Distribution of orexinergic neurons and their terminal networks in the brains of two species of African mole rats.

Adhil Bhagwandin[1], Kjell Fuxe[2], Nigel C. Bennett[3], & Paul R. Manger[1].

[1]School of Anatomical Sciences, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown 2193, Johannesburg, Republic of South Africa.

[2]Department of Neuroscience, Karolinska Institutet, Retzius väg 8, S-171 77 Stockholm, Sweden.

[3]Mammal Research Institute, Department of Zoology and Entomology, University of Pretoria, Pretoria 0002, South Africa.

Correspondence to: Paul Manger

School of Anatomical Sciences

Faculty of Health Sciences

University of the Witwatersrand

7 York Road, Parktown, 2193

Johannesburg, REPUBLIC OF SOUTH AFRICA.

Ph: +27 11 717 2497 Fax: +27 11 717 2422

Paul.Manger@wits.ac.za

Abstract

The distribution of orexinergic cell bodies and terminal networks within the brains of two species of African mole rat (Cape-dune mole rat – *Bathyergus suillus* and highveld mole rat – *Cryptomys hottentotus*) were identified using immunohistochemistry for Orexin-A. The aim of the study was to investigate possible differences in the nuclear complement and terminal distribution of this system by comparing those of the mole rats to published studies of other rodents and mammals. The wild-caught mole rats used in this study live a subterranean lifestyle and are well known for their regressed visual system, which may lead to the prediction of differences in the distribution of the cell bodies and the terminal networks; however, we found that both species of mole rat displayed orexinergic nuclei limited to the hypothalamus in regions similar to those previously reported for other rodent and mammalian species. No immunoreactive neurons could be identified, in either species of mole rat within the anterior hypothalamic paraventricular nucleus, as has been reported for Murid rodents. The terminal networks, while remaining similar between the species, are more strongly expressed in the Cape-dune mole rat than in the highveld mole rat.

Key Words: Bathyergus; Cryptomys; orexin; hypocretin; rodent; comparative neuroanatomy.

1. Introduction

Orexin (also known as hypocretin) is a neuropeptide that has been reported to play a role in the regulation of feeding, drinking, body temperature, general activity (Lubkin and Stricker-Krongrad, 1998; Edwards et al., 1999; Hagan et al., 1999; Kunii et al., 1999; Mondal et al., 1999; Piper et al., 2000; Estabrooke et al., 2001; Hungs et al., 2001; Yoshimichi et al., 2001; Kotz et al., 2002; Berthoud et al., 2005), energy homeostasis (Mintz et al., 2001), stimulation of gastric secretion in rats (Takahashi et al., 1999), increasing metabolic rate in rats (Lubkin and Stricker-Krongard, 1998), altering luteinising hormone release in rats (Pu et al., 1998) and in the regulation of the sleep-wake cycle specifically associated with increased wakefulness and inhibition of REM sleep (Sakurai et al., 1998; Chemelli et al., 1999; Siegel, 1999; Bourgin et al., 2000; Kilduff and Peyron, 2000; Thannickal et al., 2000; van den Pol, 2000).

The distribution of orexin-immunopositive (Orx +) cell bodies and terminal networks within the brain has been reported in a range of mammals including: humans (*Homo sapiens*, Moore et al., 2001); domestic cat (*Felis catus*, Zhang et al., 2001, 2002); domestic sheep (*Ovis aries*, Iqbal et al., 2001); six species of rodent (laboratory rat, *Rattus norvegicus* – Broberger et al., 1998; Peyron et al., 1998; Chen et al., 1999; Cutler et al., 1999; Date et al., 1999; Hagan et al., 1999; Nambu et al., 1999, Risold et al., 1999; Baldo et al., 2003; Chou et al., 2004; Espana et al., 2005; Kirouac et al., 2005; Nixon and Smale, 2007; Nile grass rat, *Arvicanthus niloticus* – Novak and Albers, 2002; Nixon and Smale, 2007; golden or Syrian hamster, *Mesocricetus auratus* – McGranaghan and Piggens, 2001; Mintz et al., 2001; Vidal et al., 2005; Nixon and Smale, 2007; laboratory mouse, *Mus musculus*, C57B1 strain, Broberger et al., 1998; Siberian or Djungarian hamster, *Phodopus sungorus* – McGranaghan and Piggins, 2001; Khorooshi and Klingenspor, 2005; degu, *Octodon degus* – Nixon and Smale, 2007); five microchiropteran species (Kruger et al., 2010); and the Eastern grey kangaroo (*Macropus giganteus*, Yamamoto et al., 2006). The Orx + neuronal cell bodies were invariably localized within the hypothalamus and while for most mammals they were represented as a rather homogenous loosely packed large cluster of neurons located in the perifornical and lateral hypothalamus (see above references), in certain rodents there may be up to four clusters, or nuclei, of orexinergic neurons – the two described above, plus one

cluster located in the anterior hypothalamic paraventricular subnucleus and one in the lateral ventral hypothalamic supraoptic area (LVHA) (Nixon and Smale, 2007).

