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The superior colliculus of the camel: a neuronal-specific nuclear protein (NeuN) and neuropeptide study

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Abstract

In this study we examined the superior colliculus of the midbrain of the one-humped (dromedary) camel, *Camelus dromedarius*, using Nissl staining and anti-neuronal-specific nuclear protein (NeuN) immunohistochemistry for total neuronal population as well as for the enkephalins, somatostatin (SOM) and substance P (SP). It was found that, unlike in most mammals, the superior colliculus is much larger than the inferior colliculus. The superior colliculus is concerned with visual reflexes and the co-ordination of head, neck and eye movements, which are certainly of importance to this animal with large eyes, head and neck, and apparently good vision. The basic neuronal architecture and lamination of the superior colliculus are similar to that in other mammals. However, we describe for the first time an unusually large content of neurons in the superior colliculus with strong immunoreactivity for met-enkephalin, an endogenous opioid. We classified the majority of these neurons as small (perimeters of 40–50 μm), and localized diffusely throughout the superficial grey and stratum opticum. In addition, large pyramidal-like neurons with perimeters of 100 μm and above were present in the intermediate grey layer. Large unipolar cells were located immediately dorsal to the deep grey layer. By contrast, small neurons (perimeters of 40–50 μm) immunopositive to SOM and SP were located exclusively in the superficial grey layer. We propose that this system may be associated with a pain-inhibiting pathway that has been described from the periaqueductal grey matter, juxtaposing the deep layers of the superior colliculus, to the lower brainstem and spinal cord. Such pain inhibition could be important in relation to the camel's life in the harsh environment of its native deserts, often living in very high temperatures with no shade and a diet consisting largely of thorny branches.

Key words dromedary; enkephalin; midbrain; nociception; periaqueductal grey.

Introduction

The mammalian superior colliculi form laminated eminences on the rostral half of the tectum of the midbrain. Although studies have established the architecture of the mammalian superior colliculus in different species, notably the rat (Altman & Carpenter, 1961; Brodal, 1972; Harting, 1977; Graybiel, 1978), rabbit (Barker & Dreher, 1998; Gonzalez-Soriano et al. 2000; McHaffie et al. 2001), hamster (Kang et al. 2002), ferret (Behan et al. 2002; Cirone et al. 2002), cat (Garey & Powell, 1968; Garey et al. 1968; Edwards et al. 1979; Mize, 1999; Krauzlis, 2001; McHaffie et al. 2001; Cirone et al. 2002), monkey (Krauzlis, 2001; McHaffie et al. 2001; Soares et al. 2001; Soares-Mota et al. 2001) and humans (Leuba & Saini, 1997), there is a paucity of information on such large mammals as the camel. The camel is held in Arabian folklore to possess exceptional visual capabilities and this is supported by scientific evidence (Harman et al. 2001). By macroscopic examination of the camel brain we found it to possess a superior colliculus several times the volume of the inferior colliculus, whereas in most species, including humans, the two are of approximately the same size. This could be significant because dolphins (order Cetacea), closely related to the order Artiodactyla, to which camels belong (http://www.ezresult.com/article/Ungulate; Luo,

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2000), are known to have an elaborate auditory system and possess an inferior colliculus that is much larger than the superior. The superficial layers of the superior colliculus receive afferents from the retina and the visual cortex. Portions of the visual cortex and superior colliculus receiving input from particular regions of the retina are interconnected, and related cortical and retinal projections coincide in the superior colliculus (Garey, 1965; Garey & Powell, 1968; Garey et al. 1968).

The mammalian superior colliculus is involved in processing sensory information from other modalities than just visual, including feedback from the auditory, somatosensory and motor regions of the cerebral cortex to its deeper layers (Garey et al. 1968). This is consistent with the superior colliculus being an important integrative centre for the control of orientation of head and eye (Wurtz & Albano, 1980).

The superior colliculus is composed of six layers of alternating grey (largely composed of neuronal cell bodies) and white matter (containing mainly nerve fibres). From the surface, the following layers can be recognized: stratum zonale, superficial grey, stratum opticum (in which retinal fibres enter the superior colliculus), then intermediate grey and white followed by deep grey and white layers (Kanaseki & Sprague, 1974). This cytoarchitecture of the superior colliculus has been established using simple methods such as Nissl staining, but which has the disadvantage that it is unable to distinguish neurons, especially small interneurons, from the numerous supporting glial cells present throughout the central nervous system, hampering demarcation between neuronal layers. More recently, neurons have been demonstrated using an antibody to a neuronal marker that does not stain glia, neuronal-specific nuclear protein (NeuN) (Mullen et al. 1992).

The first objective of this study was therefore to establish the neuronal lamination as well as the number, sizes and types of neurons in the superior colliculus using NeuN immunohistochemistry and Nissl staining. The latter technique has the advantage of displaying the cytoplasmic structure, whereas NeuN immunohistochemistry preferentially stains neuronal nuclei.

Several neuropeptides have been implicated in the functions of the superior colliculus. Enkephalins, the endogenous ligands for opiate receptors, are naturally occurring neuropeptides that have been implicated in reinforcement of the analgesic effects of opiates. They do this by suppressing central neural pathways of pain via mu opioid receptors found in the periaqueductal grey of the midbrain (PAG), an area associated with the analgesic effect of opiates (Basbaum & Fields, 1984; Graybiel et al. 1984; Mize, 1989; Miguel-Hidalgo et al. 1990; Okamoto et al. 1990; Berson et al. 1991; Humphrey et al. 1995). In addition to suppression of central neural pain pathways, opiates are known to be involved in the control of visual reflexes including saccadic eye movements. Studies in rats (Okamoto et al. 1990; Miguel-Hidalgo et al. 1990) and cats (Mize, 1989; Berson et al. 1991) have demonstrated enkephalins in a minor population of small neurons located almost exclusively in the superficial layers with a few patches of positive fibres in deeper layers. In the light of the adaptation of the camel to its harsh desert environment, we hypothesized that the role of an analgesic system would be advantageous for survival. We have therefore examined the distribution of leucine- and methionine-enkephalin (leu- and met-enk) in the camel superior colliculus by immunohistochemistry. We have also examined the distribution of substance P (SP) and somatostatin (SOM), both of which have been shown to be present in the superficial layers of the superior colliculus (Spangler & Morley, 1987; Ogawa-Meguro et al. 1992; Harvey et al. 2001).

