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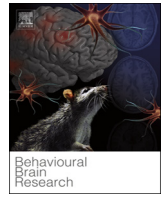
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Stress sensitivity and cutaneous sensory thresholds before and after neuropathic injury in various inbred and outbred rat strains

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ABSTRACT

Chronic pain is associated with altered affective state, stress, anxiety and depression. Conversely, stress, anxiety and depression can all modulate pain perception. The relative link between these behavioural constructs in different inbred and outbred rat strains, known to be variously hypo/hyperresponsive to stress has not been determined. Hindpaw sensory thresholds to repeated mechanical (von Frey filament and electronic Randall Selitto) and thermal (Hargreaves, cold plate and hot plate) stimulation were routinely assessed over three weeks in non-injured male rats of the following strains; WKY, LEW, F344, Hsd:SD and CrI:SD. Thereafter, threshold responses to Sparing Nerve Injury (SNI) were assessed using von Frey, pin prick and Hargreaves testing in the same strains over a three month period. Finally, anxiolytic efficacy of the benzodiazepine drug diazepam was assessed using the Elevated Plus Maze (EPM), as a surrogate index of functional plasticity of circuits involved in affective processing. Repeated nociceptive testing was associated with distinct strain-dependent changes in sensory thresholds in naïve rats; stress-hypo-responsive LEW rats presented with a mechanical/thermal hyperalgesia phenotype, whereas stress-hyper-responsive WKY rats presented with an unexpected heat/cold hypoalgesia phenotype. After SNI, LEW rats showed minimal signs of neuropathic sensitivity. Diazepam was anxiolytic in all tested strains with the exception of LEW rats reflecting distinct inherent affective processing only in this strain. The contribution of stress reactivity to nociceptive sensory profiles appears to vary in the absence or presence of neuropathic injury. Intriguingly, the functional responsiveness of affective state prior to injury may be a predisposing factor to developing chronic pain.

1. Introduction

Pain, emotionality and stress in animals and humans, are tightly linked behavioural constructs each of which are determined by a constellation of genetic and environmental factors [1]. Patients with high levels of anxiety are reported to experience elevated pain [2,3], whilst pre-surgical anxiety has been found to be a risk factor for developing chronic postsurgical pain [4]. Consistent with these observations, reducing experimental anxiety has been shown to have antinociceptive actions in humans [5], as well as in rodents [6]. Stress in turn, can have a profound influence on pain sensitivity and anxiety levels. Whilst the physiological response to acute stress has evolved to promote survival via recruitment of the flight or fight response, when unremitting and chronic, stress can have debilitating consequences for physical and mental functioning [7]. Accordingly, stress may elicit pain relief [8] or

exacerbate signs and symptoms of chronic pain in the clinical setting [9–11]. Furthermore, in addition to promoting hyperalgesia pre-clinically [12], ongoing or chronic stress has also been shown to be a key cause of depressive- [13] and anxiety-like behaviour in animals [14].

The majority of preclinical pain research uses traditional outbred rat strains, typified by the Sprague Dawley and Wistar strains, presumably based on facets of reproductive fecundity, low unit costs and methodological reproducibility [15]. Although other controllable factors including sex and age of experimental subjects have also recently been highlighted as experimental confounds [16,17], the specific strain of rat appears to have been largely neglected as a relevant consideration. This is puzzling based on the increasing appreciation that genetics and environment closely interact to modulate pain processing both prior to, and after injury [1,18]. For example, the inbred Wistar Kyoto (WKY) rat

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possesses a 'depressive-like' phenotype [19], exhibits distinct changes in hypothalamo-pituitary-adrenal (HPA) axis function in response to stress [20], and has been reported to exhibit a lowered pain threshold in the absence of injury compared with other inbred strains [21,22].

In light of the above observations, we decided to characterize hindpaw cutaneous sensory profiles, response to neuropathic injury and anxiolytic sensitivity of a number of outbred and inbred rat strains, all sourced from the same vendor to help mitigate any impact of nature versus nurture, and chosen on the basis of their well characterised reactivity to stress, and inherent anxiety or depressive phenotypes [19,23,24]. The sensory tests were performed routinely over a 3 week period to help consolidate habituation to the procedures used. We then chose to focus primarily on mechanical sensory thresholds, and to examine the effects of peripheral nerve injury across strains on this feature of neuropathic hypersensitivity. Finally, to help gauge any intrinsic involvement of putative differences in emotional response of the chosen strains to experimental outcomes in pain experiments, the functional efficacy to anxiolytic actions of the benzodiazepine drug diazepam were assessed.

2. Materials and methods

2.1. Animals and housing

All experiments were performed in accordance with Danish legislation (Law no. 474 of 15/05/2014 and Order no. 12 of 07/01/2016) regulating experiments on animals which complies with the European Directive 2010/63/EU. Experimental protocols for the different testing modalities at H. Lundbeck A/S were approved by the Animal Experiments Inspectorate in Denmark.

For all experiments male rats from the strains Lewis (LEW), Fischer (F344) and Wistar Kyoto (WKY) from Harlan / Envigo UK, Hsd:SD, Harlan / Envigo, Netherlands and CrI:CD(SD) from Charles River Laboratories, Germany, were used. Unfortunately, the F344-strain could not be supplied for the diazepam study, as the vendor discontinued the strain. Note that they were purposely sourced from the same vendor (with the exception of CrI:SD) to help facilitate the impact of nature vs nurture to (e.g. housing, weaning and dietary conditions) on the behavioural endpoints used to describe sensory phenotypes and potential impact of emotionality. They were ordered home at seven weeks of age and allowed to acclimatize for 1–2 weeks before performing any baseline testing or enrolment into experiments. Upon arrival, animals were pair-housed in transparent Techniplast polycarbonate macrolone type III-high open cages (42.5*26.6*18.5 cm) from Scanbur, Denmark, with environmental enrichment consisting of aspen wood chewing blocks (S-Bricks from Tapvei, Estonia), paper-wool shavings (LBS Biotechnology, UK) for nesting material and red Rat Retreats™ (Bio-Serv, Flemington, US) for hiding. For bedding, aspen chips (Tapvei, Estonia) were used, and cages were changed twice a week, and never on the days of testing. Food (Altromin 1324, Brogaarden, Denmark) and water were available ad libitum, and acidified (pH: 3.6 ± 0.5) drinking water was changed on a weekly basis, according to standard procedure for the animal facility. The light-dark cycle was 12:12 h with lights on from 6.00 to 18.00 h. Animals were moved to the testing laboratory at least three days prior to baseline testing and remained in the same room during the entire study period.

2.2. Study design

The overall study consisted of three distinct experiments each of which was performed using separate groups of rats from each of the strains described above.

In Experiment 1, naïve rats (n = 10 of each strain) were routinely exposed to a series of different nociceptive assays as shown in Table 1, which were performed over a three week period in order to characterize hindpaw sensory thresholds across the different strains. Accordingly,

each testing modality was performed once per week, and with one week between repetitions of the same modality. The experiment was performed in one cohort at the same time that included all strains and subjects, and with randomized order of testing for the individual subjects and strains.

