^m_{i,d,g,t}.$$

The coefficients $\mathcal{A}^m_{d,g,t}$ and $\mathcal{B}^m_{d,g,t}$ were supposed to decompose into a systematic ($A^m_{d,g,t}$ and $B^m_{d,g,t}$) and a random part ($a^m_{d,g,t}$ and $b^m_{d,g,t}$, respectively), similarly to the gender study. Coding, variable selection procedure, selection criteria and model-fitting method were the same as outlined above; the reference categories were the sham group, day -4, and tibial territory. The best fitted model can be summarized as follows:

- For sham and $SNIv_{(t)}$ animals in both nerve territory and on days -4, -2, 14 and 21, for SNI and $SNIv_{(s,cp)}$ animals in both territory on days -4 and -2, finally for SNI and $SNIv_{(s,cp)}$ animals in the tibial nerve territory on days 7,14

and 21, the fixed-effect part $A^{m}_{d,g,t}$ of the intercept $\mathcal{A}^{m}_{d,g,t}$ can be described by $A^{m}_{d,sham,t} = \alpha_0$ with parameter value given in Table IV.

- The same group and territory combinations on day 7 have $A^{m}_{d7,g,t} = \alpha_0 + \alpha_{d7}$ with parameter values given in Table IV.
- The only group, territory and day combinations which are significantly different from the previous ones are the SNI and the $SNIv_{(s,cp)}$ animals measured on the sural territory on days 7,14 and 21 and the $SNIv_{(t)}$ animals measured on the sural territory on day 7. The fixed-effect part of the intercept for these combinations are $A^{m}_{d7,g,Sural} = \alpha_0 + \alpha_{d7} + \alpha_{d7,g,Sural}$ with group index $g =$ SNI or $SNIv_{(s,cp)}$; $A^{m}_{d,g,Sural} = \alpha_0 + \alpha_{d,g,Sural}$ with group index $g =$ SNI or $SNIv_{(s,cp)}$ and day index $d = d14$ or $d21$; and $A^{m}_{d,g,Sural} = \alpha_0 + \alpha_{d7} + \alpha_{d7,SNIv(t),Sural}$ with the parameters given in Table IV.
- For every group, the fixed-effect part of the slope is always $B^{m}_{d,g,t} = \beta_0$.

Improved estimation procedures for generalized linear mixed-effects models are a current research topic in statistics: more refined analyses than those described above are certainly possible. Mixed-effects models (Venables and Ripley 2002;Pinheiro and Bates 2002) may be fitted using the data analysis packages R or S-PLUS.

Figure legends

Figure 1 : Spared nerve injury (SNI) surgical procedure (D-E) and variants of the SNI procedure $SNIv_{(t)}$ (G, H) and $SNIv_{(s,\ cp)}$ (I, J). A: Anesthetized mouse positioning and incision mark on left hind thigh. The paw is immobilized in an extended and slightly elevated position. B: The *biceps femoris* muscle is exposed and the artery *genus descendes* is used as a landmark for direction of muscle incision. C: Exposure of the sciatic nerve and peripheral branches: common peroneal, tibial and sural nerves. D: The 6.0 silk thread is slipped under the common peroneal and the tibial nerves. The nerve dissection is minimal and any contact with the sural nerve is avoided. E: Ligature of the common peroneal and the tibial nerves. F: The ligated nerves are transected distally and a 2 mm section is removed. Care is taken to avoid the sural nerve completely. G and H: The ligation is placed around the common peroneal and sural nerves, leaving the tibial nerve intact in the $SNIv_{(t)}$. I and J: The tibial nerve is injured in the $SNIv_{(s,\ cp)}$, while the sural and common peroneal nerves are spared by the procedure. K: Plantar view of the left hindpaw. The colored area on the photograph corresponds to the sural/tibial nerve skin territory that is stimulated with the von Frey monofilaments. Notice that glabrous/hairy border is carefully avoided.

bfm, *biceps femoris* muscle; agd, *genus descendes* artery; CPN, common peroneal nerve; TN, tibial nerve ; SN, sural nerve.

Figure 2 : Mechanical withdrawal threshold of response decreases significantly in ipsilateral paws (filled symbols) of the SNI group compared to the sham group (n = 9 and 6 male mice respectively, * $p < 0.001$). No significant change occurs in contralateral paws (open symbols) after SNI or in the sham group.

Figure 3: Mechanical withdrawal threshold decreases significantly in ipsilateral paws (filled symbols) of both male (triangles) and female (circles) mice after spared nerve injury (SNI) (n=6 for each group, *$p < 0.001$). No significant change occurs in contralateral paws (open symbols) of male and female mice and there is no significant difference between male and female groups ($p > 0.05$).