A preliminary version of this paper was presented to the Anatomical Society in 2004 (Mensah-Brown & Garey, 2004).

Materials and methods

The brains of 12 adult male camels aged between 2 and 4 years were obtained from a commercial abattoir in Al Ain, United Arab Emirates, and were fixed by immersion in 4% paraformaldehyde within 15 min of death. Initial fixation continued overnight at 4 °C. Blocks containing the superior colliculus were removed from the midbrain and fixed for a further 24 h. Fixed specimens were subsequently kept in 30% or 15% sucrose in 0.1 M phosphate-buffered saline (PBS) until cryostat sectioning.

Nissl staining

Blocks from superior colliculi stored in sucrose in PBS were slowly freeze-embedded using cryomatrix mountant gel (Shandon, Pittsburgh, PA, USA) on the cryobar of a motorized cryostat (Shandon, Cheshire, UK). Coronal sections 15 μm thick were then cut and cold mounted on gelatin-coated slides, and stained with 1% toluidine blue.

JB4 embedding

To investigate further the profile of cells identified as neurons of the mesencephalic trigeminal nucleus, other blocks were cut into 300-μm sagittal sections with a vibratome and each section further divided into two equal pieces in the coronal plane. The posterior pieces were routinely dehydrated and transferred to JB4 infiltration fluid for 2 × 90 min, then overnight at 4 °C. The infiltration fluid comprised a mixture of 1.25% benzoyl peroxide (catalyst) in JB4 solution A (monomer) (Polysciences, Warrington, PA, USA). The specimens were then embedded in a mixture of 25 mL freshly made infiltration fluid and 1 mL JB4 solution B (accelerator) at room temperature under vacuum in polyethylene moulding trays. Sections 2 μm thick were cut from each block, mounted on gelatin-coated slides, air dried and stained with methylene blue basic fuchsin.

Immunohistochemistry

Other blocks were freeze-embedded as described above. Cryostat sections 50–60 μm thick were cut and placed immediately in 50% ethanol to aid penetration. The free floating sections were washed in three 5-min changes of 0.1 M PBS (pH 7.4). They were then incubated in 3% hydrogen peroxide in absolute methanol for 30 min to block endogenous peroxidase. They were again washed in three 5-min changes of 0.1 M PBS and subsequently incubated at room temperature with antisera to mouse NeuN (Chemicon, Temecula, CA, USA) diluted 1 : 10 000, and rabbit leu-enk and met-enk, SP and SOM (Peninsula Laboratories, USA) all diluted 1 : 40 000 in 0.3% triton X in 0.1 M PBS. The sections were washed for 3 × 5 min and then incubated with the link antibody comprising biotinylated anti-rabbit IgG for the neuropeptides and anti-mouse IgG for NeuN (Jackson ImmunoResearch Laboratories, USA) diluted 1 : 500 in 0.3% triton X in 0.1 M PBS for 1 h and then peroxidase-labelled extravidin (Sigma, St Louis, MO, USA) diluted 1 : 1000 in 0.3% triton X in 0.1 M PBS for 1 h, the sections being washed 3 × 5 min in 0.1 M PBS buffer between the link antibody and the extravidin. The sections were then washed in two 5-min changes of 0.1 M PBS and one 5-min change in 0.1 M PB. Peroxidase activity was demonstrated with diaminobenzidene. One microlitre of diaminobenzidene hydrochloride in phosphate buffer (PB) was diluted to 50 mL with 1 mL of 3.5% nickel chloride, 7.5 μL 30% hydrogen peroxide and distilled water. The mixture was filtered if the final solution appeared cloudy. The sections were incubated in the diaminobenzidene for 3–5 min in a hooded incubator. The reaction was stopped with 0.1 M PB and the sections mounted on gelatin-coated slides. After air drying, they were dehydrated in ethanol up to 100%, cleared in xylene and coverslipped using Cytoseal 60 mounting medium (Stephens Scientific, Riversdale, USA).

For each primary antibody used, control experiments were performed with substitution of primary antibody by 0.1 M PBS and substitution of primary antiserum by antiserum (with the exception of NeuN) adsorbed with the purified antigen 10^{-6} M of sera of the various antibodies. Anti-met-enk was also adsorbed to leu-enk peptide to exclude cross-reactivity between anti-leu- and anti-met-enk. Peptides were obtained from the same source as the antisera.

Sections were examined using a Zeiss Axiophot photomicroscope, and micrographs taken using an AxioCam HRC digital camera with AxioVision 3.1 software to capture images (Carl Zeiss, Jena, Germany).

Quantification

Images of toluidine-blue-stained sections were captured with the AxioVision 3.1 software and the perimeters of neurons whose nuclei were clearly visible (20 per layer in four camels, using every third section) were determined.

Similarly, the perimeters of all neurons in four consecutive sagittal sections immunostained for SOM, SP, leu-enk and met-enk from three camels were measured. The total numbers of neurons measured were 368 for SP, 337 for SOM and 178 for leu-enk. For met-enk, 25, 204, 209 and 134 neurons were measured in the stratum opticum and the superficial, intermediate and deep grey layers, respectively.