In Experiment 2, baseline testing of mechanical (von Frey and pin prick, Days -3 and -1) and thermal thresholds (Hargreaves, Day -2) was assessed in rats (n = 12 of each strain) prior to Spared Nerve Injury (SNI) surgery which was performed at Day 0. Thereafter, hindpaw sensory thresholds were assessed for mechanical (von Frey and pin prick) on Days 4, 7, 10, 13, 15, 18, 20, 23, 25, 28, 35, 47, 69, 83 and 95, and thermal (Hargreaves) on Days 5, 8, 12, 19, 24 and 29 post-surgery. In addition, animals were examined for signs of distress, wound dehiscence and wound infection post-operatively and daily afterwards. The experiment was performed in one cohort at the same time that included all strains and subjects, and all animals were operated on the same day. Testing was performed in a randomized order, and each round of von Frey/pin prick or Hargreaves testing included subjects of each strain.

Finally, in Experiment 3, naïve rats (n = 12 of each strain with the exception of F344 which were not included due to an abrupt discontinuation of the strain by the vendor) were used to assess the acute anxiolytic effects of the benzodiazepine site ligand diazepam (0.3, 1, 3 mg/kg) which was administered i.p. using a 30 min pre-treatment time. These rats had been used 4–7 days previously for a locomotor activity experiment which assessed the acute effects of morphine or vehicle (0.3–6.0 mg/kg) (data included in Hestehave et al. [25]). Experiment 3 was divided into five cohorts that included animals from each strain and treatment group, and was performed over five consecutive days.

All injections and testing were performed between 7.30 and 16.00, in a randomized order by the same female researcher. The experimenter was blinded to the treatments, but not to rat strains, due to marked differences in behaviour, anatomical features and body size between strains.

2.3. Assessment of hindpaw mechanical thresholds to low and high intensity stimulation

Low intensity mechanical sensitivity was assessed by using a series of calibrated von Frey monofilaments (North Coast Medical, Inc., Morgan Hill). Animals were placed in individual Plexiglas (10.8*13.8*17.0 cm) enclosures on an elevated wire grid located in the same room in which they were routinely housed. They were given at least 15 min to acclimate to the enclosure and the experimenter's presence prior to stimulation of the lateral plantar surface of the hindpaw, at different anatomical locations in the area innervated by the intact sural nerve, with a series of calibrated von Frey filaments (0.4, 0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10.0, 15.0, 26.0 g). Briefly, to initiate testing a filament with a bending force of 4.0 g was first applied to the hindpaw with uniform pressure for 5 s. A brisk withdrawal was considered a positive response whereupon the next lower filament in the series was applied, until the filament inducing no response was determined. In the absence of a positive response the neighbouring higher filament was applied until determination of the filament inducing a positive response. After the first change in response pattern, indicating the threshold, four additional applications were performed; when no response, the next filament with a higher force was tested, and when a positive response, the next lower force filament was tested. The 50% threshold was determined by the following equation: 50% threshold (g) = $10^{\log(\text{last filament}) + k \cdot 0.3}$. The constant, *k*, was found in the table by Dixon [26], and determined by the response pattern.

Following measurements with the von Frey filaments, and while rats were still in the Plexiglas chamber, high intensity mechanical sensitivity to pin prick stimulation was performed as described previously by Kristensen, et al. [27]. A blunt safety pin was applied to the lateral area

Table 1

Pain phenotyping schedule. Testing was performed over three consecutive weeks similar to the test week as shown in the table. Marked in parenthesis is the time of day that the testing was performed.

Week day	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday/Sun-day
Pain-test	Von Frey & Pin Prick (8.00–13.00)	Hargreaves (8.00–13.00)	No testing	Hot plate (9.00–10.00) Cold plate (15.00–17.00)	Randall Selitto (9.00–10.30)	No testing

of the plantar surface of the hindpaw with enough force to produce a brisk reflex withdrawal response in naïve rats, but without penetrating the skin, and the withdrawal duration (s) was measured. The maximal withdrawal time was set to 30 s. Two measurements were applied with approximately 5 min in between and the mean duration was calculated.

High intensity mechanical sensitivity was also measured via the use of an electronic Randall Selitto algometer [27] (IITC Life Science, U.S.A.) as described previously [25,27]. The investigator gently restrained the rat prior to the application of progressively increasing mechanical pressure (g) to the mid region of the left hindpaw using the device (cut-off = 450 g). Pressure was discontinued at the precise moment the rat attempted to make a reflex hindpaw withdrawal, which in some instances was accompanied by vocalization, and the paw pressure threshold (g) was recorded. Two additional measures were made with approximately 20–30 s intervals on adjacent regions of the hindpaw, and the average of the three measurements was designated as the threshold.

2.4. Assessment of hindpaw thresholds to heat and cold stimulation

The hot plate procedure used in these experiments has been described previously (Hestehave, et al. [28] and Hestehave, et al. [25]). For all experiments, we used a hot plate (Ugo Basile Srl 7280, Gemonio, Italy), with a pre-set plate temperature of 52.5 °C as recommended for rats [29]. Time was recorded immediately from when the rat was placed on the hot plate until the first sign of discomfort from the thermal stimulus (e.g. licking, shaking, or stepping of the hindpaws) was observed. A cut-off-time was set at 60 s, and animals were removed immediately after either the positive response or cut-off time was reached to prevent thermal damage to the paw. A similar procedure was used for measuring hindpaw sensitivity to cold stimulation on a cold plate (IITC Life Science, U.S.A.) which was set to 0 °C. The time was recorded until similar signs of discomfort (e.g. licking, shaking, or stepping of the hindpaws) was observed.

Thermal sensitivity was also assessed using Hargreaves radiant heat stimulation [30]. The animals were placed in individual Plexiglas (10.8*13.8*17.0 cm) enclosures on an elevated glass floor, under which the radiant heat source (Ugo Basile 7370 Plantar Test Unit, Gemonio, Italy) was located. They were allowed to habituate to the environment for 10–15 min on tests 1 and 2, and for 20 min on the last test. The radiant heat source was placed under the hindpaw, and the time to withdrawal of the stimulated hindpaw was measured automatically when the radiant beam was no longer interrupted by the paw. Three recordings were performed with approximately 5 min in between and the mean latency was calculated.

2.5. Spared nerve injury

Neuropathic pain was induced using the Spared Nerve Injury (SNI) procedure as described by Decosterd and Woolf [31]. The methodology used was similar to previously reported by our group [32] and the same experienced technician performed all surgeries. Anaesthesia was induced with 5.0–5.5% sevoflurane (SEVOrane®; AbbVie Inc.) delivered in a mixture of 70% O₂ and 30% N₂O in a Plexiglas induction chamber and maintained by 2–3% sevoflurane in a 70:30-mixture of O₂/N₂O supplied via a face mask for spontaneous breathing. The anaesthetic depth was monitored by observing respiration rate and skin-color

indicating level of oxygenation, and testing the hindpaw withdrawal reflex. Each rat was provided with a single dose of antibiotics (Amoxicillin, Noromox Prolongatum®, 150 mg/kg), and analgesia (subcutaneous buprenorphine, Temgesic®, 0.03 mg/kg) as validated in Hestehave, et al. [32]. The rat was placed in left lateral recumbency, the fur was shaved on the lateral surface of the left thigh and the area was swabbed with chlorhexidine to secure aseptic conditions. A longitudinal incision was made through the skin caudal to the femur, and the underlying musculature (*musculus biceps femoris*) was opened with scissors and blunt dissection to reveal the sciatic nerve and the three terminal branches; the sural, common peroneal and tibial nerves. A gentle pinch was performed with forceps on the common peroneal and tibial nerves before ligation to verify that they were the intended nerves. Pressure of the common peroneal nerve provoked a brief flick of the toes, whereas the tight ligation of the tibial nerve provoked twitches in the surrounding musculature [33]. The common peroneal and the tibial nerves were ligated with non-absorbable 5-0 Prolene ligatures (Jørgen Kruuse A/S, Denmark), and sectioned distally to the ligation, removing approximately 2 mm of the distal nerve stump; thereby performing both axotomy and ligation of the two nerves, while leaving the sural nerve intact. Great care was taken not to irritate, stretch or pull the intact sural nerve. The musculature was re-approximated and the skin closed with tissue glue (Vetbond™, Jørgen Kruuse A/S, Denmark). The duration of the procedure from induction of anaesthesia until the animal regained consciousness was approximately 15–20 min.