Figure 4: Relative frequency of paw withdrawal increases significantly in both males and females following SNI (n = 6 for each group). The relative frequency of paw withdrawal after ten stimulations of the sural nerve skin territory was determined for each of eleven von Frey monofilaments (0.008g, 0.02g, 0.04g, 0.070g, 0.16g, 0.40g, 0.60g, 1.0g, 1.4g, 2.0g, 4.0g) on the indicated days. Frequency of paw withdrawal is modified in the ipsilateral paw ($p < 0.001$) after SNI, but there is no significant difference between groups ($p > 0.05$).

Figure 5: Analysis of the effect of spared nerve injury (SNI) in female and male mice using mixed-effects model. A: Time-dependence of the intercept $A_{d,s,p}$ in the fitted mixed-effects model. Parameter values for ipsilateral paws of female $(A_{d,0,1})$ and male $(A_{d,1,1})$ mice are plotted against time post-surgery. (—●—) female mice; (---▲---) male mice. The same type of line without symbols shows 95% confidence

intervals. The intercept values for the contralateral paws ($A_{d,0,0}$ and $A_{d,1,0}$) remained unchanged over time for both genders; baseline values are plotted for comparison at left with their 95% confidence intervals. (○) female contralateral paw; (Δ) male contralateral paw. B: Time-dependence of the slope parameter $B_{d,s,p}$ in the fitted mixed-effects model (filled circles) together with its confidence interval (line without symbol).

d, s, p are the indices for day (-4, -2, 4, 7, 14, 21 and 28 days after SNI), sex (0, female; 1, male), paw (0, contralateral; 1, ipsilateral to SNI).

Figure 6 : Comparison of the intercepts of individual response profiles measured on the sural and the tibial nerve territory of the same animal, by groups. Points represent the two intercepts (sural and tibial) of a mouse on one day. Baselines values for tibial and sural intercepts (○) are similar and clustered along the x=y line. At 7 ( ), 14 (●) and 21 (●) days after surgery, the four groups showed different clustering tendencies. An increase in mechanical sensitivity is present in the sural territory for SNI and $SNIv_{(s,cp)}$ mice which have much larger sural than tibial intercept values and therefore group in the lower right triangle on the graphs, while sham and $SNIv_{(t)}$ mice have similar or larger tibial than sural intercepts, clustering around the x=y line and above it.

Figure 7: Mixed-effects analysis of the sham and SNI-variant procedures. The fixed-effect part of the intercept is plotted in function of time for the sural nerve territory in the different surgery groups. The sham group is represented on every plot (---●---) for

comparison. SNI (—●—) and $SNIv_{(s,cp)}$ (···●···) groups show an increase in mechanical sensitivity at 7, 14 and 21 days after surgery as shown by the increase of intercept value. Except for a lower value at 7 days post-surgery in the sural territory, curves for the $SNIv_{(t)}$ group (-·-●-·-) are identical to the sham group suggesting no hypersensitivity in this group. 95% confidence intervals are shown with the same line type without symbols.

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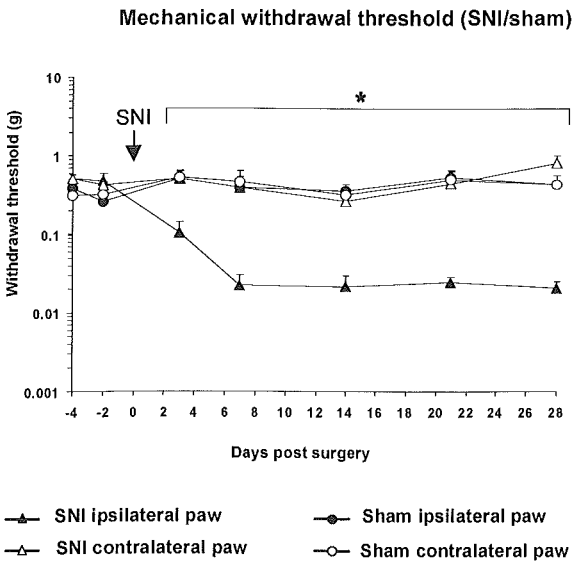

Mechanical withdrawal threshold (SNI/sham)
SNI
*
Withdrawal threshold (g)
10
1
0.1
0.01
0.001
-4 -2 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Days post surgery
SNI ipsilateral paw
SNI contralateral paw
Sham ipsilateral paw
Sham contralateral paw