Data were analysed using one-way ANOVA and *P*-values < 0.05 were considered significant.

Results

Immunolabelling was absent in all the control experiments. Pre-adsorption of antiserum with antigen completely abolished staining. Leu-enk and met-enk peptides did not cross-react, so leu-enk did not abolish met-enk immunoreactivity and vice versa. Immunoreactivity to the various antisera was detectable in neurons and fibres and varicose axon terminals.

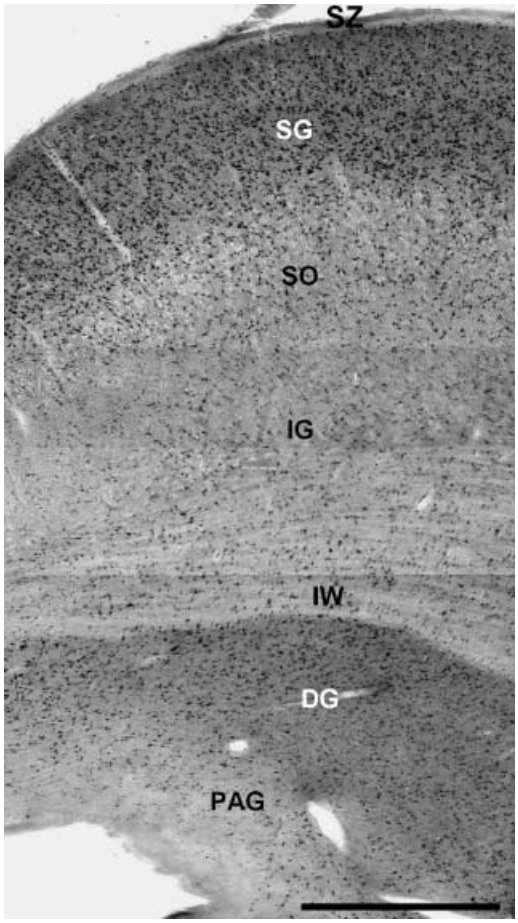

Fig. 1 Low-power micrograph of the laminae of the superior colliculus of the camel showing the stratum zonale (SZ), superficial grey layer (SG), stratum opticum (SO), intermediate grey (IG), intermediate white (IW), deep grey (DG) as well as the periaqueductal grey (PAG). Scale bar = 500 μm.

NeuN immunohistochemistry and Nissl staining revealed distinct lamination of the superior colliculus, comprising alternating layers of neurons and fibres, but the distinction between the deep grey layer and the PAG was less obvious than in most mammals studied so far. The PAG, indeed, appeared relatively small (Fig. 1). Using Nissl-stained sections, we subdivided the neurons into three groups, namely small (perimeters of 10–50 μm), medium (50–100 μm) and large (> 100 μm), to aid description. Small neurons predominated in all layers. However, a number of large cells in the intermediate grey made the mean size of neurons there significantly higher than in other layers ($P < 0.005$) (Figs 2a–c and 3a–f; Table 1). Large unipolar neurons were observed at the dorsal border of the deep grey layer but were not included for quantification as they were considered as sufficiently different from other neurons to warrant separate consideration. JB4-embedded sections demonstrated thick fibres between the intermediate and deep layers emanating from these unipolar neurons (Fig. 4a–c).

Table 1 Perimeters of Nissl-stained neurons in the laminae of the superior colliculus of the camel

Lamina	Neuronal size (μm)
SZ	35.12 ± 0.52 (range 16.28–119.1)
SG	38.8 ± 0.76 (range 15.5–77.6)
SO	40.03 ± 1.21 (range 19.97–86.54)
IG	55.8 ± 4.72 (range 45.92–241.9)*
DG	40.34 ± 3.72 (range 34.3–87.54)
ME5	111.48 ± 6.68 (range 74.78–251.27)

*$P < 0.008$ compared with SZ value.
Abbreviations: S = stratum zonale; SG = superficial grey; SO = stratum opticum; IG = intermediate grey; DG = deep grey; ME5 = trigeminal mesencephalic nucleus; SZ = stratum zonale.

Immunohistochemistry of leu-enk, met-enk, SOM and SP

Immunoreactivity to leu-enk, SOM and SP was essentially restricted to the upper third of the superficial grey layer where they were discernible predominantly in small to medium neurons (Fig. 5a–d). Neurons immunoreactive to SOM and SP were present throughout the superficial grey, sometimes extending into the superficial portion of the stratum opticum (Fig. 7e–h). SOM neurons possessed predominantly ovoid nuclei and dendrites extending over a wide field (Fig. 7i). SP neurons had a variety of dendritic fields, including horizontal and vertical orientations, with wide and narrow dendritic fields observed with equal frequency. Some of these neurons were pyramidal in shape. Met-enk immunoreactivity was detectable in all layers. In the superficial grey, and extending into the stratum opticum, mainly small and medium met-enk cells with oval nuclei but poorly discernible dendrites were discernible. Neurons with narrow dendritic fields, some of which formed a double 'Y' (giving an overall X shape) were commonly visible in the stratum opticum. Pyramidal cells with wide dendritic field were also discernible (Figs 6a–d and 7a–d). Met-enk immunoreactivity was also observed in large multipolar pyramidal-like cells located mainly in the intermediate grey and white layers. Although these neurons were present in all regions, most were detected in the lateral region of the intermediate white layer (Fig. 8a,b). Lying at the dorsal