Body-weight was measured every test day, and welfare parameters related to the presence of wounds, general condition, porphyria, appetite, and body posture were also scored daily, using a previously published protocol with scores of 0, 0.1 or 0.4 depending on severity [32]. The facility veterinarian was consulted if the welfare compromise reached an overall score of 0.4.

SNI is characterized by an increased sensitivity in the lateral area of the paw, innervated by the sural nerve [31], and naturally, the assessment of response to nerve injury by both mechanical (von Frey and Pin Prick) and thermal sensitivity (Hargreaves) was performed by stimulation of this lateral area of the affected left hindpaw. Besides this, the testing was completely similar to testing during naïve conditions.

2.6. Assessment of anxiety-like behaviour

An Elevated Plus Maze (EPM) was used to assess anxiety-like behaviour [34]. The maze consisted of four arms, each 45 cm long * 10 cm wide, arranged in a cross-like disposition, with two opposite arms with 50 cm high walls on each side as enclosure, and the other two arms without walls. Connecting the open and closed arms, was a central 10*10 cm square, giving access to any of the four arms. The surface of the maze was covered in black rubber flooring in order to increase the contrast between the white fur of the rat and the background, as well as to provide a non-slippery surface for the animal to walk on. The animal was placed in the centre of the EPM at the junction between the open arm and closed arms, enabling it to visualize both open and closed options from the beginning. The experimenter backed quietly away from the maze and left the room, while recording was performed for 5 min per animal by use of a camera placed above the maze, and movement between zones was tracked using Ethovision XT 9 (Noldus Information Technology). In the Ethovision system, the traditional zones were added (open, closed and centre), as well as the very distal

(10*10 cm) part of the open arms. The EPM was swiped with 70% ethanol between each rat to minimize odour clues from the previous animals. Great care was made to allow sufficient time for the ethanol to evaporate, and ventilation-hoods were located above the maze to minimize the smell of ethanol. The EPM was located in a separate room adjacent to the housing room. Rats were brought directly from their housing to be placed on the maze with no habituation to the EPM room, where the light-intensity was measured to be; centre: 276 lux, open: 283–388 lux (near centre - distal end of open arm) and closed: 19–82 lux (distal end of closed arm – near centre).

2.7. Drugs and administration

Diazepam was administered i.p. in a volume of 5 ml/kg. In order to dissolve the powder-formulation of diazepam (Sigma-Aldrich), we first added the majority of the vehicle (HP- β : hydroxypropyl-beta-cyclodextrin 10% + glucose monohydrate 4.4%) and some $\text{CH}_3\text{SO}_3\text{H}$ 1 M was added to dissolve the powder-formulation, after which the solution was slowly titrated with NaOH until a pH > 6.5 was reached, and the remainder of the vehicle added. Then the different concentrations were made with HP- β so that all groups would receive their dose in 5 ml/kg solutions, and the pH was confirmed to be above 6.5 before i.p. administration. Buprenorphine (Temgesic®; 0.03 mg/kg) was administered s.c. in a volume of 5 ml/kg. Amoxicillin trihydrate was administered s.c. in a volume of 1 ml/kg = 150 mg/kg; Noromox Prolongatum Vet®, 150 mg/ml (ScanVet, Fredensborg, Denmark).

2.8. Statistical analysis

Animals were randomly allocated to treatment groups, and treatments were blinded for the experimenter performing the testing. The same experimenter performed all behavioural tests and handling during the study. Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical comparisons between strains were made using either Two Way ANOVA (Repeated Measures, when applicable), or One Way ANOVA for comparisons of Area Under the Curve (AUC), with Bonferroni's post test for comparison between groups. Statistical analysis on overall treatment effects of diazepam between groups of the same strain, were made using One Way ANOVA, with Dunnett's post test comparison to vehicle-treated group. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Experiment 1 - Assessment of hindpaw sensory thresholds in naïve rats

In order to characterize nociceptive thresholds in normal naïve rats of the different strains, a series of repeated tests were performed. Fig. 1A + B shows the response to repeated application of low intensity mechanical stimuli using manual von Frey monofilaments. Two Way Repeated Measures ANOVA on data presented in Fig. 1A revealed a significant effect of strain ($F[4,135] = 5.69$, $P = 0.0009$, 9.4% of the variation) and time / repeated testing ($F[3,135] = 9.87$, $P < 0.0001$, 10.9% of the variation), and an interaction between the two effects ($F[12,135] = 2.49$, $P = 0.006$, 11.0% of the variation). For several strains, there was a marked decrease in threshold from baseline 1 to 2, especially for the stress-hyperresponsive strains, F344 and WKY. When comparing baseline means for the four test days (Fig. 1B), One Way ANOVA showed the same overall effects of strain, and Bonferroni's post tests revealed that Crl:SD and F344 showed a significantly higher 50% response threshold than LEW, and that Crl:SD also was significantly higher than WKY ($P < 0.05$), (Fig. 1B).

High intensity mechanical testing was performed using an electronic Randall Selitto device, and revealed clear strain differences in response to this stimulus (Fig. 1C + D). There was a significant effect of strain, ($F[4,90] = 10.06$, $P < 0.0001$, 26.7% of variation, Two Way Repeated

Measures ANOVA), but no effect of the repeated testing / time, though a significant effect of test subjects ($F[45,90] = 1.52$, $P = 0.047$, 29.9% of the variation) (Fig. 1C). Bonferroni post test showed that LEW rats had a significantly lower threshold on two (vs. F344 and WKY) or all (vs. Hsd:SD and Crl:SD) test days compared with the other strains. There were no significant differences between the other strains tested. Comparing mean mechanical thresholds for the three tests days (Fig. 1D), confirmed the strain differences, and that LEW rats had a significantly lower threshold than all the other strains tested ($P < 0.01-0.001$), indicating a hyperalgesic phenotype for the LEW strain.

Thermal sensitivity was first assessed by obtaining three repeated measures of response latency on a hot plate at 52.5 °C (Fig. 2A + B). Two Way Repeated Measures ANOVA revealed a significant effect of strain ($F[4,90] = 4.24$, $P = 0.0054$, 14.2% of variation), and time / repeated testing ($F[2,90] = 5.20$, $P = 0.0073$, 4.1% of variation). Additionally, there was an interaction between the effect of time/test-day and strain ($F[4,90] = 2.80$, $P = 0.0081$, 8.8% of variation), indicating that the effect of time depended on the specific strain. Repeated testing also produced a marked decline in response latency, especially for the F344 strain. On the other hand, there was a significant effect of the subjects ($F[45,90] = 2.14$, $P = 0.0011$), which accounted for 37.7% of the variation (Fig. 2A). Comparing the average hot plate response latency for the three tests, One Way ANOVA and Bonferroni's post test demonstrated that WKY had significantly higher response latencies than LEW, Crl:SD and Hsd:SD ($P < 0.05$), but not compared with F344 (Fig. 2B).

