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# Reaction time and replenishment time of SP and CGRP after incision in rat skin

**Research Article**

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**Abstract:** Background. The skin neurogenic inflammation is mainly related to Substance P (SP) and Calcitonin Gene-related Peptide (CGRP). There is no data on their availability in the dynamics of skin nerve endings, concerning their release and replenishment after a nociceptive stimulus, so this was investigated. Materials and methods. 25 rats were randomly distributed in 5 groups. The animals of the control group (CG) determined the baseline levels of neuropeptides in the skin. The groups S0 and S30 did not receive any cutaneous stimulus at 30 and 60 minutes, respectively. In the group S1, an "incision stimulus" was made at 30 minutes. In the group S31, a nociceptive stimulus was performed by subdermal scratching at 30 minutes and, at 60 minutes, the "incision stimulus" was carried out in the same location ("nociceptive hyperstimulation"). The skin samples of the other animals were harvested from the back 1 minute after their death. SP, pro-CGRP and CGRP were quantified by Western Blotting. Results. The "incision stimulus" released SP, S1 compared to S0 ($p < 0.05$) detected in the first minute, and the replenishment time was more than 30 minutes. Also, it cleaved pro-CGRP, S1 compared to S31 ($p < 0.05$) in the first minute, and its replenishment time less than 30 minutes. Release of CGRP was not detected. Conclusion. The incision released SP already detected in the first minute; its replenishment time is more than 30 minutes. The incision decreased pro-CGRP, also detected in the first minute; and its replenishment time is less than 30 minutes.

**Keywords:** *SP • CGRP • Neurogenic Inflammation • Neuropeptides • Reaction Time • Temporal Distribution*



## 1. Introduction

The main neuropeptides related to skin neurogenic inflammation are the Substance P (SP) and the Calcitonin Gene-related Peptide (CGRP) [1-3]. The SP is a tachykinin with potent vasodilator properties because it stimulates the nitric oxide release by endothelial cells [4]. It is often found in sweat glands and in fibers and nerve endings of the dermis and epidermis. The SP plays important roles in the immune system, in cellular and humoral levels, by participating in inflammatory processes [5].

CGRP is an amino acid and the most abundant neuropeptide in the skin [6]. It is also synthesized from the posterior spinal ganglion [7], but in pro-CGRP (15 kDa) form and, after its cleavage, the CGRP is released in nerve endings, in its reactive form (5KDa) [8,9]. There are two CGRP isoforms described, α-CGRP and β-CGRP [10], although they are functionally quite similar. It is the most potent vasodilator known and does

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not involve protein extravasation [11,12]. CGRP also modulates cellular and humoral immune properties in the skin [13], and its receptors are expressed on many inflammatory cells [14], confirming its immunomodulatory potential [15,16].

Both SP and CGRP are frequently present in the same nerve fiber, and they are dependent on the Nerve Growth Factor [17]. SP induces the co-release of CGRP which, in turn, enhance the action of SP although CGRP may have long-lasting effects [18,19]. Thus, the release of these neuropeptides in the early phase of neurogenic inflammation, and its local actions, can determine the intensity of the whole inflammatory process of the wound healing [18,20,21]. The action of these neuropeptides can be ceased, by enzymatic degradation or endocytosis (internalization), by the neuropeptide-receptor complex in the postsynaptic membrane [22-24].

The neurogenic inflammation has been increasingly researched in experimental studies related to wound healing [5,25], focusing on these two neuropeptides. In the literature there is no satisfactory information about the availability of these neuropeptides in the skin. It is necessary to understand how is the dynamics of nerve ending in the release and replenishment of them after a nociceptive stimulus, such as a skin incision. Therefore, the present study aims to investigate the time required for reaction and replenishment of SP and CGRP, so that the nerve endings are functionally able to a new nociceptive stimulus in rat skin.

# 2. Materials and methods

This study is experimental, controlled, randomized, and blinded for laboratory analysis. The procedures were performed in the Experimental Surgery Laboratory, Department of Surgery, Universidade Federal de São Paulo–Unifesp, and the biochemical analysis were performed at the Neurosurgical Pathophysiology Laboratory of the Faculdade de Medicina da Universidade de São Paulo-LIM45–FMUSP.

Twenty five male Wistar EPM-1 rats aged 8 weeks, weighing between 260 and 300 grams, from the Development Center of Experimental Models for Medicine and Biology of Unifesp were used. All rats were housed in individual cages, receiving appropriate common ration and water *ad libitum.* They remained in sleep-wake cycle of 12 hours, with a constant temperature of 22°C.