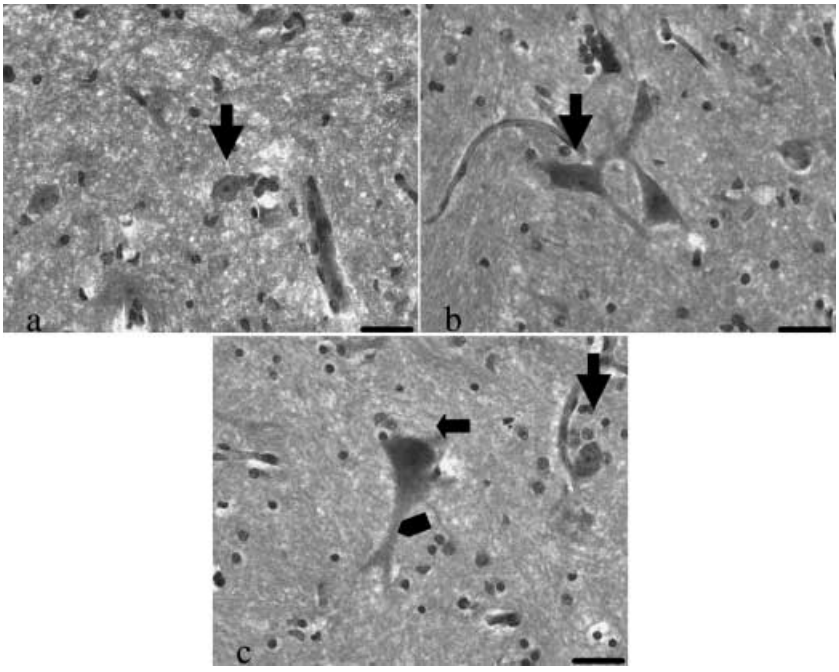

Fig. 2 Micrograph of Nissl-stained sections of the superior colliculus showing small and medium neuronal somata in the superficial (a,b) and deep (c) grey layers and a large neuron with a bifurcating process in the deep layer. Scale bar = 20 μm.

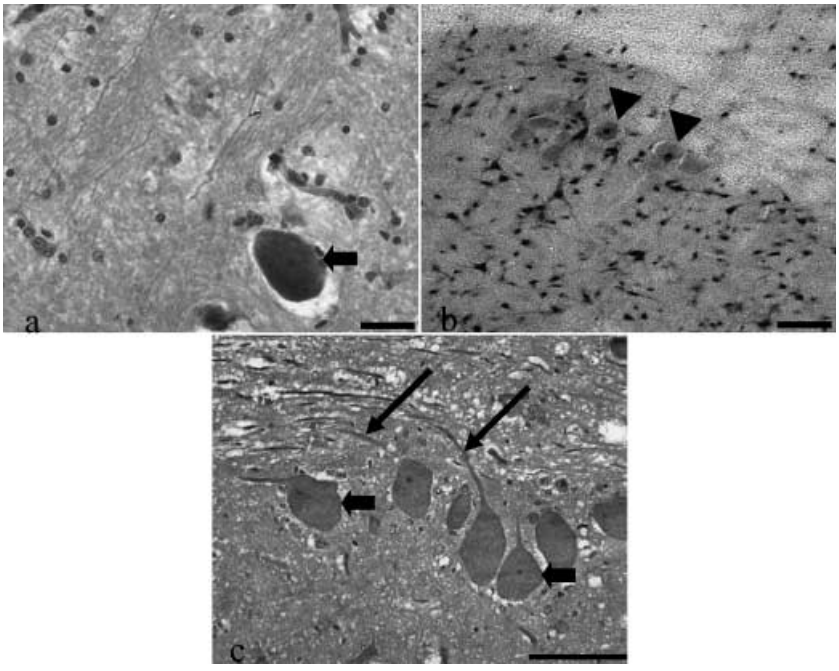

Fig. 3 Micrographs of Nissl-stained (a) (block arrow), NeuN-immunostained (b, arrowheads) and JB4-embedded (c) sections showing neurons of the mesencephalic trigeminal nucleus in the dorsal part of the deep grey. Note the fibres emanating from neurons and extending between the intermediate white and deep grey (c). Scale bars: a = 20 μm; b,c = 100 μm.

border of the deep grey and often extending into the zone of thick met-enk immunoreactive fibres were groups of large neurons that were mainly unipolar in profile, although some multipolar neurons were also observed. These neurons were identified as comprising the mesencephalic trigeminal nucleus (Fig. 9a–d). The deep layer was indistinguishable from the PAG and contained few small-sized met-enk neurons and several varicose profiles that we interpreted as axon terminals (Fig. 11a).

Nerve fibres

For all the neuropeptides, the superficial layer had a strongly immunoreactive neuropil, but while projections of neurons immunoreactive to leu-enk, SOM and