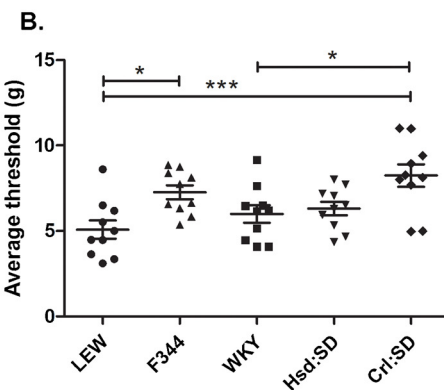
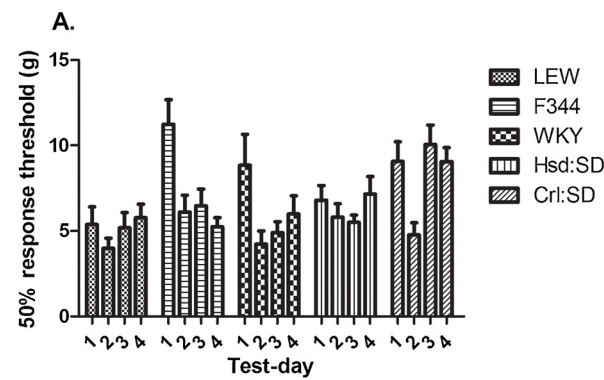
Next, thermal response latencies were measured over three tests using a Hargreaves radiant heat device (Fig. 2C + D). Two Way Repeated Measures ANOVA demonstrated a significant effect of strain: ($F[4,90] = 8.88$, $P < 0.0001$, 24.1% of variation), and repeated measures / time ($F[2,90] = 3.40$, $P = 0.0377$, 3.1% of variation) (Fig. 2C). Comparison of the average response latencies confirmed the effect of strain, and Bonferroni's post tests revealed significant lower latency thresholds for LEW than WKY, F344 and Crl:SD, and that Hsd:SD had lower thresholds than WKY and F344 (Fig. 2D).

Finally, thermal sensitivity to cold stimulation was assessed by measuring response latency on the cold plate (Fig. 2E + F). Two way Repeated Measures ANOVA on the three test days showed significant effects of strain ($F[4,90] = 12.20$, $P < 0.0001$, accounting for 35.5% of the variation), and time / test day ($F[2,90] = 11.39$, $P < 0.0001$, 6.0% of the variation), but no interaction. A significant effect of the subjects ($F[45,90] = 2.750$, $P < 0.0001$) accounted for 32.7% of the variation (Fig. 2E). Comparison of the average response latencies confirmed the effect of strain, and Bonferroni's post test showed that both F344 and WKY had longer response latencies than LEW ($P < 0.01-0.001$). WKY also had longer response latencies than both SD strains ($P > 0.01-0.001$), while only the Hsd:SD and LEW strains were lower than F344 (Fig. 2F).

3.2. Experiment 2 - Assessment of hindpaw sensory thresholds in SNI rats

Development of neuropathic pain following SNI surgery was primarily assessed by use of manual von Frey monofilaments (Fig. 3). Analysis of untransformed mechanical response thresholds failed to show any differences between strains, but did show significant effects of time: $F[16,848] = 85.76$, $P = < 0.0001$, interaction: $F[64,848] = 4.06$, $P < 0.0001$, and subject: $F[53,848] = 6.299$, $P < 0.0001$, Two Way Repeated Measures ANOVA (Fig. 3A) confirming that SNI induced mechanical allodynia. However, as the various strains presented with different baseline levels prior to surgery, the data was also transformed and compared to baseline (Fig. 3C–F). Two baselines were performed prior to surgery as the phenotyping study (Fig. 1) had revealed a drastic decline from baseline 1 to 2 in naïve rats. Therefore, the transformation was performed in relation to both baseline 1 and 2. When analysing the transformed data sets, Two Way RM ANOVA revealed significant effects

Von Frey



Randall Selitto

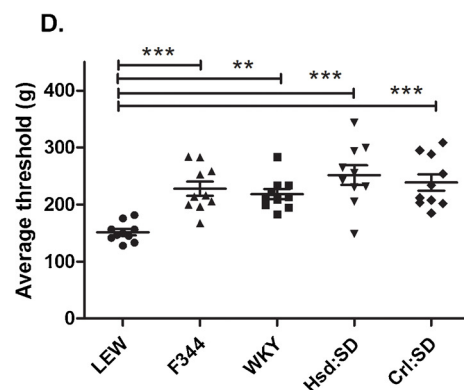
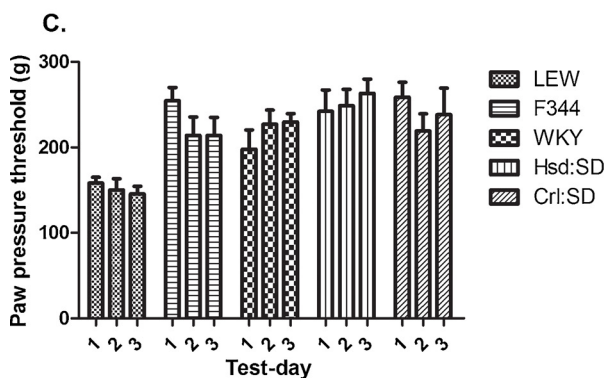


Fig. 1. Assessment of mechanical thresholds in naïve male rats of each strain. **A + B.** Low intensity mechanical threshold (g) measured by von Frey monofilaments. **A.** 50% response threshold to von Frey filaments, measured in subsequent testing sessions over 4 weeks. **B.** Average von Frey threshold during the four testing sessions shown in **A** presented as a scatter plot. **C + D.** Electronic Randall Selitto device, measuring high intensity mechanical threshold (g). **C.** Randall Selitto measurements indicating paw pressure threshold (g), measured in three subsequent testing sessions. Two Way Repeated Measures ANOVA, with Bonferroni post test showed that LEW had a significantly lower threshold on 2 (F344 and WKY) or all (SD:Hsd and SD:Cri) test days compared with the other strains. **D.** Average paw pressure threshold during the three testing sessions shown in panel **C** presented as a scatter plot. For all graphs data is presented as mean \pm SEM. All groups, $N = 10$ male rats. Statistically significant differences between groups are presented using following symbols: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as determined by One Way ANOVA and Bonferroni's post test.

of strain (baseline 1; $F[4,848] = 11.74$, $P < 0.0001$. baseline 2: $F[4,848] = 5.79$, $P = 0.0006$), as well as time (baseline 1; $F[16,848] = 54.13$, $P < 0.0001$. baseline 2: $F[16,848] = 52.10$, $P < 0.0001$) and interaction (baseline 1; $F[64,848] = 2.39$, $P < 0.0001$. baseline 2: $F[64,848] = 2.59$, $P < 0.0001$) (Fig. 3C–E). Calculating the area under the curve for each subject was made to enable further comparison of the strains. Although the untransformed data indicated no strain differences (Fig. 3B), when the data was transformed in relation to the pre-surgical baselines, all strains developed a significantly higher degree of mechanical allodynia than LEW ($P < 0.01$ – 0.001) (Fig. 3D and F).