Figure 2

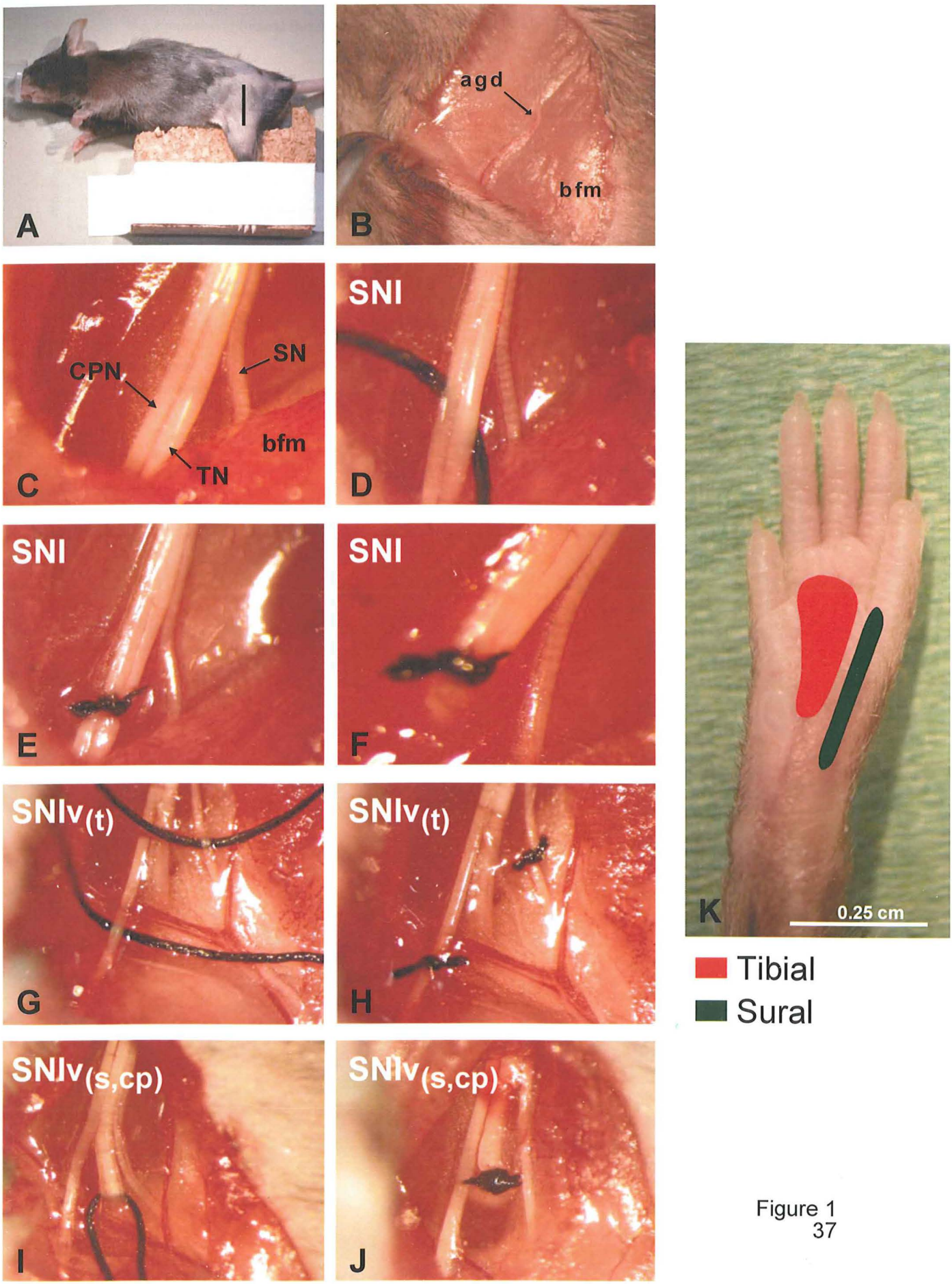

A
B
agd
bfm
C
CPN
SN
TN
bfm
D
SNI
E
SNI
F
SNI
G
SNIv(t)
H
SNIv(t)
I
SNIv(s,cp)
J
SNIv(s,cp)
K
0.25 cm
Tibial
Sural

Figure 1

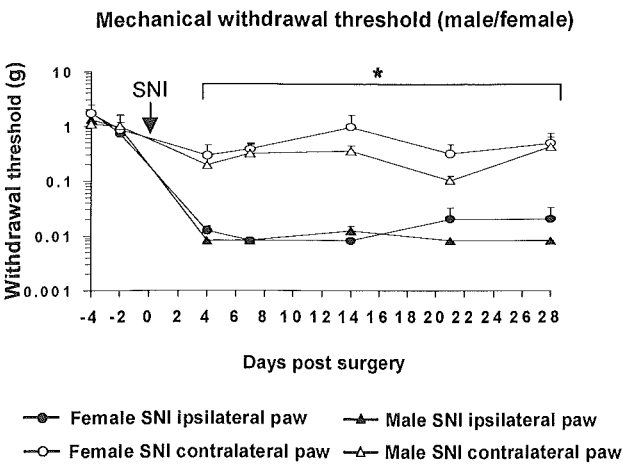

Mechanical withdrawal threshold (male/female)
Withdrawal threshold (g)
10
1
0.1
0.01
0.001
SNI
*
-4 -2 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Days post surgery
Female SNI ipsilateral paw
Male SNI ipsilateral paw
Female SNI contralateral paw
Male SNI contralateral paw