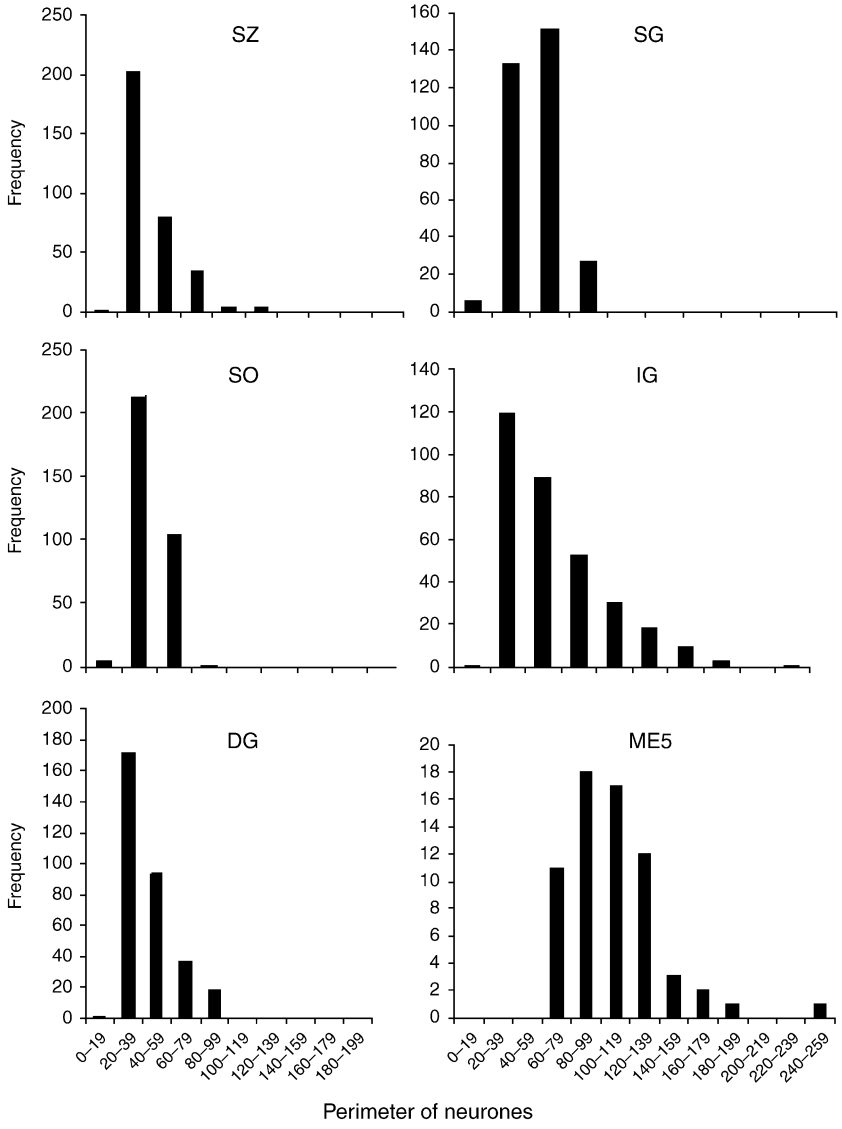

Fig. 4 Frequency graphs of perimeters of neurons in the different layers of the camel superior colliculus.

SP appeared limited to the layers in which their cell bodies were found, thick met-enk immunoreactive fibres were observed between the intermediate and deep grey layers (Fig. 10a). These fibres appeared similar to those emanating from the mesencephalic trigeminal nucleus (Fig. 3c). In addition, bridging met-enk fibres were observed between the superficial and deep layers with some extending into the PAG (Fig. 10b). Although the PAG was immunoreactive to all neuropeptides studied, met-enk and SP were remarkable for their intensity (Fig. 11a–d).

Discussion

This study has demonstrated that the superior colliculus of the camel (dromedary) is a laminated structure similar architecturally to the superior colliculus of other mammals, but with notable differences. The study also demonstrates for the first time the lamination of the superior colliculus with NeuN immunohistochemistry and confirms the advantage of the technique over the usual Nissl stain in that neurons are reliably distinguishable from glia. Its usefulness in determining numerical density of neurons is obvious. NeuN immunohistochemistry is, however, disadvantaged by the fact that it cannot be used to determine the size of cells as it does not stain cytoplasm. We have found no studies on laminar numerical density of cells in the superior colliculus in the literature and therefore we are unable to state definitely whether there are any quantitative differences between the camel and other mammals, which might explain the remarkable size of the camel superior

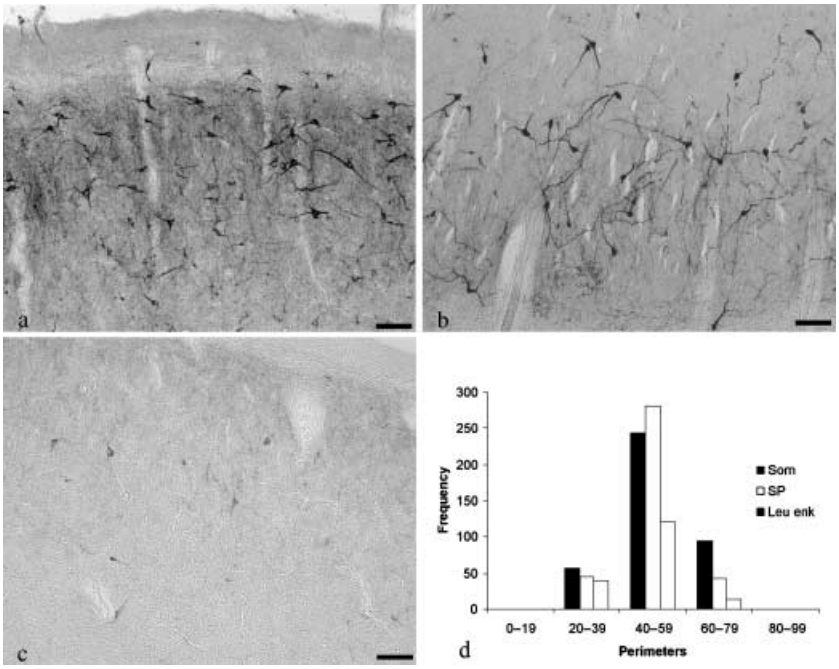

Fig. 5 Micrograph showing substance P (a), somatostatin (b) and leucine enkephalin (c). The neurons are restricted mainly to the upper portions of the superficial grey. Scale bar = 100 μm. (c) Perimeters of immunoreactive neurons.