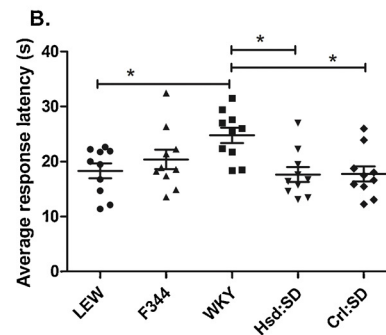
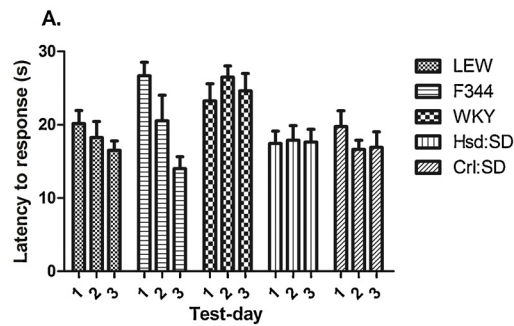
Mechanical hypersensitivity was also measured in SNI rats via use of the pin prick test (Fig. 4A + B). Two Way Repeated Measures ANOVA showed significant effects of both strain ($F[4,848] = 2.81$, $P = 0.0346$) and time ($F[16,848] = 9.86$, $P = < 0.0001$) (Fig. 4A), but when comparing the AUC, Bonferroni's post test revealed no difference between strains *per se* (Fig. 4B).

Thermal sensitivity was also assessed following nerve injury by use of Hargreaves radiant heat (Fig. 4C + D). Two Way Repeated Measures ANOVA showed a significant effect of strain only ($F[4,54] = 7.57$, $P < 0.0001$), but no effect of time, indicating that the response latency to thermal stimuli was not altered by SNI (Fig. 4C). When calculated as AUC values (Fig. 4D) an unaltered threshold to heat was confirmed by the striking resemblance with the naïve thresholds presented in Fig. 2D. In view of the lack of change in threshold sensitivity to heat for all strains following SNI the assessment was discontinued after day 29 post

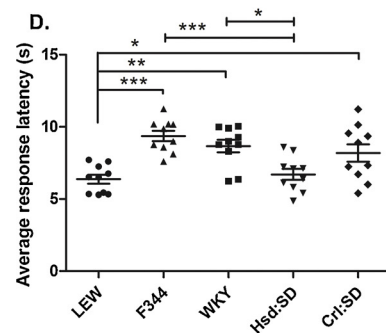
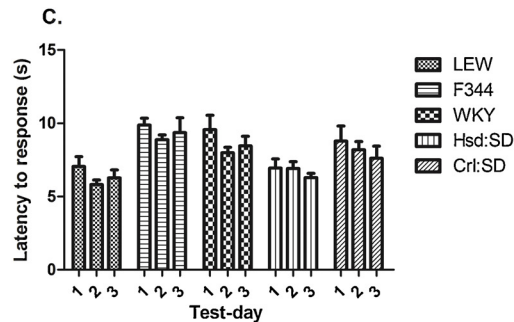
injury.

Finally, routine welfare observation of all SNI rats revealed the presence of superficial wounds over the first days after the surgery for the F344 strain (5/12 rats) compared with the other strains indicating that they had an increased tendency to bite or self-mutilate the affected paw. Of the other strains, only one Hsd:SD rat developed similar self-mutilated wounds; the behaviour subsided and the superficial wounds healed. However, at approximately two months after surgery three SNI-injured Hsd:SD rats had developed superficial wounds, and one of them had to be euthanized due to wound infection. Body weight was followed during the study, and not surprisingly, statistical analysis showed clear effects of both strain ($F[4,54] = 213.3$, $P < 0.0001$) and time ($F[26,54] = 1946$, $P < 0.0001$), as well as an interaction between the two factors ($F[104,54] = 15.29$, $P < 0.0001$, as determined by Two Way Repeated Measures ANOVA). The CRL:SD strain had a significantly higher body weight than all other strains at all timepoints ($P < 0.001$), despite all strains being ordered home at the same age. The starting-weights at the first baseline for the individual strains were: Mean \pm S.E.M.; LEW; 205.5 ± 4.2 g, F344; 163.4 ± 1.9 g, WKY; 173.8 ± 4.2 g, HSD:SD; 225.5 ± 2.4 g, CRL:SD; 299.0 ± 3.7 g, and at the final measurement on day 95; LEW; 338.6 ± 7.4 g, F344; 315.4 ± 3.8 g, WKY; 358.1 ± 5.9 g, HSD:SD; 395.9 ± 8.1 g, CRL:SD; 506.4 ± 11.2 g.

Hot plate



Hargreaves



Cold plate

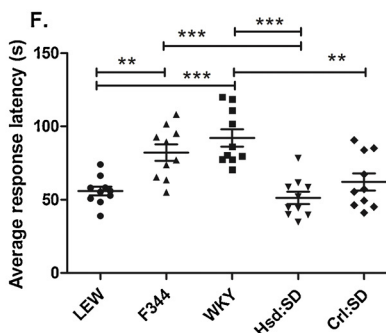
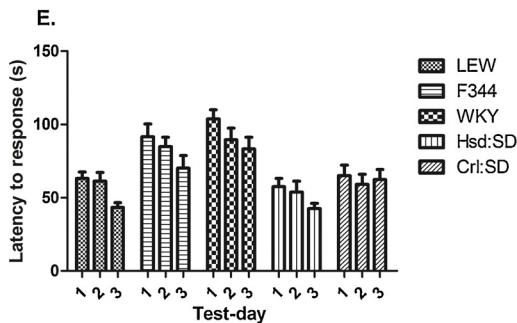


Fig. 2. Assessment of thermal thresholds in naïve male rats of each strain. A + B. Hot plate response latency. A. Response latencies to thermal stimulation were recorded over three exposures to a hot plate (52.5 °C) and presented in seconds (s). B. Average hot plate response latency (s) during the three test days shown in A presented as scatter plot. C + D. Hargreaves response latency to radiant heat. C. Response latencies (s) measured on three test days. D. Average response latency (s) of the three days test's in C presented as scatter plot. E + F. Cold Plate. Response latency (s) to cold stimulation (0 °C). E. Response latencies (s) measured on three test days. F. Average response latency (s) of the three baselines shown in panel E presented as scatter plot. For all graphs data is presented as Mean \pm SEM. All groups, N = 10, male rats. Statistically significant differences between groups are presented using the following symbols: *P < 0.05, **P < 0.01, ***P < 0.001, determined by One Way ANOVA and Bonferroni's post test.

3.3. Experiment 3 - Assessment of anxiety-like behaviour in naïve rats

The anxiolytic effect of diazepam (0.3–3 mg/kg, i.p.) was assessed in a third group of animals by tracking behaviour in the Elevated Plus Maze (EPM). Unfortunately, the F344 strain could not be supplied for this experiment, due to the vendor discontinuing the strain.

The most prominent effects were detected in the open arms of the maze, where all strains except for LEW showed significant effects of diazepam (Fig. 5). Increasing doses lead to increased time spent in the open arms for all strains, except LEW; WKY: F [3,44] = 6.45, P = 0.0010. Hsd:SD: F [3,44] = 6.97, P = 0.0006. Crl:SD; F [3,44] = 3.23, P = 0.031, LEW: F [3,44] = 0.933, P = 0.4330 = not significant. Dunnett's post test revealed that for both SD strains, the 3.0 mg/kg dose resulted in significantly more time spent in the open arms than vehicle treatment (P < 0.01–0.001), while the Minimum Effective Dose (MED) for WKY was evident at 1.0 mg/kg (P < 0.05) (Fig. 5).