Orexin immunoreactive terminal networks have been found in differential relative densities throughout the varying brain regions previously examined (see references cited above). A high relative density of Orx+ terminals have been consistently observed within the paraventricular nucleus of the epithalamus, the noradrenergic locus coeruleus complex and the serotonergic dorsal raphe nuclear complex. The majority of the nuclei of the hypothalamus as well as the septal region, cholinergic nuclei of the pons, the ventral tegmental area, the nuclei of the solitary tract, the remaining serotonergic nuclei and both limbs of the diagonal band of Broca were consistently described as having a medium relative density of Orx+ terminals. A low to absent density of Orx+ terminals have been recorded in regions such as the cerebral cortex, major nuclei of the dorsal thalamus and other regions of the central nervous system (see references above).

Recent studies have demonstrated that in rodents and other species, the complexity of the nuclear organization of the diffusely projecting cholinergic, catecholaminergic and serotonergic systems remains consistent within an order despite differences in brain size, phenotype, lifestyle or evolutionary distance (e.g. Manger, 2005; Maseko et al., 2007; Bhagwandin et al., 2008; Dwarika et al., 2008; Limacher et al., 2008, Gravett et al., 2009, Pieters et al., 2010; Bux et al., 2010). In terms of the orexinergic system this appears to hold true for most mammals; however, the observations of variance in the number of potential orexinergic nuclei in rodents (Nixon and Smale, 2007) may indicate greater organizational variance within an order for this diffusely projecting system than other systems previously studied.

In contrast to this order level consistency in nuclear organization, the pattern of terminal networks of these diffusely projecting neurotransmitter systems appears to be more variable within the order. For example, when the innervation patterns of the serotonergic (Raghanti et al., 2008a) and cholinergic (Raghanti et al., 2008b) terminal networks within the cerebral cortex of humans, chimpanzees and macaque monkeys were compared, there were no species differences in the primary motor cortex (Brodmann's area 4), but significant quantitative and qualitative differences were observed between macaques on the one hand and chimpanzees and humans on the other (which were

similar) in two pre-motor cortical areas (Brodmann's areas 9 and 32). These studies are suggestive of family level consistencies and inter-family differences in the terminal networks of the diffusely projecting neural systems. Comparative studies of the orexinergic projections in rodents are also suggestive of certain qualitative differences in terminal network densities within and between families despite many overall similarities (McGranaghan and Piggins, 2001; Nixon and Smale, 2007).

In the current study, whole brains of two species of African mole rat, the highveld mole rat (*Cryptomys hottentotus*) and the Cape dune mole rat (*Bathyergus suillus*) were examined immunohistochemically for orexin-A. Both species studied have a greatly reduced visual system (Oelschlager et al., 2000; Cernuda-Cernuda et al., 2003; Nemec et al., 2004), are subterranean, and appear to have a free-running circadian activity oscillator (Lovegrove and Papenfus, 1995; Lovegrove and Muir, 1996; Negroni et al., 2003; Oosthuizen et al., 2003; Gutjahr et al., 2004). These atypical rodent features combined with the distant (but familial) relation to each other and to the non-familial laboratory rat (Adkins et al., 2003), provide an interesting model to examine changes in nuclear organization and terminal network patterns that may be related to phenotype, life history and behaviour.

2. Materials and Methods

The brains of three adult highveld mole rats (*Cryptomys hottentotus*) (average body weight: 86.5 g; average brain weight: 1.5 g) and three adult Cape dune mole rats (*Bathyergus suillus*) (average body weight: 965 g; average brain weight: 3.4 g) were used in the current study. All animals were treated and used according to the guidelines of the University of the Witwatersrand Animal Ethics Committee, which parallel those of the NIH for the care and use of animals in scientific experimentation. The mole rats were placed under deep barbiturate anaesthesia (Euthanaze, 200mg sodium pentobarbital/ kg, i.p.), and then perfused intracardially upon cessation of respiration. The perfusion was initially done with a rinse of 0.9% saline solution at 4°C, followed by a solution of 4% paraformaldehyde in 0.1M phosphate buffer (PB) (approximately 1l/kg of each solution). Brains were then removed from the skull and post-fixed overnight in 4% paraformaldehyde in 0.1M PB, and then allowed to equilibrate in 30% sucrose in PB. The brains were then frozen and sectioned into serial coronal and sagittal sections of 50 µm thickness. A one in three series of stains was made for Nissl, myelin and orexin A. Sections kept for the Nissl series were mounted on 0.5% gelatine coated glass slides, cleared in a solution of 1:1 chloroform and absolute alcohol, then stained with 1% cresyl violet. Myelin sections were stored in 5% formalin at 4°C for a period of two weeks and were then mounted on 1% gelatine coated glass slides and subsequently stained with a modified silver stain (Gallyas, 1979).