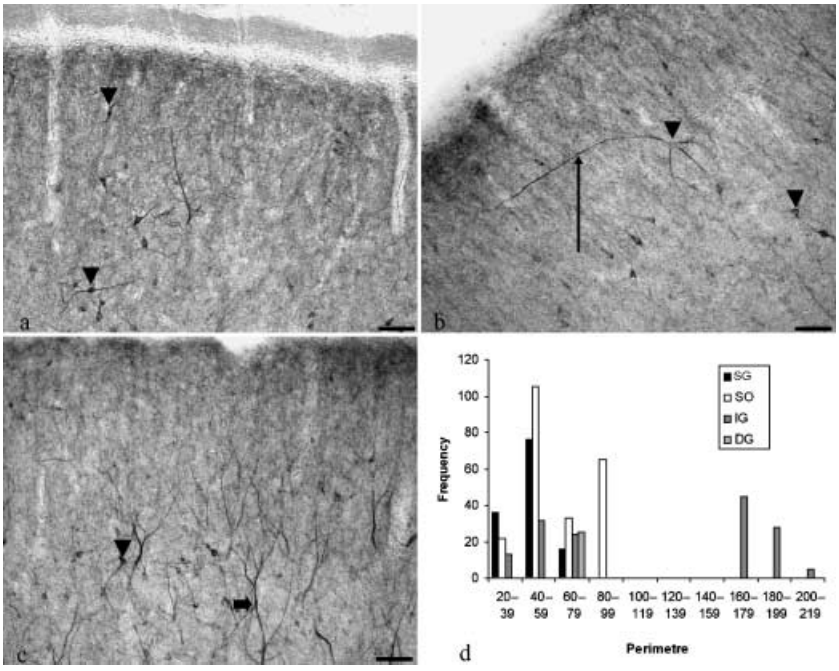

Fig. 6 Micrograph showing small met-enk immunoreactive multipolar neurons (arrows) in the deep portion of the superficial grey (a,b) and medium neurons in the stratum opticum (c). Note the long dendrite (b, arrow) extending toward the surface and also the X-shaped neurons of the stratum opticum (c). (d) Perimeters of met-enk neurons. Scale bar = 100 μm.

colliculus compared with the inferior colliculus. Interestingly, the camel also possesses a relatively smaller PAG than in most mammals.

This size difference could be important on functional grounds due to a well-developed visual system and also due to the necessity for the camel to co-ordinate eye and head movements accentuated by its long, muscular neck. The large size of the superficial portion of the colliculus and the fact that the commonest dendritic morphology of neurons immunoreactive to the various peptides was of the narrow field variety both support the concept of a well-developed visual system in the camel. Hilbig et al. (2000) suggested that narrow field neurons are visual relay cells, whereas the wide field neurons may represent relay cells involved not only in vision. Our observation of large, predominantly unipolar neurons identified as in the mesencephalic trigeminal neurons and extending to the caudal portion of the