Figure 3

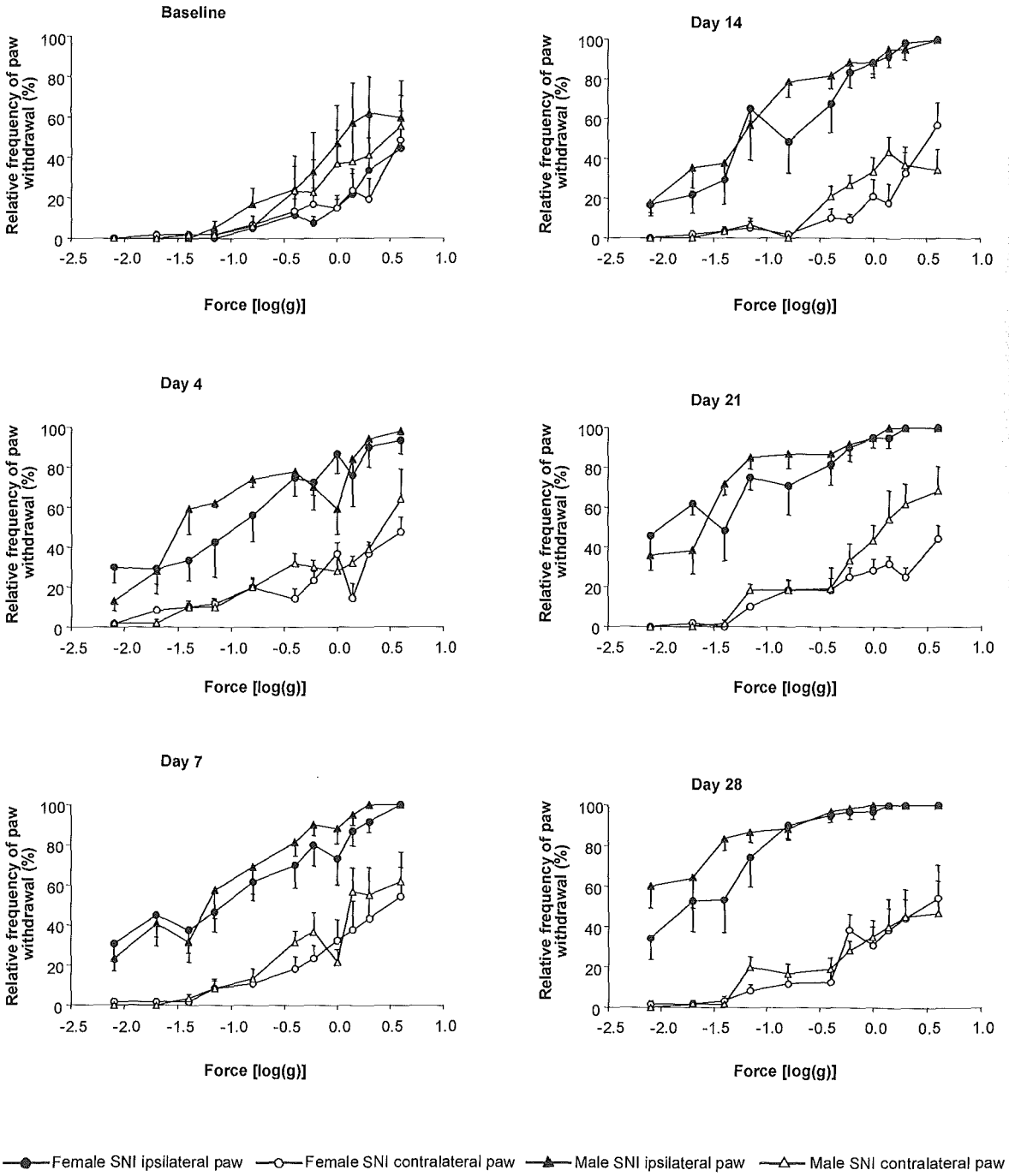

Baseline
Day 14
Day 4
Day 21
Day 7
Day 28
Relative frequency of paw withdrawal (%)
Force [log(g)]
Female SNI ipsilateral paw
Female SNI contralateral paw
Male SNI ipsilateral paw
Male SNI contralateral paw