Only LEW rats showed significant effects of treatment on the time spent in the closed arms (LEW: F [3,44] = 4.72, P = 0.0061, One Way ANOVA), with increasing doses leading to increased time spent in this zone, indicating that the increased amount of time spent in the open areas was at the expense of time spent in the centre zone. In the centre zone, all strains showed significant effects of the treatment (LEW: F

[3,44] = 7.739, P = 0.0003. WKY: F [3,44] = 10.33, P < 0.0001. Hsd:SD: F [3,44] = 12.34, P < 0.0001. Crl:SD; F [3,44] = 12.81, P < 0.001, One Way ANOVA). Dunnett's post test revealed that all strains administered 3.0 mg/kg diazepam spent significantly less time in the centre zone compared with vehicle.

Diazepam treatment also diminished locomotor activity / distance travelled, as measured by the Ethovision software. There were significant effects of both strain (F[3,176] = 13.84, P < 0.0001) and treatment (F[3,176] = 24.89, P < 0.0001), with strain contributing less to the total variation (13.9%) than treatment (24.9%, Two Way ANOVA). Overall, WKY were less active than the other strains. Analysing the effects on the different strains independently showed significant effects of the treatment for all strains except Crl:SD, indicating that diazepam had similar pharmacokinetic and sedative properties across the majority of the strains, including LEW, but that this did not correlate directly to anxiolytic effects (LEW: F [3,44] = 11.30, P < 0.0001. WKY: F [3,44] = 12.73, P < 0.0001. Hsd:SD: F [3,44] = 9.984, P < 0.0001. Crl:SD; F [3,44] = 2.731, P = 0.0551 = not significant, One Way ANOVA).

4. Discussion

The current study further confirms that various rat strains that

Mechanical allodynia

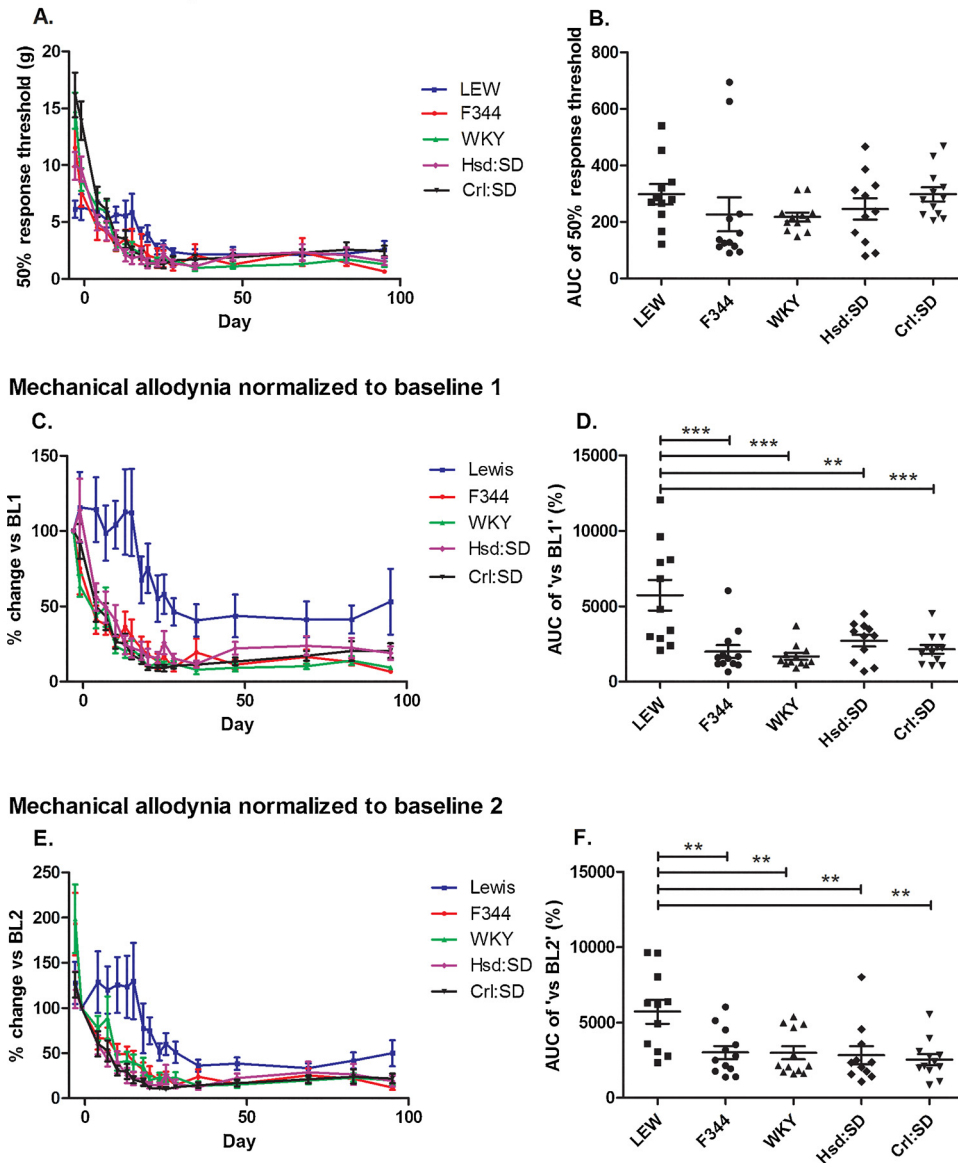


Fig. 3. Development of low threshold mechanical allodynia following Spared Nerve Injury. **A.** 50% response threshold to mechanical von Frey stimuli, from day -3 to 96 days post-surgery. **B.** Area under the Curve (AUC) for the mechanical allodynia presented in Fig. A, presented as scatter plot. **C-F:** Development of mechanical allodynia, compared with pre-surgical baselines: **C.** The mechanical threshold presented in panel A, presented as percentage of baseline 1 (%). **D.** AUC of the mechanical response normalized to pre-surgical baseline 1 shown in panel C presented as scatter plot. **E.** The mechanical threshold shown in panel A presented as percentage of baseline 2 (%). **F.** AUC of the mechanical response normalized to pre-surgical baseline 2 shown in panel E, presented as scatter plot. For all graphs data is presented as mean \pm SEM. N = 12, male rats of each strain (N = 11 for LEW). Day 0 = SNI surgery. Statistically significant differences between groups are presented using the following symbols: **P < 0.01, ***P < 0.001 as compared to LEW, determined by One Way ANOVA and Bonferroni's post test.

incorporate diverse facets of stress reactivity can present with distinct sensory profiles upon cutaneous noxious stimulation. Surprisingly, we observed that the development of neuropathic hypersensitivity in the strains tested did not appear to correlate with a high sensitivity to nociceptive stimulation at baseline or inherent sensitivity to stress. To help reconcile this unexpected disconnect, we then proceeded to show that the functional responsiveness of affective state in the absence of injury, as assessed via differential strain sensitivity to the anxiolytic drug diazepam in the EPM, may be one of a number of predisposing factors that contribute to the development of neuropathic pain.

