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# Simultaneous Measurement of Behavior and the Somatosensory Evoked Potential in a Rat Model

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## Abstract

Studies have shown that specific characteristics of somatosensory evoked potentials (SEPs) reflect nociception in both animals and humans. A relationship between SEPs and the unpleasantness of noxious stimulation in rats has recently been demonstrated using Pavlovian fear conditioning, consisting of a training phase in which a conditioned stimulus (CS) is paired with an unconditioned stimulus (US) to elicit the SEPs. After the training, CS-induced fear-conditioned behavior serves as a readout parameter for aversion to the US (i.e., the SEP stimulation paradigm). To prevent the animals from gnawing the stimulation cables that are necessary for generating SEPs, investigators have used a tight-fitting jacket that restrains the rats but also inhibits behavioral measurement. The use of a neck collar is an alternative technique that not only prevents cable gnawing but also allows the animals unrestricted movement while enabling investigators to assess fear-conditioned behavior and measure SEPs. The current study explores the effects of the tight-fitting jacket and the neck collar on SEPs. A within-subjects design was used for recording the SEP of each rat while the animal wore the collar or jacket. Both conditions show a similar SEP morphology, but data from the collar-wearing rats indicated an increase of the N150 peak amplitude (associated with emotional arousal) and peak latencies that appeared to be shorter. Thus the collar will be useful in future studies as it allows the simultaneous evaluation of SEPs and behavior.

**Key Words:** amplitude; behavior; Elizabethan collar; latency; nociception; rat model; refinement; somatosensory evoked potential (SEP)

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## Introduction

Alleviation of pain in animals is negatively influenced by limited knowledge about its recognition and effective treatment (Marris 2009), necessitating the generation of more knowledge about animal pain (patho)physiology. The somatosensory evoked potential (SEP<sup>1</sup>), a time- and stimulus-locked fragment of the electroencephalogram, represents the processing of noxious stimuli (Bromm and Lorenz 1998) that can be recorded and analyzed in a highly standardized and objective way. The SEP waveform is described by the latency (the time of occurrence) and amplitude (height) of peaks of interest (the specific occurrence and latency depend on the SEP-generating stimulus, species, and filter settings used). Because the SEP correlates well with both subjective pain ratings in humans (Arendt-Nielsen and Bjerring 1988; Chen et al. 1979; Kanda et al. 2002; Ohara et al. 2004) and aversion to pain in animals (Van Oostrom et al. 2005, 2007), and because the SEP is altered by anesthetic and analgesic treatment in both humans (Banoub et al. 2003) and animals (Stienen et al. 2004, 2006; Van Oostrom et al. 2009), it may serve as a readout parameter in pain research.

Pain consists of a sensory component, which refers to the encoding of the stimulus type, intensity, and spatiotemporal localization, and an affective component, which refers to the experienced pain unpleasantness (Price 2002). In animal pain research, the SEP is of special interest because of its potential to quantify and differentiate the sensory and affective component of acute pain. Stienen and colleagues (2006) have suggested that SEPs recorded from the primary somatosensory cortex (S1<sup>1</sup>-SEPs) represent the sensory component and those from the vertex (Vx<sup>1</sup>-SEPs) the affective component. However, the neural origin of the two types of SEPs and their role in the sensory or emotional component of pain are not yet fully understood.

The relationship between the SEP and sensory and affective pain components can be studied using Pavlovian fear conditioning (Van Oostrom et al. 2005), in which animals are trained to associate a neutral stimulus (conditioned stimulus [CS<sup>1</sup>]; a tone) with an aversive one (unconditioned stimulus [US<sup>1</sup>]). After the training, aversive behavioral responses (e.g., freezing) evoked by the CS can serve as a measure for aversion to the US and thus an expression of pain induced by the US

<sup>1</sup>Abbreviations that appear  $\geq 3x$  throughout this article: CS, conditioned stimulus; S1, primary somatosensory cortex; SEP, somatosensory evoked potential; US, unconditioned stimulus; Vx, vertex

(Fanselow and Bolles 1979; Van Oostrom et al. 2007). Studies have shown that SEP-producing stimuli can be used as the US (Van Oostrom et al. 2005, 2007) and that, in rats, the administration of electric stimuli to the tail base is particularly effective (Stienen et al. 2003; Van Oostrom et al. 2009). However, the rats tend to gnaw on the stimulation cables used for evoking SEPs. The use of a tight-fitting jacket (Stienen et al. 2003) prevented the animals from gnawing the cables but also considerably restrained their movement, making it extremely difficult to assess freezing behavior while measuring SEPs.