Figure 4

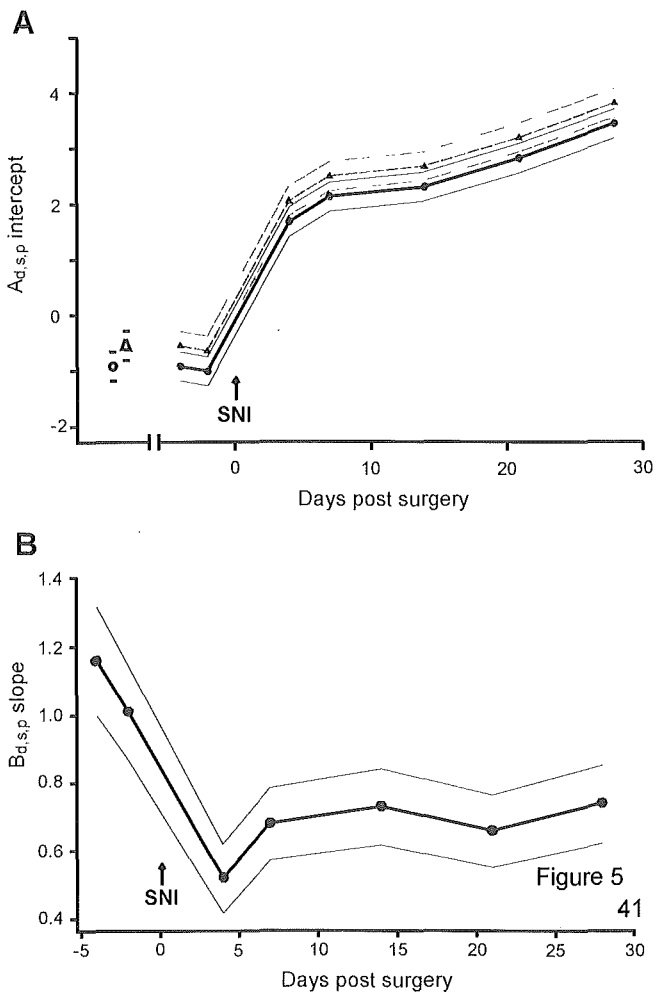

A
4
2
0
-2
$A_{d,s,p}$ intercept
SNI
0
10
20
30
Days post surgery
B
1.4
1.2
1.0
0.8
0.6
0.4
$B_{d,s,p}$ slope
SNI
-5
0
5
10
15
20
25
30
Days post surgery