4.1. Nociceptive reflexes in naïve rats

We selected the inbred strains for this study based on characteristics related to stress reactivity and affective phenotypes. The WKY rat has a 'depressive-like' phenotype [19] and exhibits distinct changes in HPA axis function in response to stress [20]. The LEW and F344 strains are often compared as a model of HPA axis function in which LEW is relatively hypoactive compared to the stress-hyperresponsive F344 [24]. We purposely sourced all the inbred strains from the same vendor-specific site (with the exception of Crl:SD and Hsd:SD) to help us to

assess the impact of nature rather than nurture (e.g. housing, weaning and dietary conditions) on the behavioural endpoints used to describe sensory phenotypes and potential impact of emotionality.

The application of low intensity mechanical stimulation to the hindpaw of naïve rats reduced threshold responses from first to second baseline in all strains. This was particularly striking in the two stress hyperresponsive strains, F344 and WKY. Similarly, in the hot plate test the effects of repeated testing were most prominent in the stress-sensitive F344 strain. Although this latter finding contrasts with some other studies [35,36], it confirms our previous observations [25] and is consistent with the concept of stress-induced analgesia upon initial testing [37,38]. Thereafter, we believe that habituation to the repeated handling/testing inherent in our study design in a strain such as F344, can sufficiently diminish stress reactivity to manifest as shorter response latencies. In contrast, high intensity Randall Selitto mechanical stimulation was not affected by repeated testing, implying that pain tolerance as opposed to pain detection is less sensitive to acute stress. Previously, a positive correlation has been shown between thermal and mechanical stimuli on hindpaw nociceptive responses in naïve male rats representing eight inbred and outbred strains [39]. This relationship was recapitulated here only in stress hypo-responsive LEW rats,

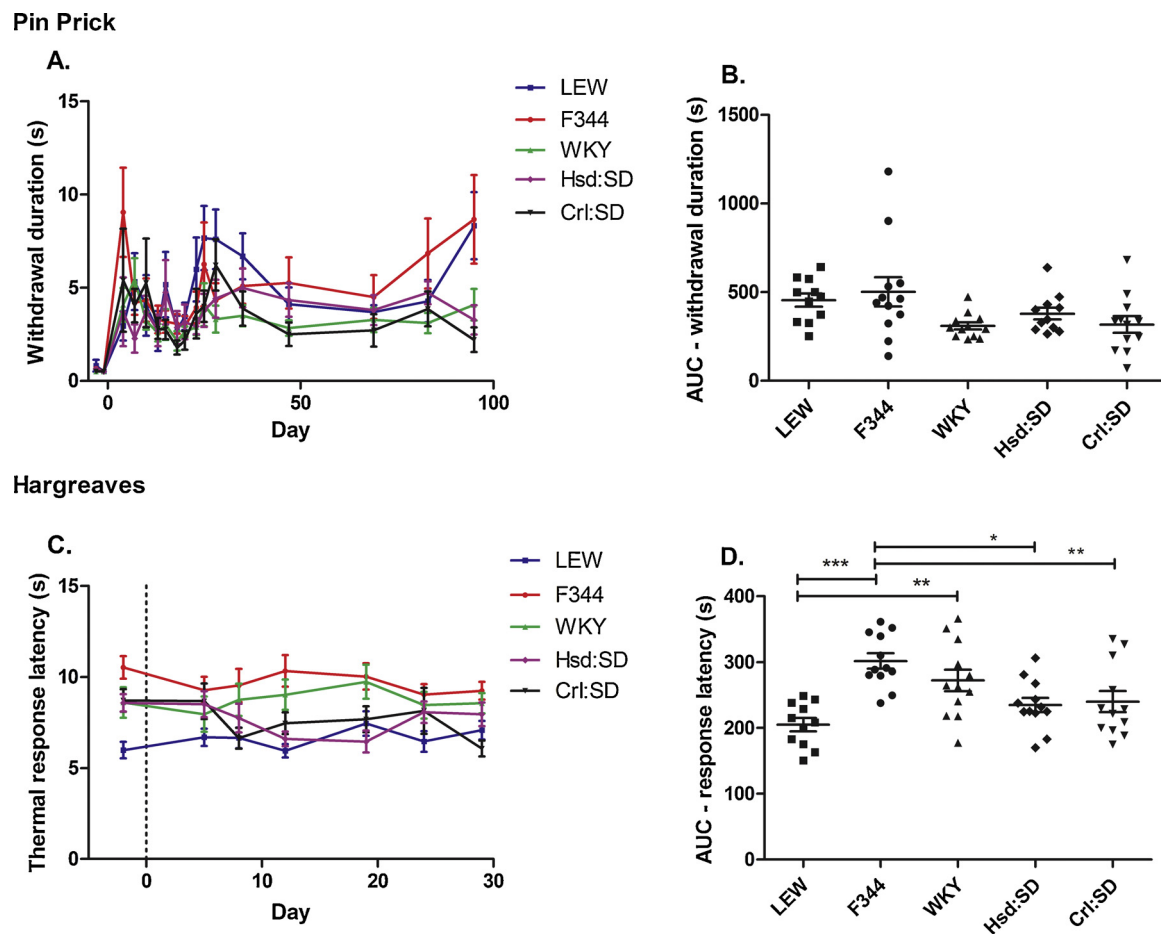


Fig. 4. Development of high threshold mechanical hypersensitivity and sensitivity to noxious heat following Spared Nerve Injury. **A.** Mechanical hypersensitivity, measured by response duration (s) following Pin Prick stimulation. **B.** AUC of the mechanical hypersensitivity shown in panel A presented as scatter plot. **C.** Thermal sensitivity from day -2 to 30 post surgery, measured by response latency (s) to Hargreaves radiant heat. **D.** AUC of thermal sensitivity shown in panel C presented as scatter plot. For all graphs: data is presented as mean \pm SEM. N = 12 of each strain (N = 11 for LEW). Day 0 = SNI-surgery. Statistically significant differences between groups are presented using the following symbols: *P < 0.05, **P < 0.01, ***P < 0.001, determined by One Way ANOVA and Bonferroni's post test.

which appeared as 'hyperalgesic' compared with the other strains tested [39,40]. Conversely, WKY rats appeared to be 'hypoalgesic' in thermal tests, which was unexpected given that they have previously been reported to be sensitive to somatic thermal [41–43] or visceral stimuli [44–46]. From a simple practical perspective, these data clearly highlight the need to pay careful attention to the number of baseline tests performed with specific nociceptive tests when using different rat strains in experimental pain research.

4.2. Nociceptive reflexes in neuropathic rats

Strain differences in nociceptive responses after peripheral nerve injury have been reported previously [47–50]. We were surprised by the complete lack of hypersensitivity to low intensity mechanical stimulation displayed by LEW rats compared with the other strains after SNI. Although other studies have shown that LEW rats can express some degree of neuropathic hypersensitivity after either SNI or Chronic Constriction Injury, the onset occurs later than in F344 and SD rats [40,51], and as such is not entirely discordant with our findings. Several studies have indicated a marked development of hypersensitivity in WKY rats when exposed to nerve injury [52,53], albeit with different responsiveness depending on the neuropathic model used [53]. Possibly reflecting a differential contribution of inflammatory components to neuropathic hypersensitivity between nerve trauma models [54], we have previously observed that WKY rats have a diminished mechanical

response to inflammatory hyperalgesia compared with the other strains tested here [25]. Alternatively, it could also reflect differences in experimental testing conditions used between laboratories [55]. Stress has been reported to exacerbate chronic pain conditions in the clinical [9,10] and preclinical setting [56,57]. Notably, we found that the two stress-hyperresponsive strains (F344 and WKY) developed a markedly higher degree of neuropathic pain than the stress-hypo-responsive LEW strain.