This study was approved by the Research Ethics Committee of the Unifesp (number 1910/10), and was performed in accordance with the ethical standards by the Brazilian Society of Sciences in Laboratory Animals.

## 2.1 Settings

All animals were randomly distributed into five groups, undergoing general anesthesia and hair removal on the back, in a standardized way, except those belonging to the Control Group (CG). The hair removal of the CG was performed after the animals´ sacrifice and then the skin samples were harvested. In the animals of groups S0 and S30, the skin samples were harvested 30 and 60 minutes after hair removal, without another prior cutaneous stimulus. In the group S1, the animals were subjected to “incision stimulus” 30 minutes after hair removal. In the group S31, the animals were subjected to hyperstimulation of the “incision stimulus” 60 minutes after hair removal, and then the skin samples were harvested (Figure 1).

## 2.2 Experimental Procedure

All animals underwent general anesthesia by intramuscular application of xylazine hydrochloride / ketamine solution in the ratio of 1:2, respectively, and 1 ml $kg^{-1}$. Then each animal was placed in the prone position, immobilized on a board of surgery, and the hair removal on dorsal region was performed, in a rectangular region centered on the dorsal midline. The cranial limit is the transverse plane at the scapular superior angle. The caudal limit is the transverse plane in the lower portion of iliac crests, comprising an area of 12 cm x 6 cm (Figure 2). The hair removal was performed using an electric hair cutting machine (8267 Pro Basic, Wahl® brand, 60Hz, 10W).

The sacrifice of the animals (assisted painless death) belonging to CG was performed and, only then, they were submitted to hair removal; and after one minute, the skin sample was harvested up. In the other groups, hair removal was performed *in vivo* and the skin sample was harvested one minute after the sacrifice of animals, and after each procedure related.

The animals belonging to the group S0 were sacrificed 30 minutes after hair removal. The skin sample was harvested one minute later. A similar procedure was carried out in relation to the Group S30, but with a hold time of 60 minutes after hair removal. In the group S1, it was held the “incision stimulus” 30 minutes after hair removal, by surgical blade (number 15), in a straight line segment 3 cm long on the dorsal midline starting from 5 cm of the transverse line between the scapular inferior angles, in the cranial-caudal direction. The skin of interest extends until the superficial fascia (Figure 3). The animals belonging to the group S31 were submitted to hyperstimulation of “incision stimulus”, which