Figure 5

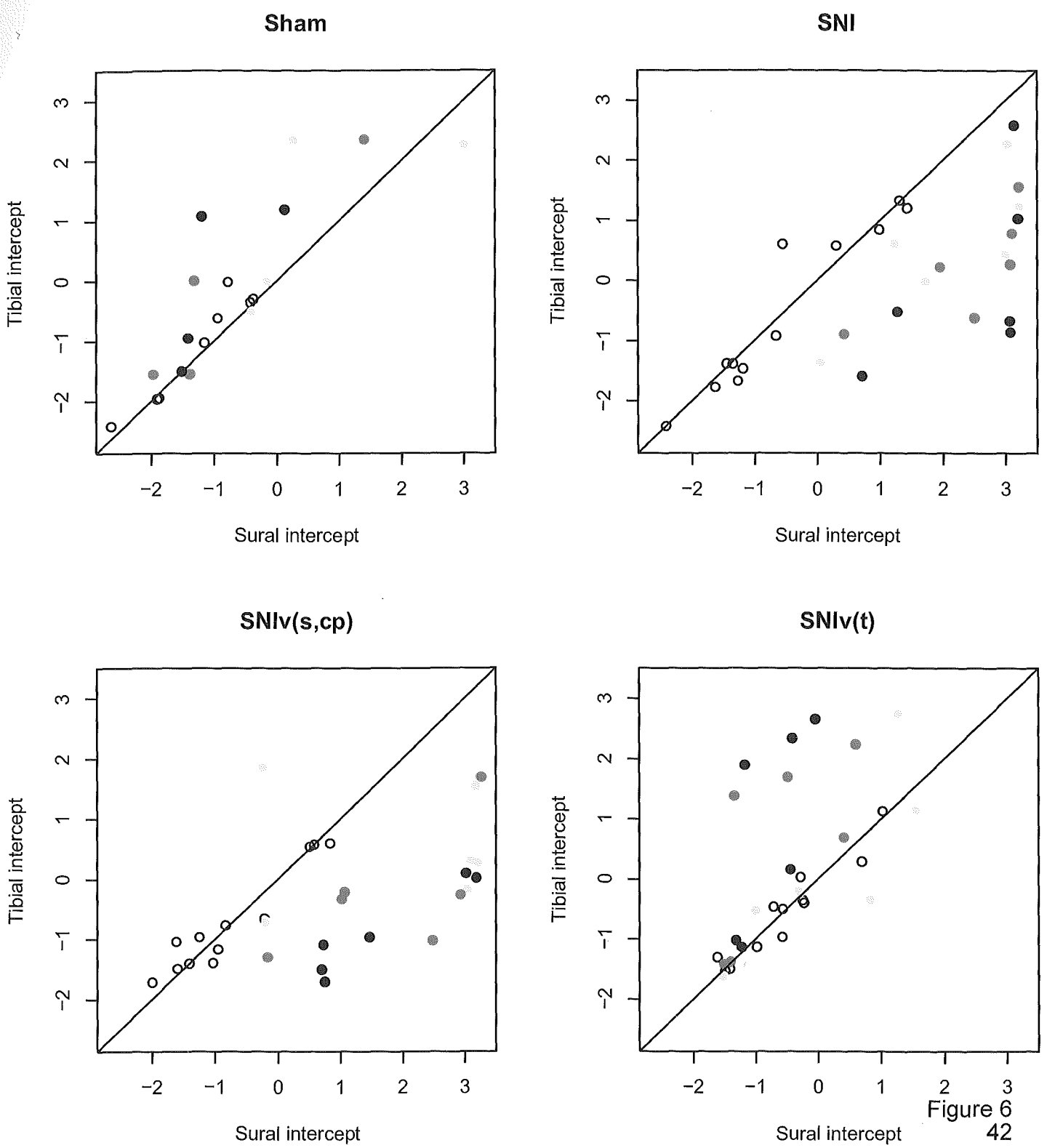

Sham
SNI
SNIv(s,cp)
SNIv(t)
Tibial intercept
Sural intercept
−2
−1
0
1
2
3

Figure 6

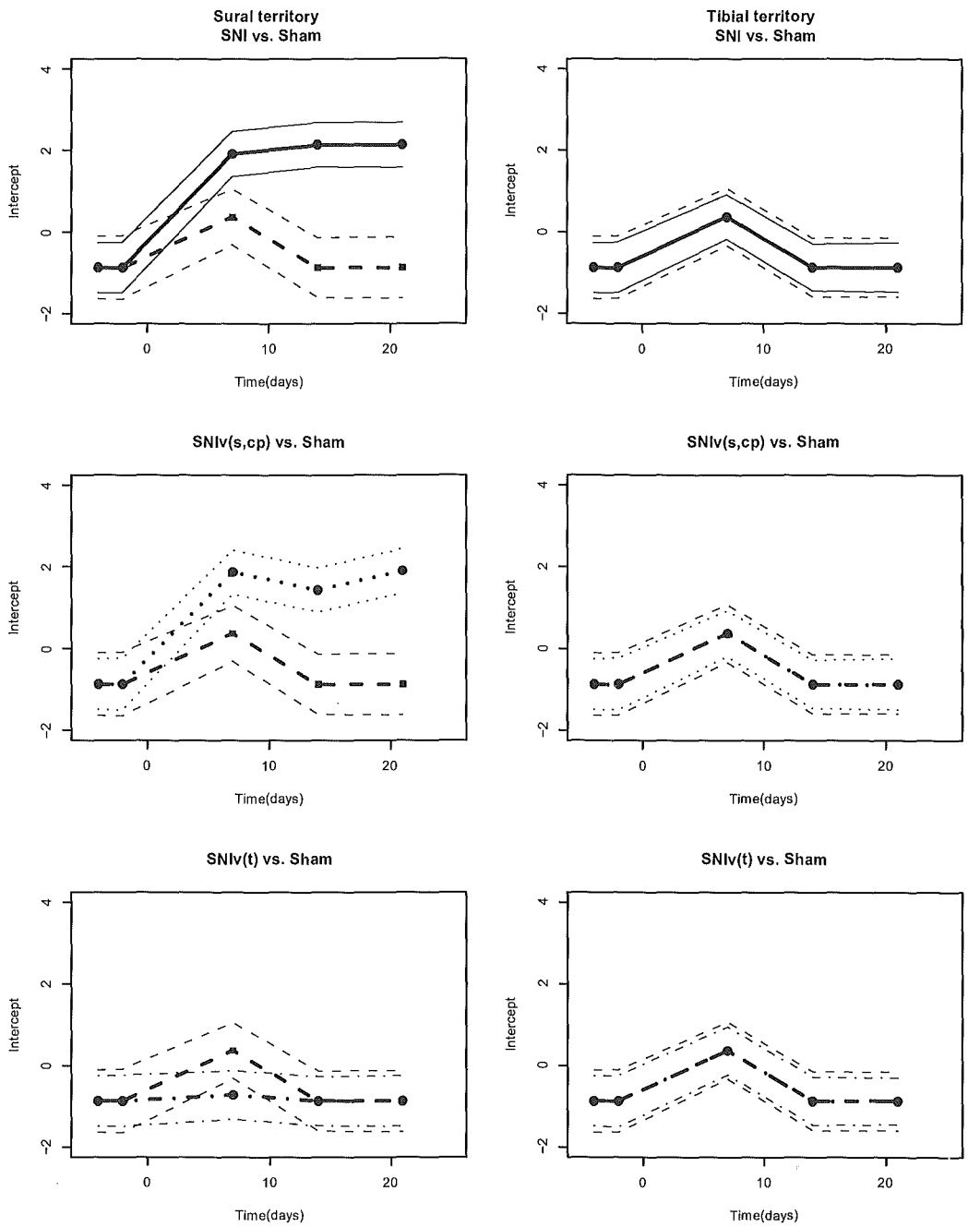

Sural territory
SNI vs. Sham
Tibial territory
SNI vs. Sham
SNIv(s,cp) vs. Sham
SNIv(s,cp) vs. Sham
SNIv(t) vs. Sham
SNIv(t) vs. Sham
Intercept
Time(days)
-2
0
2
4
0
10
20

Figure 7
43

Table I - Comments for movie1

	Description of events
Setting	The restrainer consists of three serial individual transparent Plexiglas boxes (10x10x13cm each) placed on an elevated platform with soft wire mesh floor (0.2x0.2cm). Mice are gently placed in the box before testing and can move freely in the box during testing.
Von Frey monofilaments	A series of eleven von Frey monofilaments (0.008g, 0.02g, 0.04g, 0.07g, 0.16g, 0.40g, 0.60g, 1.0g, 1.4g, 2.0g, 4.0g) will be used to evoke paw withdrawal response. Testing starts with the lowest filament of the series and the glabrous skin territory of the sural nerve (Figure 1G) is stimulated 10 times at a low frequency (> 0.2-0.3 Hz). The filament is applied perpendicularly to the skin surface until it bends. The number of withdrawals is recorded and then the next filament (in ascending order) is used. Number of withdrawal response is recorded for each filament of the series.
SNI ipsilateral paw: withdrawal response	SNI induces in the ipsilateral paw a high frequency of withdrawal response by an intermediate force filament. The stimulus evokes a very brisk withdrawal response that is shown at normal (@ 32", 35") and slow motion (@ 40"). The stimulus may also evoke abnormal exaggerated response (@43s, 47s) that follows the withdrawal by brief licking of the paw. Notice the area stimulated (the lateral plantar area of the paw) and the bending of the filament. The investigator observes the animal from underneath the grid. For clarity, a lateral view is presented with stimuli @ 59', 1'02" and 1'05", the later evoking again an exaggerated response with paw shaking and licking