As an alternative to the tight-fitting jacket we propose the Elizabethan collar, which prevents cable gnawing, allows the animal to move freely, and permits both the assessment of freezing behavior and measurement of SEPs. The simultaneous recording of neurophysiological and behavioral parameters enables in-depth evaluation of the relationship between the specific SEP characteristics and the sensory and affective component of pain. The collar technique further refines the rat model for studying animal pain through SEPs by yielding more information without increasing the number of animals.

We explored the effects of the tight-fitting jacket and the Elizabethan neck collar on different characteristics of the SEP.

## Animals

Adult male Wistar rats (HsdCpb:WU, Harlan Netherlands BV, Zeist, NL; 8 weeks old, body weight 250–300 g at the time of arrival,  $n = 20$ ) were paired in a divided system with two rats in one clear 1500 U Eurostandard Type IV S cage ( $48 \times 37.5 \times 21$  cm) separated by a wire-mesh fence (a “living apart/together system”). The rats, which are social animals (Hrapkiewicz and Medina 2007), were able to engage in some forms of social interaction through sight, smell, and sound without damaging each other’s head-mounted SEP-measuring receptacle (see Surgery, below). The animals were provided with bedding material (Aspen chips), ad libitum access to food and water, and paper tissues as cage enrichment. The environment was controlled (temperature,  $21 \pm 2^\circ\text{C}$ ; humidity,  $47 \pm 3\%$ ), with an inverse 12-hour light-dark cycle (lights off from 6:00 AM to 6:00 PM) and a radio on constantly at a low volume as background noise. Animals were handled daily by the personnel who performed the experiment.

## Materials and Methods

### Ethical Note

The scientific committee of the Department of Animals in Science and Society of Utrecht University provided peer review of this experimental protocol (DEC-DGK 2009.I.02.011). In addition, the protocol received the approval of the Animal Experiments Committee of the Academic Biomedical Centre in Utrecht, based on De Wet op de Dierproeven (the Dutch Experiments on Animals Act of 1996; EU 1996a) and on the Dierproevenbesluit (the Dutch Experiments on Animals

Decision of 1996; EU 1996b). Furthermore, all animal experiments followed the “Principles of Laboratory Animal Care” (EU 1996a) and referred to the *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research* (NRC 2003).

## Surgery

After an acclimation period of 2 weeks, the animals underwent surgery for permanent implantation of epidural electrodes. Animals were transported individually from their housing to a separate room, where anesthesia was induced under red lighting with 0.25 mg/kg of fentanyl (intraperitoneally, i.p.; Fentanyl Janssen, Janssen-Cilag BV, Tilburg, NL; 0.05 mg/ml of fentanyl citrate) and 0.15 mg/kg of dexmedetomidine (i.p.; Dexdomitor, Pfizer Animal Health BV, Capelle a/d IJssel, NL; 0.5 mg/ml of dexmedetomidine hydrochloride). After each animal’s pedal reflex disappeared, the animal was transported to the surgery room and, after intubation, was anesthetically maintained with isoflurane in 100% O<sub>2</sub>. The animals received 8 ml of saline (subcutaneously, s.c.) to support normal fluid balance, 5 mg/kg of enrofloxacin (i.p., Baytril 2.5%, Bayer BV, Mijdrecht, NL), and eye ointment (Ophtosan Oogzalf, Produlab Pharma Raamsdonkveer, ASTfarma BV, Oudewater, NL; 10,000 IE of vitamin A palmitate per gram).