Whilst the SNI procedure is generally associated with a robust mechanical response post-injury, thermal responses are typically more difficult to reliably measure due to the lateral location of the terminal nerve projections of the intact sural nerve [31]. We did not detect any effect of nerve injury on the thermal threshold to radiant heat. We cannot discount that LEW rats might have developed other sensory abnormalities or have been functionally affected by SNI. However, given that F344 rats present with increased autotomy scores compared with LEW after complete denervation [39], any sensory abnormalities if present would have been unlikely to have incorporated facets of dysesthesia or paraesthesia. For future studies, alternative measures like acetone drop for cold allodynia [58], conditioned place preference [59] or functional gait alterations [60] would potentially extend on the sensory phenotypes that these strains may exhibit.

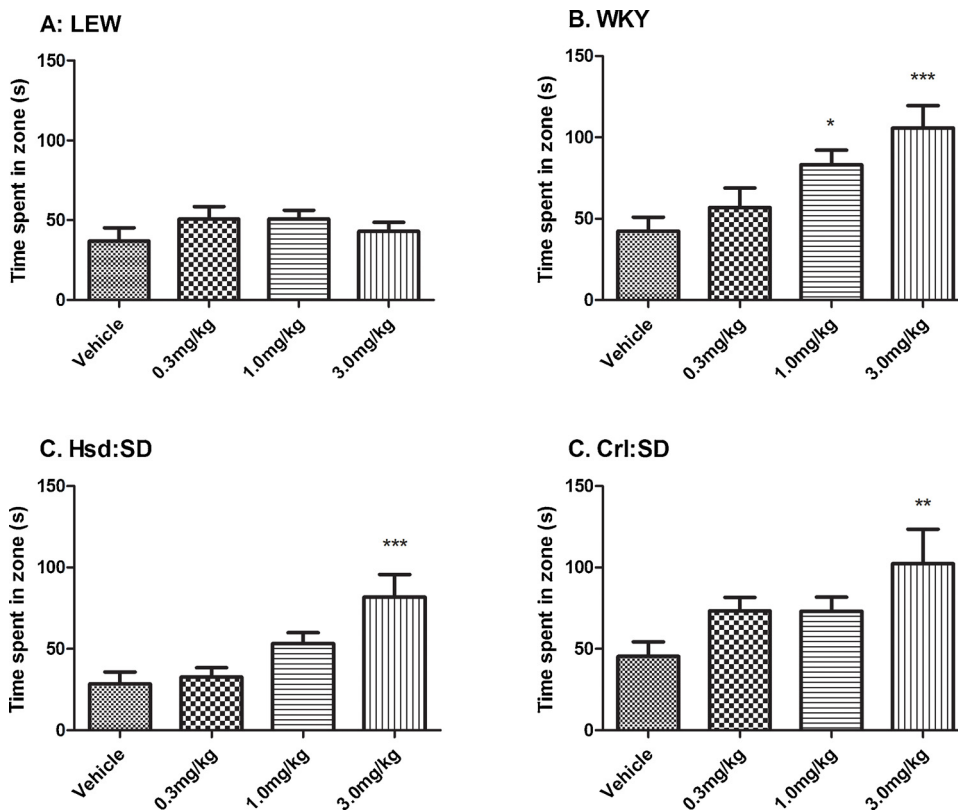


Fig. 5. Anxiolytic effects of diazepam in the elevated plus maze. Graphic show time spent in the open arms of the maze (s), for each dose of diazepam (0.3–3.0 mg/kg) and presented for each strain; A. LEW, B. WKY, C. Hsd:SD, D. Crl:SD. For all graphs data is presented as mean \pm SEM of the amount of time spent in the open arms. N = 12. Statistically significant differences between groups are presented using the following symbols: *P < 0.05, **P < 0.01, ***P < 0.001, determined by One Way ANOVA and Dunnett's post test compared to vehicle treated group.

4.3. Anxiety-like behaviours in naïve rats

Pain appreciation is a multidimensional process that integrates sensory discriminative and affective motivational components. This latter feature as it pertains to pain shares a number of common neurobiological mechanisms with emotional disorders such as anxiety [61]. Indeed, several studies assessing the influence of anxiety on pain perception in humans suggest that pain sensitivity is higher in people experiencing increased anxiety [62–65]. Conversely, reducing experimental anxiety has antinociceptive actions [5], a relationship that persists also in rodents [6].

Based on the lack of an obvious correlation between putative stress reactivity and nociceptive thresholds of the strains included here, we wondered if a facet of emotionality might potentially predispose or protect particular strains from developing neuropathic hypersensitivity. To test this additional post-hoc hypothesis, rather than simply assessing anxiety-like behaviour of naïve rats from each strain in the EPM (which similar to nociceptive testing has also been reported to be subject to test/retest variability [66,67]), we decided to assess their functional responsiveness to the anxiolytic drug diazepam. Much as expected the different strains increased their exploration of the open arms of the EPM after administration of diazepam. Again, LEW rats were the notable exception. Although anxiolytic sensitivity to diazepam should not be discounted for this specific strain [68], other groups have reported similar findings to ours using either the EPM [69] or impulse choice tests [70]. Furthermore, LEW rats are also less sensitive to the anxiolytic actions of other benzodiazepines such as chlordiazepoxide [71]. Whether this lack of functional response to diazepam indicates that affective processing is diminished in LEW rats and serves to protect them from some of the negative consequences of peripheral nerve injury remains to be established in future studies. Note that we would advocate that any measures of anxiety and nociception pre- and/or post-injury are preferably obtained in the same animals to facilitate correlational analysis. A limitation of the latter experiment arising as a consequence of a post-hoc design is that the various experiments were performed in

different cohorts of rats, albeit they were sourced from the same vendor and of a similar age.

4.4. Conclusions

In the current study, we observed clear strain-dependent differences in hindpaw nociceptive sensory thresholds in the absence of injury. However, we were unable to reconcile these differences to the inherent stress reactivity of the inbred strains tested. Notably, stress-sensitive F344 and WKY rats presented with a hypoalgesic sensory phenotype whereas LEW rats paradoxically presented with a hyperalgesic phenotype. Thereafter, only LEW rats failed to show a robust neuropathic hypersensitivity after SNI. Despite the apparent complexity of the interplay between stress and nociceptive response profile in the absence or presence of neuropathic injury, we found that the functional responsiveness of affective state prior to injury may be a predisposing factor to developing chronic pain. Our findings indicate that further comparative investigations such as ours are required to help facilitate a better translational appreciation of specific rat strains for purposes of experimental pain research.

Contributions

The study was conceptualized by SH, KA, TBP and GM. Experiments were designed by SH and GM and discussed thereafter with KA and TBP. SH performed all experiments and statistical analysis. SH and GM drafted the manuscript and all authors discussed the results and finalized the manuscript.

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The Authors declare that there are no conflicts of interest. Two of the authors are/were employed by the pharmaceutical company, H.

Lundbeck A/S, and one by Hoba Therapeutics, but as both companies have no commercial interests in the compound (or related compounds) tested in the current study, these affiliation does not result in conflicting interests.

Declaration of Competing Interest

The Authors declare that there are no conflicts of interest. Two of the authors are/were employed by the pharmaceutical company, H. Lundbeck A/S, and one by Hoba Therapeutics, but as both companies have no commercial interests in the compound (or related compounds) tested in the current study, these affiliation does not result in conflicting interests.

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