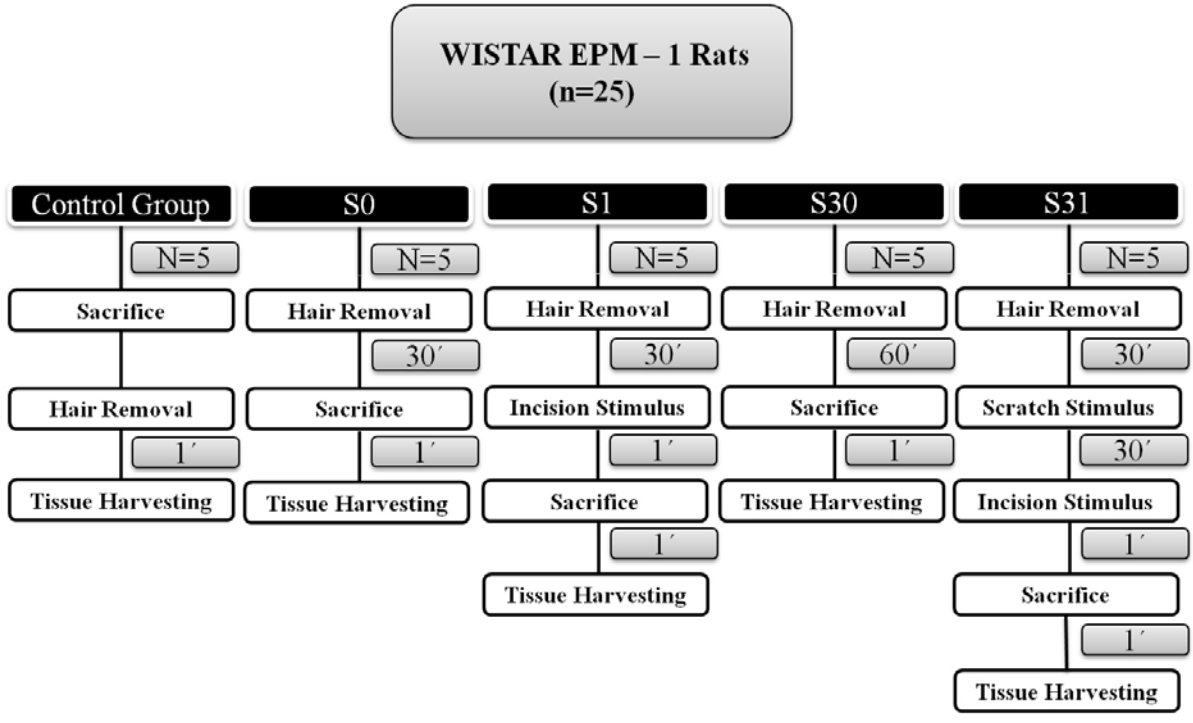


**Figure 1.** Allocation of the study groups.

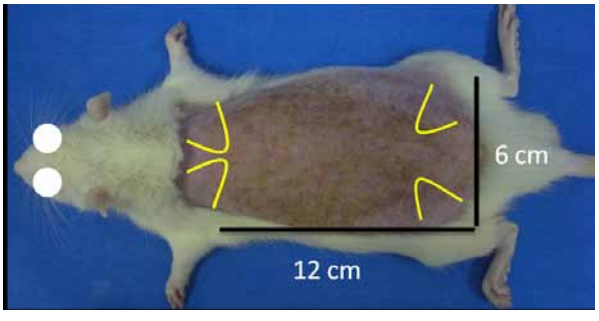


**Figure 2.** Model of rat hair removal.

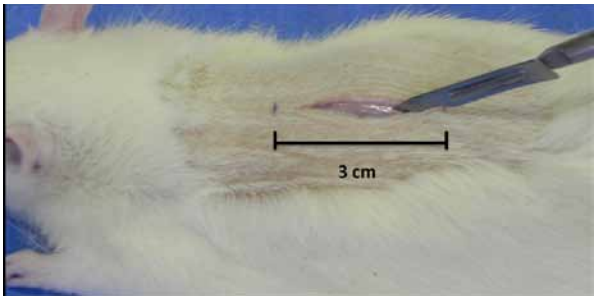


**Figure 3.** Model of "incision stimulus".

consisted of a linear scratch of the subdermal plane, in the same location of the line segment described above, 30 minutes after hair removal, and the "incision stimulus" itself after 60 minutes. The scratch was performed by the bezel of a hypodermic needle 40 x 12 mm (18G x 1½") introduced into the caudal point of the line segment, after conducting three abrasion incursions in the cranial – caudal direction, without transposing the needle through the epidermis (Figure 4).

## 2.3 Obtaining samples

After sacrifice each animal was firmly held on the caudal and cranial extremities of the trunk, arching it so as to keep the dorsal skin stretched out and exposed. Using an electric dermatome, a partial skin fragment with a thickness of 500 µm was removed, measuring 2x2 cm. To obtain the skin sample, an 8 mm punch was used at the center of this fragment. The samples were immediately placed in dry 2.0 ml Eppendorff tubes, immediately frozen on dry ice, and kept at -20°C until analysis of these neuropeptides in the obtained samples.

## 2.4 Western blot analysis

Tissues samples were individually homogenized in lyses buffer containing 1% NP40 (Sigma), 0.5% sodium deoxycholate (Sigma), 1% SDS (Sigma), 1mM $L^{-1}$ EDTA (Sigma), 1mM $L^{-1}$ EGTA (Sigma), and 1% protease inhibitor cocktail (Sigma) diluted in phosphate buffered saline (PBS, pH 7.4) and then sonicated.

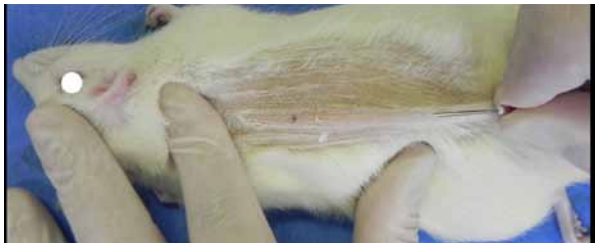

**Figure 4.** Linear scratch of the subdermal plane in the same location of the previous "incision stimulus": hyperstimulation of "incision stimulus".

The homogenate was centrifuged (14,000 rpm) for 30 min at 4°C [26,27]; supernatants were transferred into new tubes and stored at -70°C until use.

Protein concentrations were determined according to the method described by Bradford [28]. The samples (60μg of protein/lane) were separated on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide (Bio-Rad) by electrophoresis gel at 100 V for one hour.

Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad) for one hour to 100 V. After transference the membranes were blocked with 10% milk diluted in TBS-T (mixture of Tris-Buffered Saline and 0.05% Tween 20) for 15 minutes under slight agitation at room temperature. Then, all membranes were incubated with goat antibody to SP (1:100 in 3% milk/TBS-T, Sigma) or rabbit antibody to CGRP (1:300; Sigma) overnight at 4°C.

Membranes were washed twice for 10 minutes in TBS-T and incubated at room temperature for 1 hour with anti-goat or anti-rabbit conjugated secondary antiserum (1:10,000-1:2,000, respectively; Amersham Biosciences, UK). Blots were washed two times with TBS-T and once TBS. After final washes, the membrane was incubated with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Science, USA) for 1 minute. The membranes were exposed to an X-ray film for imaging (Hyperfilm™ ECL, Amersham Biosciences, USA) to visualize protein bands. After this blots were stripped, blocked and incubated with rabbit antibody to β-actin (1:30,000 in 3% milk/TBS-T, Sigma) during 1 hour at room temperature and developed as previously described.

The films were scanned (HP Scanjet G4000 series) and SP and CGRP protein levels were quantified by densitometry by means of a computer-assisted image analyzer using Image J software (version 1.43u, National Institute of Health, USA). Density normalization was done dividing the proteins by β-tubulin III density values

## 3. Results

The stimulation in 30 minutes (S1) released SP ($p < 0.05$) compared to the same period without stimulation (S0), and the replenishment time of this neuropeptide was greater than 30 minutes (Figure 5).

As for CGRP, the stimulation in 30 minutes (S1) and the hyperstimulation (S31) at 60 minutes consumed the pro-CGRP (15 kDa) ($p<0.05$), but there was no difference in the amount of this neuropeptide between the groups (graphic 2). The replenishment time of this neuropeptide was less than 30 minutes (Figure 6). The reactive form (5 kDa) had no significant difference between the groups (Figure 7).

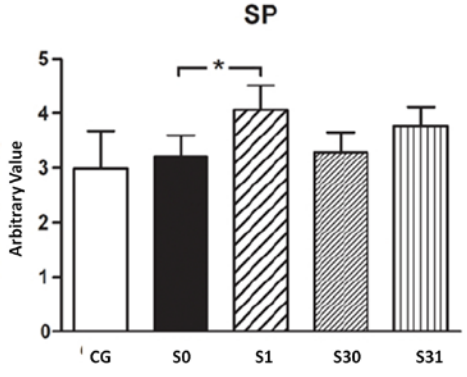


**Figure 5.** SP values found for each group.

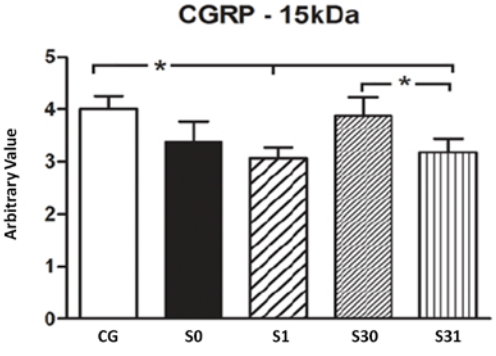


**Figure 6.** Pro-CGRP values found for each group.

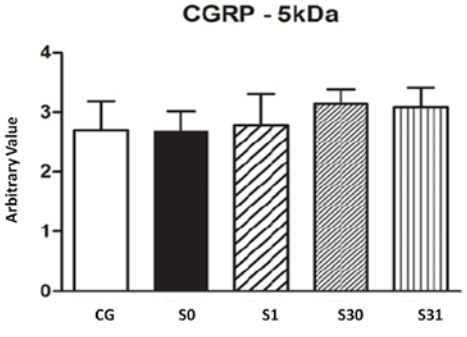


**Figure 7.** CGRP values found for each group.

## 4. Discussion

The neurogenic inflammation is triggered by different types of nociceptive stimuli on the skin by the activation of dermal-epidermal sensory nerve fibers [5,25,29]. Didactically it can be divided into neuroelectric and neuropeptidergic components. The first occurs immediately after the skin wound, with the conduction of electrical impulses from the injury site, in dromic direction, by the "current of injury" generation. The second component consists of neuropeptides release through nerve endings in the skin, in dromic direction [30,31].

Neuropeptides which are in the presynaptic terminals, within the nerve ending, are considered, *a priori*, ready to undergo exocytosis. The axonal reflex, when released, moves in dromic direction more neuropeptides to be released again, recharging the nerve fiber [23,32]. This reflex takes place in an interval in which, even the skin receiving new stimuli, probably there is no release of neuropeptide anymore until the fiber receives a minimum replenishment, adequate for a new response [33,34]. This reaction and replenishment time were the topics that guided the present study.