For the surgery, the prepared animal was positioned in the stereotactic apparatus (model 963, Ultra Precise Small Animal Stereotaxic, David Kopf Instruments, Tujunga, CA). Body temperature was monitored using a rectal probe thermometer and maintained at  $37\text{--}38^\circ\text{C}$  with an adjustable electrical heated mattress. In addition to clinical assessment (i.e., pedal reflexes), respiratory rate, heart rate, and respired CO<sub>2</sub> and spO<sub>2</sub> were monitored continuously and anesthetic administration was adjusted appropriately. After the skin incision but before detachment of the periosteum from the neurocranium, 3 mg/kg of lidocaine solution (Alfacaine 2% plus adrenaline, Alfasan BV, Woerden, NL), 20 mg/ml of lidocaine hydrochloride, and 0.01 mg/ml of adrenaline were applied. Four small wired stainless steel screws (tip diameter 0.6 mm, impedance 300–350  $\Omega$ ; Fabory DIN 84A–A2, Borstlap BV, Tilburg, NL) were implanted epidurally over the vertex (4.5 mm caudal to bregma, 1 mm right from midline), S1 (2.5 mm caudal to bregma, 2.5 mm right from midline), and left and right frontal sinus (10 mm rostral to bregma, 1 mm lateral from midline). An electromyography (EMG) electrode was implanted in the trapezoid muscle using suture material and the EMG wire tunneled under the skin to the receptacle. All electrodes were wired to an eight-pin receptacle (Mecap Preci-Dip 917-93-108-41-005, Preci-Dip Durtal SA, Delémont, Switzerland) and fixed to the skull with dental cement (Simplex Rapid, Associated Dental Products, Ltd, Swindon, UK). The skin was closed in a single layer around the receptacle.

After the surgery, anesthesia was antagonized with 0.6 mg/kg of atipamezole (i.p.; Antisedan, Pfizer Animal Health BV), 5 mg/ml of atipamezole hydrochloride, 0.05 mg/kg of

buprenorphine (i.p.; Buprecare, AST Farma BV, Oudewater, NL), and 0.3 mg/ml of buprenorphine outside the surgery room in a separate room under red lighting. Rats were returned to their home cages once they regained purposeful locomotion.

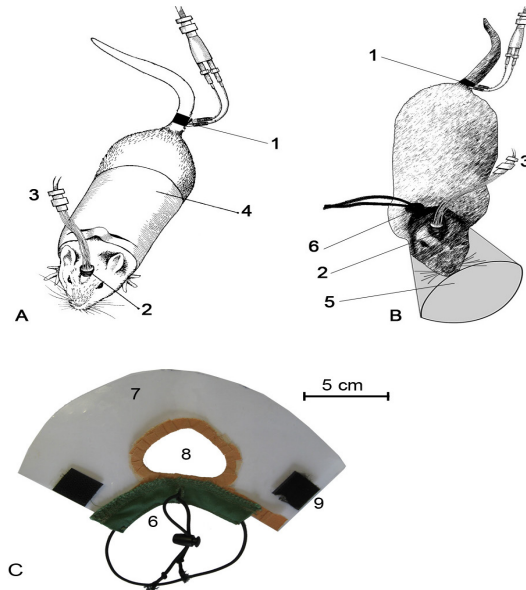
Postoperative analgesia was provided by 0.05 mg/kg of buprenorphine (s.c.) at 12-hour intervals for 3 days after surgery and 0.2 mg/kg of meloxicam (s.c.; Metacam, Boehringer Ingelheim, Alkmaar, NL, 5 mg/ml) at 24-hour intervals for 2 days after surgery. Animals recovered for at least 2 weeks (including habituation) before the start of the SEP measurements.

## Stimulation and Electrophysiological Recording Procedures

Electrical stimuli (Stienen et al. 2003, 2004, 2006; Van Oostrom et al. 2005, 2007, 2009) were administered to the epidermis of the medial part of the left tail base using a set of two bar electrodes (2 mm diameter brass) tapered toward the contact site and spaced 3 mm apart. The electrodes were fixed in a piece of plastic tube that enclosed the tail and was tightened by adhesive tape for optimal fixation. SEPs consisted of 32 square-wave pulses of 2 ms duration with a stimulus frequency of 0.5 Hz and a stimulus intensity of 3 milliamperes generated with a Grass stimulator (Model S-88, Grass Medical Instruments, Quincy, MA) and triggered by dedicated software built in-house in a Labview environment (Labview 7.2, National Instruments Netherlands BV, Woerden, NL). The stimuli were delivered to a Grass stimulation isolation unit and a constant current unit controlled the stimulus intensity. For SEP measurements, the rat's head-mounted receptacle was connected to the recording device via a swivel connector (SLC-2, Plastics One, Roanoke, VA).