SNI contralateral paw: withdrawal response	The frequency of response in the contralateral paw is much lower than in the ipsilateral paw, here the mouse does not respond to the stimulus (@1'19", 1'25", 1'36")
Incorrect stimuli	The territory of the sural nerve is tiny and it is sometimes difficult to coordinate the stimulation in the correct territory with animal's movements. Here we show typically a stimulus in the wrong territory, the saphenous nerve territory (@1'47"). In this case the stimulus is not considered for recordings and an additional stimulus is performed in the sural territory. Another bias is the use of filaments that are too stiff. Above 4.0g, the force produces by the filament is beyond the force that the animal can counter exerted (motor impairment due to SNI). The paw is just passively lifted by the filament (@1'51", 1'53"), without any filament' binding.

SNI: spared nerve injury, @: at that time on the video in ' for minutes and " for seconds

Table II – comments for movie 2 : general mouse behavior after SNI

	Description of events
Curiosity Standing up	Mouse curiosity is conserved, it is not frightened by the cameraman. Although its movements are slightly modified, it can stand up on its hind limbs despite the SNI lesion (left paw).
Exploration of the environment	The mouse explores its environment normally when the cage is cleaned and although careful observation can detect abnormalities in paw position and gait, overall walking is not impaired.
Climbing	Despite a neuromuscular defect due to SNI, the mouse can easily climb the cage grid. In slow motion, notice that the SNI-injured left paw is used for holding and balance, even though the digits are not gripping the bar.

Table III : Significant estimated coefficient values, standard errors and *p*-values of the fitted mixed-effects model in the spared nerve injury model (SNI).