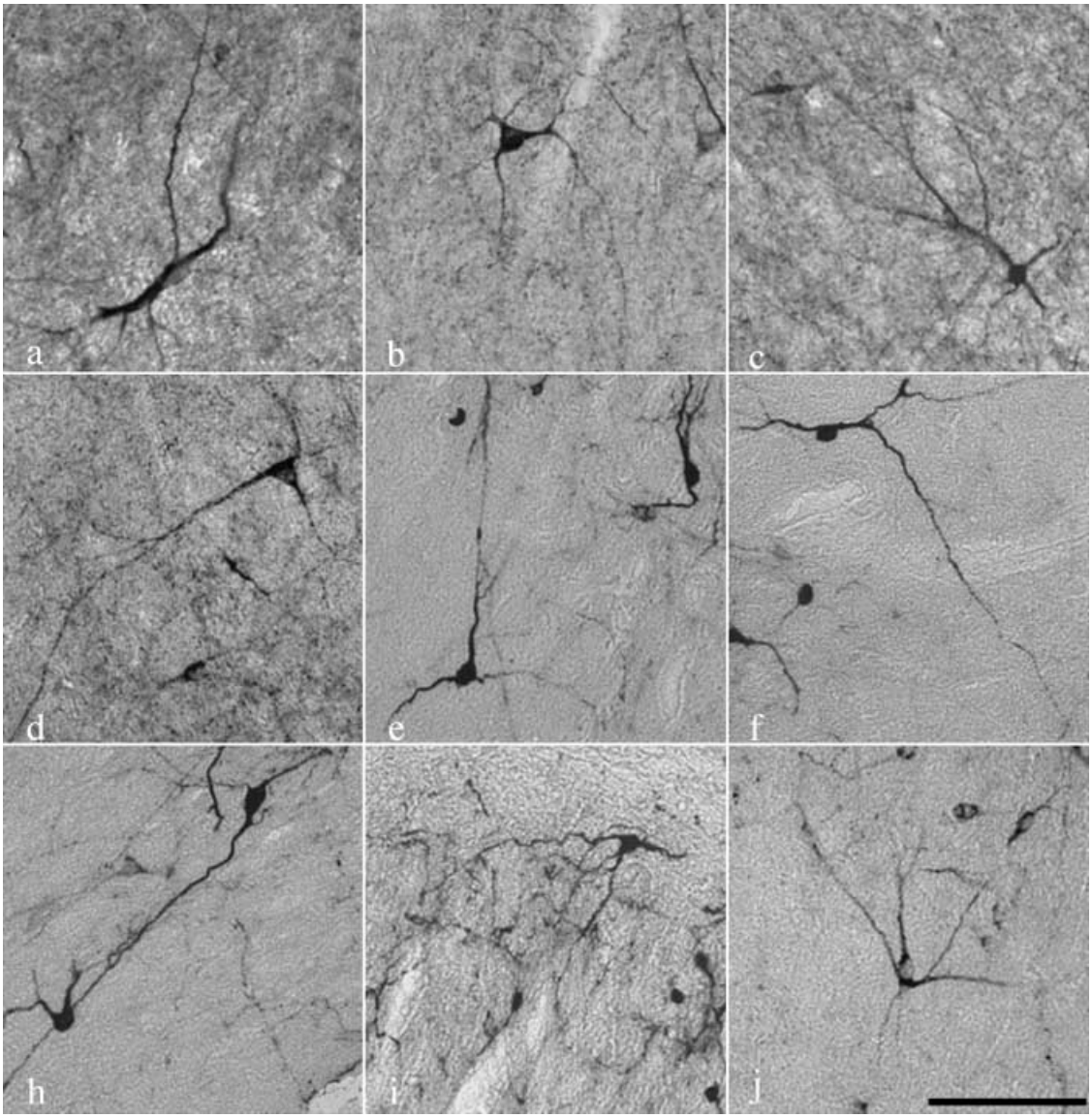

Fig. 7 Micrograph showing different types of dendritic morphologies of met-enk (a–d), SP (e–h) and SOM (i) neurons in the superficial grey and stratum opticum (a,b,c). Scale bar = 100 μm.

superior colliculus agrees with what has been described in many species (Walberg, 1984). These neurons have been associated with proprioception of the head and neck region. Ndiaye et al. (2000) described the reciprocal connections between the mesencephalic nucleus and the superior colliculus and suggested these might influence the co-ordination and stabilization of head posture and gaze, even possibly during chewing. Our observation of met-enk immunoreactivity in these large neurons may implicate the neuropeptide in this function.

Several investigators have demonstrated the presence of enkephalins in the superior colliculus (Mize, 1989; Berson et al. 1991), mainly in a minor population of small neurons located almost exclusively in the superficial layers with a few patches of positive fibres in deeper layers. Enkephalins (Mize, 1989; Berson et al. 1991) and the neuropeptides SP (Ogawa-Meguro et al. 1992) and SOM (Spangler & Morley, 1987; Harvey et al. 2001) may modulate transmission of signals from the retina, and thus affect eye movements and other visual reflexes controlled by the superior colliculus. This is consistent with our observation of small enkephalin, SP and SOM neurons in the superficial visual layers. However, in the camel a large population of met-enk neurons is found in the intermediate grey and white layers. The distribution of these met-enk neurons in the lateral regions of

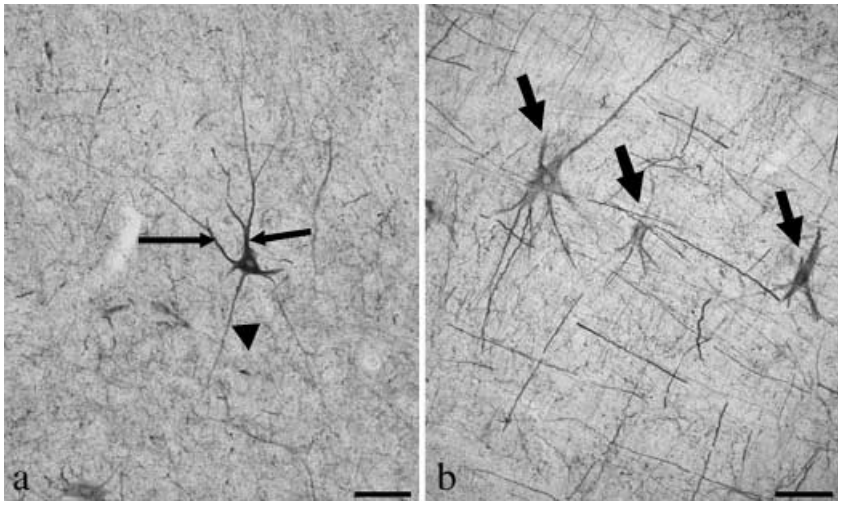

Fig. 8 Micrograph of a met-enk immunoreactive large multipolar pyramidal neuron in the intermediate grey (a) and intermediate white layers (b). Whereas dendritic processes (arrows) are distributed in all directions, axons (arrowhead) project mainly toward deeper layers. Note the bridging fibres running across the intermediate white layer. Scale bar = 300 μm.

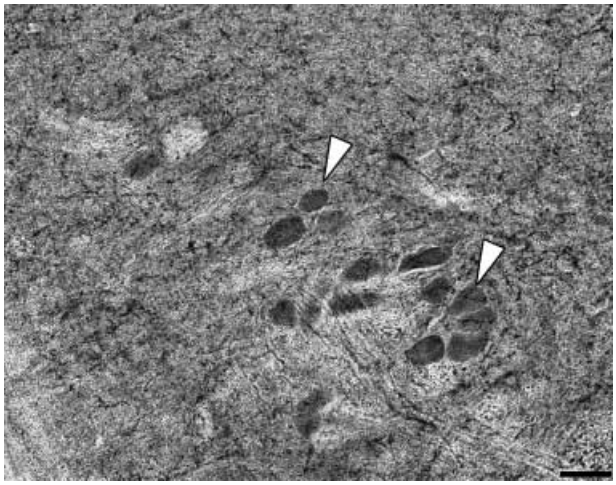

Fig. 9 Micrograph of met-enk immunoreactive neurons of the trigeminal mesencephalic nucleus. Scale bar = 100 μm.

the superior colliculus might implicate enkephalins in orientation of the head towards contralateral stimuli given that they have been identified as the probable source of the predorsal bundle fibres known to be responsible for these movements (Redgrave et al. 1986). The presence of a large band of met-enk fibres in the intermediate white layer further supports this observation. Enkephalins are important in pain suppression in central neural pathways (Basbaum & Fields, 1984). They are endogenous opiates of the nervous system, exerting their influence through opioid receptors on neurons. They may explain the well-known phenomenon of initial inhibition of pain sensation that can last for several hours after acute trauma (Melzack & Wall, 1965). A pain inhibitory pathway has been described from the PAG to the medulla in the lower brainstem, and thence