For each SEP recording, the ipsilateral frontal sinus electrodes served as a reference and the contralateral frontal sinus electrodes as signal ground. For each SEP trial, 32 400-ms segments were recorded and averaged online. All signals were amplified 1 million times, band-pass filtered between 10 and 300 Hz, and digitized online at 10 kHz by data acquisition hardware (National Instruments Netherlands BV, PCI-6251). Additionally, a 50-Hz notch filter was applied to eliminate interference from the power supply system. The SEP measurements were carried out in a 40 × 28 × 30 cm Plexiglas box

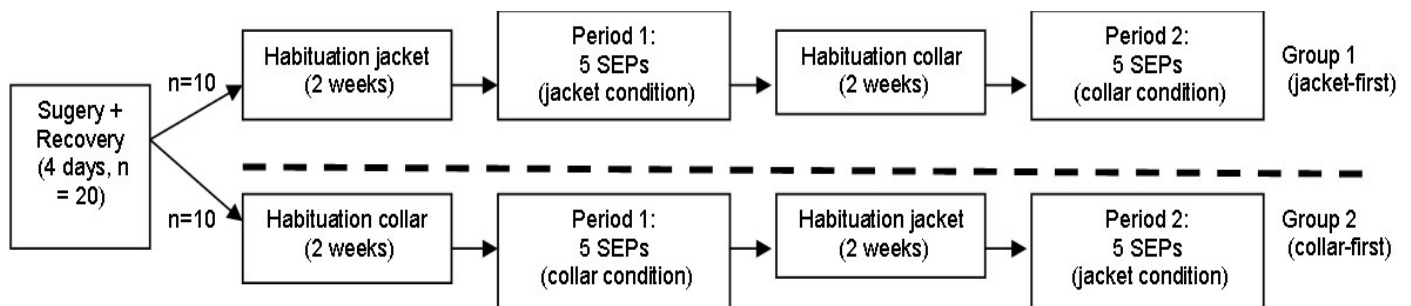
with a stainless steel electrically grounded bottom shielded by a Faraday cage. To prevent the animals from gnawing the cables, either a jacket (Stienen et al. 2003) or an Elizabethan collar was used, each developed in-house (Figure 1).



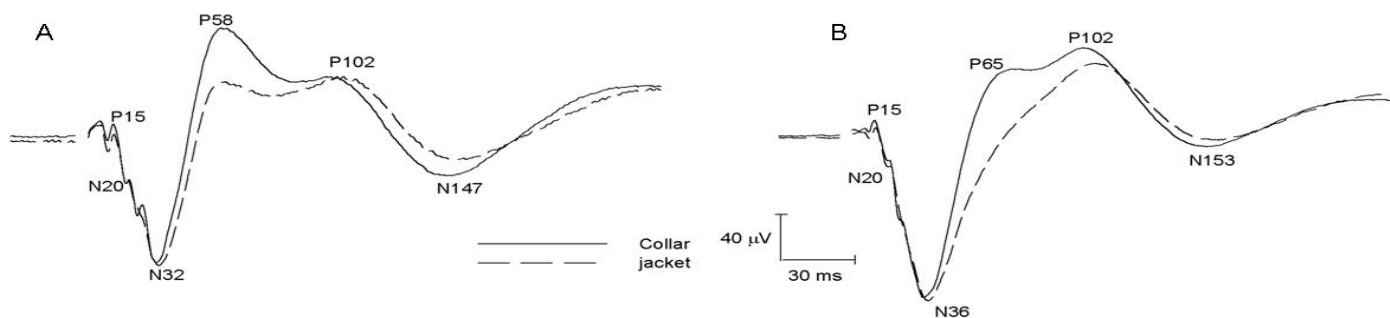
**Figure 1** Schematic representations of rat wearing jacket (A) or collar (B) during administration of SEP stimuli, and of the collar (C). (1) Tail electrode used for administering the stimuli; (2) head-mounted receptacle; (3) recording cables; (4) tight-fitting jacket; (5) Elizabethan collar; (6) elastic cord for adjusting the collar; (7) transparent overhead sheet material; (8) opening to connect the recording cables to the head-mounted receptacle; (9) Velcro tape to close the collar. Adapted from Stienen et al. (2003). SEP, somatosensory evoked potential

## Procedure

We used a two-period counterbalanced crossover design (Figure 2) and randomly assigned animals to one of two groups. Four days after surgery and before the first period of SEP measurements, animals from Group 1 were habituated to wearing the jacket for 5 minutes daily and those from Group 2 were habituated to the collar. Habituation was performed for 2 weeks in the animal housing room by the same persons carrying out the SEP measurements.