Of all the skin free nerve endings, especially type C fibers, about 95% are intradermal and 5% epidermal [35]. However, the largest neuropeptides stock is located in the deeper dermis, in the vascular plexus level, in the body of sweat glands, and in pilo-sebaceous units [36]. In a skin incision, as the release of neuropeptides occurs in small amounts by nerve endings, unlike neurotransmitters [25], the chosen option was to study only the most superficial epidermis and dermis, by using an electric dermatome in collecting skin samples. Thus, when the sample quantification was performed using the Western Blotting (WB) method, it would be avoided a flood of neuropeptides that were not directly involved to the nociceptive stimulus provoked, which could compromise the sensitivity of this method for this type of measurement. In this context, the WB would quantify the amount of neuropeptides contained in the whole sample, either inside the nerve endings or those already free in the synaptic cleft.

In the absence of any reference in the literature about the effect of hair removal in the release of SP and CGRP, it was decided to perform the trichotomy, on CG, after the animals death because the quantities of neuropeptides obtained would correspond to the baseline state of the skin. In the other groups, procedures, including trichotomy, were performed *in vivo*, and a period of 30 minutes was observed as safety margin. There is a hiatus in the literature regarding the release of neuropeptides by hair removal; however, it is assumed that it probably occurs, as well as nociceptive stimuli, mechanical stimuli also release them [38]. Therefore, in this study, 30 minutes were waited after hair removal because Grant *et al.* found that CGRP remains active and is not degraded in the skin for at least 30 minutes, even in the co-injection of SP [39].

The "incision stimulus" was enough to release SP from nerve endings (S1) in one minute; and because there was no difference between the S31 and S30 groups, it can be deduced that the scratch stimulus released SP, and 30 minutes was not enough time for a functional replenishment of the nerve ending so that reach the release level achieved in the animals belonging to group S1, after "incision stimulus". Thus, the replenishment time for SP was greater than 30 minutes. Thus, these data confirm that, as expected, the scratch stimulus was sufficiently to deplete SP; and 30 minutes, relatively long period, was not enough time for a functional replenishment of the nerve ending. This period of time supports the importance of SP nociceptive sensitization after an incision, and potentially participates directly in modulating the intensity of inflammatory response in peri-incisional tissue [37].

As for the pro-CGRP (15 kDa), it showed decreased amount after stimulus by an incision in one minute (S1), that is, it was cleaved to give the CGRP in its active form (5 kDa). Analogous to the S31 group for SP, the S31 group for pro-CGRP had no difference regarding the S1 group, therefore, the hyperstimulation did not cause further cleavage of this substance, and then cleavage levels are similar. Two hypotheses can be attributed to this fact: according to the first, and the most likely, analogously to SP, the stimulus with a needle also cleaved CGRP, and 30 minutes was enough time so that nerve endings become apt again to the cleavage of this neuropeptide to a new stimulus. The second hypothesis, the less likely, in the case of pro-CGRP, paradoxically to the SP, the scratch stimulus did not cleaved pro-CGRP and, in this case, it would not be possible to detect the replenishment time to pro-CGRP in nerve endings. However, conceptually [38,40], nociceptive stimuli release neuropeptides in a global way and, in the case of SP and CGRP one co-releases another in the same nerve ending. Thus, it would be really unlikely the second hypothesis. It can be inferred, therefore, that pro-CGRP is replenished to functional levels in a time period below 30 minutes (S31). On the other hand, in the present study, CGRP showed no changes in their amounts in several studied groups, remaining relatively constant basal levels. However, as the pro-CGRP was cleaved into CGRP, it would be expected an increased amount of this. In a different way of SP, the present study was not able to determine the moment of increased amounts thereof in 30 or 60 minutes.

This context is grounded in the literature since CGRP is one of the most potent vasodilators identified so far, with a particularly long duration, lasting several hours in human [41] and rodent skin [42]. Its intravascular half-life is only 7 minutes [43], suggesting that it is rapidly metabolized in the blood. However, it is unclear whether this difference is due to greater CGRP stability in the skin or to an alternative mechanism. More researches must be done in understanding the chronology of the availability of skin neuropeptides.

In conclusion, the skin stimulation with an incision released SP and that release was detected in the first minute; and the sufficient time for its replenishment so that nerve endings are functional to a new release of neuropeptide is greater than 30 minutes. The incision decreased the amount of pro-CGRP from nerve endings, also detected in the first minute, and its functional replenishment occurred in a period of less than 30 minutes.

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