HORN INNERVATION IN THE CALF: AN IMMUNOHISTOCHEMICAL STUDY

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Summary

The objective of the present study was to investigate the sensory receptors present in the horn buds of calves, using immunohistochemical techniques. Neurofilament and S-100 antibodies, which demonstrated nerve fibres and Schwann cell cytoplasm respectively, were utilized. Numerous free nerve endings known to be pain receptors were identified in the developing horn buds. In addition, bulbous corpuscles, Merkel cells and intraepidermal nerves were demonstrated. The wide range of well-developed sensory receptors identified suggest that the horn bud is a very sensitive area and that there is need for the application of local anaesthesia before disbudding calves.

Introduction

Although adult cattle are rarely dehorned without the use of local anaesthesia, the same does not apply to calves. This is despite the fact that several studies (Boandi et al., 1989; Wofit, 1994; Morisse et al., 1995; Hemsworth et al., 1995; Petie et al., 1996) have reported on the stress caused by disbudding. It is generally believed that the pain inflicted on the calf is minimal as the horn bud is poorly developed at this stage. No studies have been carried out to determine whether the sensory innervation of the horn bud is poorly developed in calves. The aim of this study was therefore to demonstrate the sensory receptors present in the horn buds of calves.

Materials and Methods

Eight Friesian calves, ranging in age from 2 days to 4 weeks were used in the present study. Developing horn bud was immersion-fixed in buffered neutral formalin for 5 days. After fixation, tissues were dehydrated through graded concentrations of ethanol and embedded in paraffin. The sections were immunostained to demonstrate neurofilament protein in nerves, and S-100 in Schwann cell cytoplasm. Recent reports have recognized S-100 anti-serum as a reliable marker for nerves and encapsulated nerve endings (Ramieri et al.1992,1995). Due to its high specificity for neurons, neurofilament anti-serum has been extensively utilized to demonstrate fine nerve fibres in both the dermis and epidermis (Iwanaga et al., 1982; Dalsgaard et al., 1984; Bjorklund et al., 1986; Yamamoto et al., 1995).
The immuno-staining technique was performed on 5µm sections. After the sections were deparaffinized, endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol for 30 minutes. The sections, which were to be used to demonstrate neurofilament protein, were microwaved at 800 watts for 20 minutes to enhance neurofilament immunoreactivity. The sections were then rinsed in water and treated with normal goat serum for 30 minutes.

Normal goat serum was used on the sections to be stained for neurofilament protein, whilst normal horse serum was used on the S-100 sections. The neurofilament sections were incubated for 3 hours with mouse anti-human neurofilament protein at a dilution of 1:100, whilst the S-100 sections were incubated with rabbit anti-bovine S-100 at a dilution of 1:1000. All incubations were carried out at room temperature. Control sections were incubated with normal mouse and normal horse sera. After this incubation the slides were rinsed with Tris(hydromethyl)aminoethyamine-buffered saline (TBS) and then incubated for 30 minutes with a biotinylated secondary antibody. The neurofilament sections were incubated with goat anti-mouse IgG (Vector, ABC-peroxidase staining kit, Elite), whilst the S-100 sections were incubated with horse anti-rabbit IgG (Vector, ABC-peroxidase staining kit, Elite). The sections were then rinsed in TBS and incubated at room temperature with horseradish peroxidase conjugated to Avidin-Biotin complex (Vector, ABC-peroxidase staining kit, Elite). All sections were then rinsed in TBS.

Bound antibody was visualized after the addition of a 0.06 mg/ml solution of 3, 3'-diaminobenzidine tetrachloride (Sigma Chemical Co.) in TBS, to which 0.03% hydrogen peroxide was added. Sections were counter-stained with haematoxylin.

Results

Neurofilament immunoreactivity was identified in nerve fibres in both the epidermis and dermis, as well as in Merkel cells in the epidermis. Intense S-100 immunoreactivity was demonstrated in Schwann cells and lamellar cells (modified Schwann cells).

Coiled (Krause) corpuscles

Coiled corpuscles were uncommon, only being observed in one of the eight calves. The unencapsulated corpuscles consisted of coiled nerve fibres associated with two to four lamellar cells, which had large elongated nuclei. The coiled nerve fibres were situated in the sub-epidermal dermis in contact with the epidermal ridges.

Bulbous (Golgi-Mazzoni) corpuscles

Bulbous corpuscles were the most frequently encountered sensory corpuscles. Two types of bulbous corpuscles were seen in the calves. It was unclear whether they were variants, or different stages of development of a single
bulbous corpuscle. In all the calves studied small corpuscles were generally located in the papillary dermis, whilst larger corpuscles were found in the reticular dermis.

The larger bulbous corpuscles were seen to consist of a thick central axon, a layer of presumptive lamellar cells and a capsule. The axon, which had thin and thick regions, ended in a brush-like terminal (Figure 1). In sections stained for S-100, an unstained region was seen in the central part of the corpuscle (Figure 2).