**Figure 2** Schematic overview of the procedure. See text for details. SEP, somatosensory evoked potential



**Figure 3** Grand average of the vertex (A) and primary somatosensory cortex (B) waveform per condition (collar or jacket).

The SEP measurements took place in an experimental room outside the animal housing room, to which animals were transported individually and acclimated for 30 minutes in the Plexiglas box used for the measurements. The animals were then fitted with either the jacket or collar, the electrical stimulation device was fixed at the tail base, and the head-mounted receptacle was connected to the recording device. After 15 minutes of habituation to this experimental setup, five SEPs (referred to as trials 1 to 5) were measured over 25 minutes. The animals were then returned to their home cages.

For the second period, which began 1 week after completion of the first set of trials, we reversed the groups and habituated the jacket-fitted animals to the collar and vice versa. After this second 14-day habituation period, we measured five SEPs as described above.

Because rats are nocturnal (Hrapkiewicz and Medina 2007), habituation and SEP measurements took place during the animals' active phase (lights off, 6:00 AM to 6:00 PM) under red lighting.

## Statistical Analysis

We performed calculations with Microsoft Excel 2003 and statistical analyses with SPSS 16.0;  $p$  values below 0.05 are considered significant. Using a mixed model regression, we statistically analyzed the data, obtaining the best fit by using the model with a random intercept, random slope for trial within condition, and variance components as covariance type. Fixed factors were period (period 1, period 2), condition (jacket, collar), trial (1 through 5), and their interactions. We adopted a backward strategy in which all nonsignificant interaction terms were removed. Dependent variables were the baseline corrected peak amplitudes and latencies, both of which were identified based on the grand mean. Peaks are represented as positive (P) or negative (N), with the latency of occurrence ( $\pm$  standard error of mean). The following variables were analyzed: Vx-P15 ( $\pm 1.4$ ), S1-P15 ( $\pm 0.8$ ), Vx-N20 ( $\pm 1.4$ ), S1-N20 ( $\pm 1.3$ ), Vx-N32 ( $\pm 3.0$ ), S1-N36 ( $\pm 3.1$ ), Vx-P58 ( $\pm 7.4$ ), S1-P65 ( $\pm 6.4$ ), Vx-P102 ( $\pm 6.6$ ), S1-P102 ( $\pm 6.0$ ), Vx-N147 ( $\pm 10.8$ ), and S1-N153 ( $\pm 11.9$ ). The Q-Q plots (a probability plot commonly used to check the normality assumption; e.g., Field 2009) of the residuals indicated a normal distribution for all variables.

## Missing Values

Technical problems resulted in the omission of 11 individual trials. The signals of eight trials were affected by a wet tail; in one trial there was no connection with the reference electrode (which was solved by changing the reference and ground in subsequent trials for that rat); and one rat managed to squeeze out of the jacket during measurement, resulting in two missed trials. In addition, euthanasia was necessary for two rats whose receptacle became disconnected. In total, 174 of 200 trials were analyzed.

## Results

The average waveforms per condition of the Vx and S1 are shown in Figure 3. Found effects for peak amplitudes and latencies are summarized in Tables 1 and 2, respectively, and significant effects of condition on amplitudes and latencies are shown in Figure 4. Only significant results are discussed in the text.

## Peak Amplitude

As shown in Table 1, the N20 and N32 amplitudes of the vertex increased over trials, and we identified a significant increase in period 2 for the N32 and N147 amplitudes. The P58 and N147 amplitudes were significantly higher in the collar-wearing animals than in the jacketed animals.

For the S1, the amplitudes of the N20, N36, and P102 increased significantly over trials. The N153 showed an increase in period 2, and we identified a borderline significant ( $p = 0.056$ ) increased amplitude in the collar condition. For the P65, we found a significant interaction between period and condition: simple effects showed a significant increase in P65 in the jacket condition from period 1 to 2 ( $F_{1,14.71} = 7.77, p < 0.05$ ) but no effect of period in the collar condition ( $F_{1,16.85} = 1.80, p > 0.05$ ).