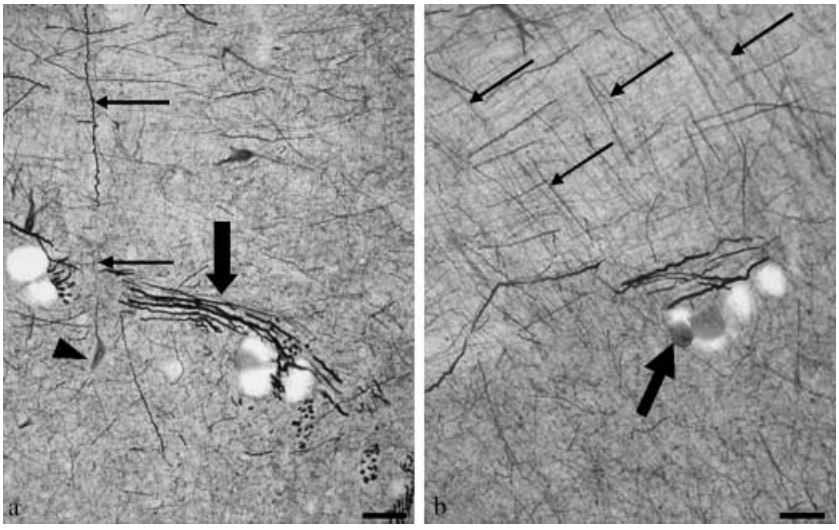

Fig. 10 Micrograph of large met-enk immunoreactive fibres (block arrow) in the intermediate white. Note also the met-enk immunoreactive bridging fibres (arrows) running across the intermediate white layer. The fibres can be seen extending into the deep grey layer. Some of the fibres appear to be dendrites of a neuron (arrowhead) lying within deep layer. Scale bar = 100 μm.

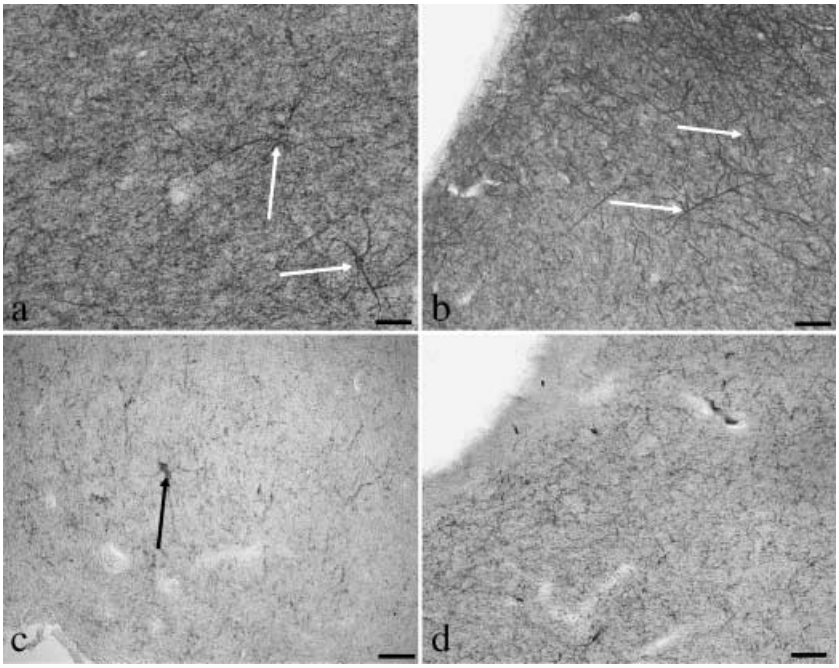

Fig. 11 Micrograph of the PAG showing neurons and neuropil immunoreactive to met-enk (a), SP (b), leu-enk (c) and SOM (d). Note the intensity of immunoreactivity to met-enk and SP in the PAG. Scale bar = 100 μm.

to the spinal cord (Liebeskind et al. 1973; Basbaum et al. 1977) and in the light of our observation of met-enk fibres extending into the deep layer and PAG, we propose that the met-enk projections that we describe here may represent the first part of that pathway in the camel. Studies by Eichenberger et al. (2002) and Osaki et al. (2003), using tracer techniques in rats, have also demonstrated connections between the deep layer of the superior colliculus and the PAG. It has been demonstrated in the rat that enkephalins that are anti-nociceptive are normally under the inhibition of GABAergic fibres. On the release of a nociceptive signal, however, the inhibitory effect of GABA is removed resulting in an increased release of enkephalins and anti-nociception (Williams et al. 1995; Stiller et al. 1996). This effect has been elicited by injecting morphine into the PAG of the rat (Williams et al. 1995).

The fact that the camel may possess a significant anti-nociceptive system is further supported by the observation of intense SP immunoreactivity in its PAG. Injecting SP in the PAG of the rat stimulates the release of endogenous opioids (Tang et al. 1983; Iadarola et al. 1986; Furst, 1999) and this activates the descending pain inhibitory pathways that mediate opioid analgesia (Rosen et al. 2004). Indeed, injecting antibodies to SP and enkephalins in the PAG of the rabbit both reduce the effect of acupuncture (Han et al. 1984).

In conclusion, the very large size of the superior colliculus of the camel brain suggests that it may have a special function in this animal. In the presence of numerous large met-enk neurons throughout the superior colliculus with fibres projecting to the PAG, the intense staining of met-enk and SP in the PAG suggests that the pain inhibiting opioid pathway, known to be present generally in mammals, may be especially well developed in the camel, perhaps helping it to withstand the extremes of temperatures and discomfort associated with its desert environment.

Acknowledgements

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