![Figure 1: A large bulbous corpuscle in the reticular dermis of a 2-day-old calf.](image)

The central neurofilament immunoreactive axon has several constrictions along its length (arrowheads). A faintly immunoreactive area (s) separates the axon from a layer of lamellar cells with elongated nuclei. A capsule (arrow) separates the corpuscle from the surrounding connective tissue. Anti-neurofilament. X 1,875.

Free nerve endings

In all the calves studied, branches from nerves which coursed between the hair follicles, extended towards the papillary dermis, where they divided into two or three preterminal nerves (Figure 3). These nerves were enclosed in cytoplasmic processes from Schwann cells. One to two of the nerve fibres extended close to the epidermis, where they divided into numerous free nerve endings which were unaccompanied by perineural cells. These free nerve endings formed intensely neurofilament-immunoreactive, club-shaped terminals which contacted groups of Merkel cells located in the stratum basale (Figure 4).

Merkel cells

Merkel cells were characterized by the presence of large irregular-shaped nuclei, which were paler than the nuclei of neighbouring keratinocytes. The Merkel
Figure 2: Transverse section through a bulbous corpuscle of a 1-week-old calf. S-100 immunoreactive inner core (I) encloses an elongated clear area (a) which is presumably the location of the axon. The inner core is formed by a mass of cytoplasmic processes from lamellar cells. A clear area (s) separates the inner core from the capsule (arrowhead). On the right is part of the preterminal axon (p) which is not enclosed in inner core lamellae. Anti-S-100. ×1,875.

Figure 3: Afferent nerves (A) divide extensively to form a terminal tree of free nerve endings, which supply an epidermal ridge, R in a 3-week-old calf. Several free nerve endings extend into adjacent dermal papillae (D). Anti-S-100. ×750.
cell nucleus was surrounded by faintly neurofilament-immunoreactive cytoplasm. Groups of four to five Merkel cells were observed in the stratum basale of epidermal pegs. Knob-like nerve terminals contacted the basal regions of these cells.

**Intraepidermal nerves**

Free nerve endings wrapped in Schwann cell cytoplasm were seen penetrating the epidermis at the tips of the dermal papillae. In anti-S-100 stained sections some of the Schwann cell processes did not extend further than the stratum basale, whereas in others the Schwann cell processes extended as far as the middle layers of the stratum spinosum, where they terminated in hook-like structures or simple tapered ends. Neurofilament-immunoreactive nerves were seen coursing as far as the stratum granulosum where they ended in plain or beaded terminals.

**Discussion**

Neurofilament and S-100 antibodies were utilized in this study to identify nerve fibres and Schwann or lamellar cell cytoplasm respectively. This enabled us to trace the course of intraepidermal nerves, as well as appreciate the inner lamellar structure of bulbous corpuscles.
A major feature of this immunohistochemical investigation was the demonstration for the first time, of the presence of sensory receptors in the horn bud of calves. This revealed that the developing horn bud in calves had numerous free nerve endings, which are generally accepted as being nociceptors. This finding emphasizes the sensitivity of the horn bud to the tissue damage that takes place during disbudding. This is supported by observations that calves disbudded without anaesthesia exhibit signs of pain such as struggling and vocalization (Hemsworth et al., 1995; Bengtsson et al., 1996). In addition, it has been shown that disbudding is associated with stress, which is most likely caused, in part, by pain inflicted on the animal (Bocandl et al., 1989; Wohlt et al., 1994; Morisse et al., 1995; Petrie et al., 1996).

Apart from the observation of free nerve endings, it was interesting to note the presence of well-developed sensory receptors in the horn bud of even the youngest animal. It was noted that bulbous corpuscles were the commonest types of corpuscular receptor encountered. Using the immunohistochemical technique the structural features of the bulbous corpuscles were clearly visible. A prominent feature of the bulbous corpuscles was the presence of an inner lamellar core, which was demonstrated using an antibody against the S-100 protein, located in the lamellar cytoplasm. Bulbous corpuscles stained in this manner were seen to have a central unstained region, which was thought to indicate the location of an axon. This central location of the axon was confirmed by using an antibody against the neurofilament protein known to be present in nerve fibres. Similar corpuscles have been observed in the skin of the mouse, rat, rabbit, cat (Cunningham and Fitzgerald, 1972) and horse (Talukdar et al., 1970).

Merkel cells and intraepidermal nerves were identified as the neural components in the epidermis. The immunohistochemical technique, using neurofilament antibodies, was able to demonstrate both the Merkel cells and intraepidermal nerves. In addition, the neurofilament antibodies labeled the expanded nerves, which terminated on the Merkel cells. This corresponds with findings by Pasche et al. (1990) who estimated that between 50 and 95% of Merkel cells in mouse epidermis were associated with expanded nerve endings.

The wide range of well-developed sensory receptors identified suggests that the horn bud is a very sensitive area. These findings emphasize the need for local anaesthesia before disbudding calves.

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References