## Peak Latency

For the Vx, the latencies of the N20 and N32 were significantly lower in the collar condition (Table 2), and for the P58 significantly decreased over periods. The P102 and N147 latencies

**Table 1 Significant effects of independent variables on peak baseline corrected amplitude**

Location	Period	Condition	Trial	Period * Condition
Vertex	N32: period 2 > period 1**	P58: collar > jacket**	N20: ↑ over trials*	
	N147: period 2 > period 1*	N147: collar > jacket**	N32: ↑ over trials**	
S1	N153: period 2 > period 1*	N153: collar > jacket ( $p = 0.056$ )	N20: ↑ over trials** N36: ↑ over trials* P102: ↑ over trials*	P65: (see Results)*

Factors are period (1 or 2), condition (jacket or collar), and trial (1, 2, 3, 4, 5). Period \* trial, condition \* trial, and period \* condition \* trial are not shown as no significant effects were found. \*significant at  $p \leq 0.05$ , \*\*significant at  $p < 0.01$ . F-values available upon request from corresponding author.

**Table 2 Significant effects of independent variables on peak latency**

Location	Period	Condition	Trial
Vertex	P58: period 1 > period 2*	N20: jacket > collar**	P102: ↓ over trials*
		N32: jacket > collar**	N147: ↓ over trials*
		N147: jacket > collar*	
S1	N20: period 1 > period 2**	N36: jacket > collar**	N36: ↓ over trials**
	P65: period 1 > period 2*	P102: jacket > collar**	P102: ↓ over trials** N153: ↓ over trials**

Factors are period (1 or 2), condition (jacket or collar), and trial (1, 2, 3, 4, 5). Period \* trial, condition \* trial, and period \* condition \* trial are not shown as no significant effects were found. \*significant at  $p \leq 0.05$ , \*\*significant at  $p < 0.01$ . F-values available upon request from corresponding author.

decreased over trials, and the N147 latency significantly so in the collar condition.

For the S1, the N20 and P65 latency showed a significant decrease over periods. Latencies of the N36, P102, and N153 decreased over trials, and those of the N36 and P102 were lower in the collar condition.

## Behavioral Observation

In contrast to the jacket condition, animals wearing the collar were able to move more freely and freezing behavior was clearly visible. Thus the collar enables scoring of freezing behavior, whereas this is not possible with the jacket.

## Discussion

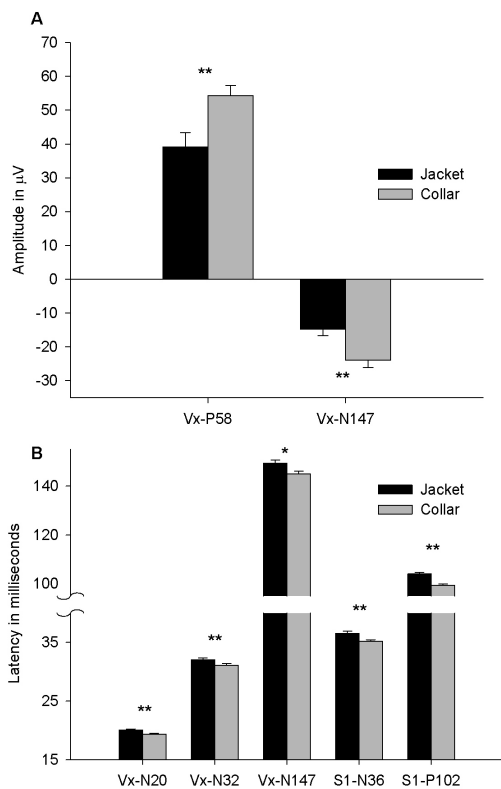
We investigated the effects of the tight-fitting jacket and the Elizabethan collar on different characteristics of the SEP in a rat model developed for measuring SEPs in the awake, freely moving rat (Stienen et al. 2003). Our primary finding is that the SEP waveform had a similar morphology in the jacket and collar condition. We therefore conclude that the neck collar

may be preferable for use in future research on the relationship between the SEP and pain perception in rats.

We found differences between the jacket and collar conditions in the latency and amplitude of some peaks. Some differences were detectable in both recording sites (Vx and S1), whereas others were detectable in only one, reflecting the involvement of different neural structures in different peaks. However, it remains unknown which specific neural structures are involved in which peaks of the SEP.

In early peaks (i.e., P15 and N20 for both the Vx and S1), which are believed to be involved in information transmission (i.e., nociception) (Chen et al. 1979; Stienen et al. 2006), we detected no difference in amplitude between the two conditions. Later peaks are believed to be involved in cognitive and emotional processing of painful stimuli (Bromm and Lorenz 1998; Chen et al. 1979), and these amplitudes (Vx-P58, Vx-N147, and S1-N153) were higher in the collar condition. Because the exact peak's latency depends on factors such as filter settings, place of measurement, and species, the Vx-N147 and S1-N153 are considered to reflect the N150.