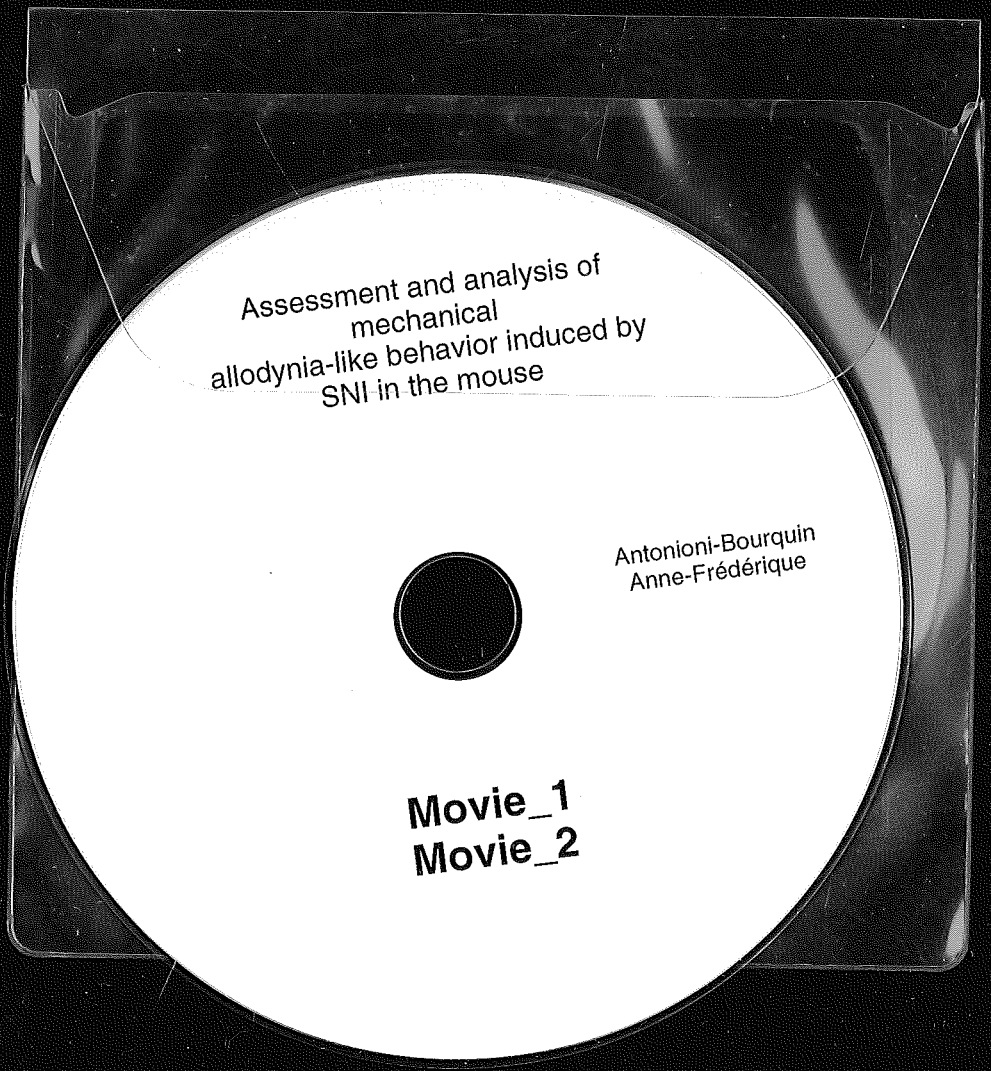
Coefficient	Value	Standard error	Significance
α_0	-0.93	0.13	<.0001
α_s	0.38	0.14	0.01
α_{BLB}	-0.08	0.34	0.82
α_{d4}	2.58	0.29	<.0001
α_{d7}	3.05	0.30	<.0001
α_{d14}	3.26	0.30	<.0001
α_{d21}	3.78	0.30	<.0001
α_{d28}	4.40	0.32	<.0001
β_0	1.19	0.08	<.0001
β_{BLB}	-0.17	0.11	0.11
β_{d4}	-0.67	0.10	<.0001
β_{d7}	-0.50	0.10	<.0001
β_{d14}	-0.45	0.10	<.0001
β_{d21}	-0.52	0.10	<.0001
β_{d28}	-0.45	0.10	<.0001

s, sex; d, day; BL, baseline

Table IV. Estimated coefficient values, standard errors and *p*-values of the fitted mixed-effects model for comparison of sham, SNI and SNI-variant procedures. No other coefficient of the model was found significant.

Coefficient	Value	Standard error	Significance
α_0	-0.87	0.39	0.03
α_{d7}	1.24	0.53	0.02
$\alpha_{d7,SNI,Sural}$	1.55	0.44	< 0.001
$\alpha_{d14,SNI,Sural}$	3.01	0.46	< 0.0001
$\alpha_{d21,SNI,Sural}$	3.01	0.47	< 0.0001
$\alpha_{d7,SNIv(s,cp),Sural}$	1.50	0.44	< 0.001
$\alpha_{d14,SNIv(s,cp),Sural}$	2.30	0.46	< 0.0001
$\alpha_{d21,SNIv(s,cp),Sural}$	2.77	0.47	< 0.0001
$\alpha_{d7,SNIv(t),Sural}$	-1.09	0.46	0.02
β_0	0.64	0.02	< 0.0001

d, day ; 7, 14, and 21 after the surgical procedure


Assessment and analysis of
mechanical
allodynia-like behavior induced by
SNI in the mouse
Antonioni-Bourquin
Anne-Frédérique
Movie_1
Movie_2