At least one study has suggested that in rats the N150 originates in the amygdala and is related to emotional arousal (Knippenberg et al. 2009). Therefore, it can be hypothesized that the jacket and collar induce differences in emotional arousal, as the N150



**Figure 4** Effect of condition (jacket or collar) on peak (A) amplitude and (B) latency. Data are represented as mean  $\pm$  standard error of the mean (SEM). \*significant at  $p \leq 0.05$ , \*\*significant at  $p < 0.01$ . S1, primary somatosensory cortex; Vx, vertex

is increased in the collar condition. However, it is not known which emotional processes are associated with an increased N150, or indeed whether this means a more negative (i.e., more aversive) or positive valence, so it is not possible to draw conclusions about the differences in emotional processing in the jacket and collar condition based on a change in the N150. Regardless of one or more specific causes of the documented effects on the S1-N153 and Vx-N147, our finding does indicate that the N150 might be of special interest in studying the relationship between the SEP and the experience of pain in the rat.

Most of the peak latencies of the Vx (N20, N32, P58, and N147) and some of the S1 peak latencies (N36 and P102) were increased in the jacket condition. An increase in latency has been linked to anesthetic and analgesic drug effects (Banoub et al. 2003) as well as a decrease in the SEP-inducing stimulus intensity (Stienen et al. 2003). The reduced latency in the collar condition could thus reflect an altered pain experience. However, the functional meaning and underlying mechanism of this effect remain elusive.

Pharmacological studies in the awake, freely moving rat (Stienen et al. 2004, 2006; Van Oostrom et al. 2007) have used the rate dispersion factor (RDF), an expression of the overall shape of the SEP waveform in a specified latency range. The RDF is especially useful in the case of disappearing (and thus undetectable) peaks, as seen under certain drug conditions (Stienen et al. 2004). Because the RDF is affected by a combination of changes in both latency and amplitude, specific changes in amplitude or latency alone do not yield clear conclusions, thus limiting direct comparison of the results of this study (which did not use the RDF) to those of studies that did use the

RDF with the same model. Additional research is necessary to consider amplitude and latency separately in order to interpret the functional meaning of SEP latency changes in the awake, freely moving rat. However, the method of restraint (i.e., collar or jacket) should be taken into account when comparing studies as it may significantly affect peak amplitudes and latencies.

Specific SEP characteristics were affected not only by condition but also by repeated measurements. A number of peaks showed an increased amplitude (Vx-N20, Vx-N32, S1-N20, S1-N36, and S1-P102) and decreased latency (Vx-P102, Vx-N147, S1-N36, S1-P102, and S1-N153) over trials. These findings are consistent with results of an earlier study (Van Oostrom et al. 2005).

In addition to an effect over trials, which are spaced at 5-minute intervals, we found an effect of repeated measurements spaced 2 weeks from each other. The amplitude of the Vx-N32, Vx-N147, and S1-N153 increased and the latency of the Vx-P58, S1-N20, and S1-P65 decreased over this 2-week period. Thus the effects on the SEP of repeated electrical stimulation over the short or long term should be taken into account when designing and interpreting experiments with repeated SEP measurements.

Of primary concern in crossover designs are carryover effects of conditions (Díaz-Urriarte 2002; Reed 2004; Senn 2002), meaning that the condition in the first period could have an effect on the condition in the second period. Because such effects are not eliminated by counterbalancing and cannot be clearly distinguished from period effects, they may bias results. There is debate in the literature about whether carryover effects should be considered in the statistical analysis at all (Díaz-Urriarte 2002; Senn 2002), and even authors who support analysis of the effects do not generally agree about which statistical analysis should be used to properly estimate them (Reed 2004). We addressed possible carryover effects by testing the period by condition interaction. The fact that this interaction was significant in only one peak amplitude (S1-P65) and in none of the peak latencies suggests that carryover effects did not significantly affect the interpretation of the results.

## Conclusion

The use of the Elizabethan collar does not affect the general morphology of the SEP and is therefore suitable for use during SEP measurements in awake, freely moving rats. This method enables the measurement of aversive behavioral responses and provides an optimal opportunity to integrate SEP and behavior. The Elizabethan collar is also an effective tool for in-depth investigation of the specific relationship between different characteristics of the SEP and either the cognitive or the emotional processing of animal pain.

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