For immunohistochemical staining the sections were first treated for 30 min with an endogenous peroxidase inhibitor (49.2% methanol: 49.2% of 0.1PB: 1.6% of 30% H_2O_2) followed by three 10 min rinses in 0.1M PB. This was followed by a 2 hour pre-incubation, at room temperature, in a solution (blocking buffer) containing 3% normal goat serum (NGS, Chemicon), 2% bovine serum albumin (BSA, Sigma), and 0.25% Triton X-100 (Merck) in 0.1M PB. The sections were then placed in a primary antibody solution containing the appropriately diluted antibody in blocking buffer, for 48 hours at 4°C. To reveal orexinergic neurons we used the anti-orexin A antibody (AB 3704, Millipore, raised in rabbit) at a dilution of 1:1500. This step was followed by three 10 min rinses in 0.1M PB, after which the sections were incubated in a secondary antibody for two hours. The secondary antibody solution contained a 1:1000 dilution of

biotinylated anti-rabbit IgG (BA-1000, Vector Labs) in 3% NGS, and 2% BSA in 0.1M PB. After three 10 min rinses in 0.1M PB, the sections were incubated for 1 hour in AB solution (Vector Labs), and again rinsed. The sections were then treated in a solution of 0.05% diaminobenzidine in 0.1M PB for 5 minutes, following which 3µl of 30% H_2O_2 was added to the 1ml of solution in which each section was immersed. Staining development was monitored visually and checked under a low power stereomicroscope until the background staining was at a level at which it could assist reconstruction without obscuring the immunopositive neuronal structures. Development was arrested by placing the sections in 0.1M PB, and then rinsed twice more in the same solution. Sections were mounted on glass slides coated with 0.5% gelatine and left to dry overnight. They were then dehydrated in a graded series of alcohols, cleared in xylene, and coverslipped with Depex. Two controls were employed in the immunohistochemistry, including the omission of the primary antibody and the omission of the secondary antibody in selected sections. The sections were observed with a low power stereomicroscope, and the architectonic borders of the sections traced according to the Nissl stained sections using a camera lucida. The immunostained sections were then matched to the drawings and the immunopositive neurons marked, in addition the density of axon terminal staining was graded from low to high for each immunostained section and medium and high marked on the drawings. The drawings were then scanned and redrawn using the Canvas 8 drawing program. The location of Orx+ neuronal structures and the corresponding orexinergic terminal network distribution were described in relation to the general neuroanatomy of the brain and the cholinergic, catecholaminergic and serotonergic systems described previously for these two speceis of mole rat (Bhagwandin et al., 2008).

Abbreviations

III – oculomotor nucleus

IV – trochlear nucleus

Vmot – motor division of trigeminal nucleus

VI – abducens nucleus

VIId – facial nerve nucleus, dorsal division

VIIv – facial nerve nucleus, ventral division

X – dorsal motor vagus nucleus

XII – hypoglossal nucleus

3V – third ventricle

A1 – caudal ventrolateral medullary tegmental nucleus

A2 – caudal dorsomedial medullary nucleus

A4 – dorsal medial division of locus coeruleus

A5 – fifth arcuate nucleus

A6d – diffuse portion of locus coeruleus

A7d – nucleus subcoeruleus, diffuse portion

A7sc – nucleus subcoeruleus, compact portion

A8 – retrorubral nucleus

A9l – substantia nigra, lateral

A9m – substantia nigra, medial

A9pc – substantia nigra, pars compacta

A9v – substantia nigra, ventral or pars reticulata

A10 – ventral tegmental area

A10c – ventral tegmental area, central

A10d – ventral tegmental area, dorsal

A10dc – ventral tegmental area, dorsal caudal

A11 – caudal diencephalic group

A12 – tuberal cell group

A13 – zona incerta

A14 – rostral periventricular nucleus

A15d – anterior hypothalamic group, dorsal division

A15v – anterior hypothalamic group, ventral division

ac – anterior commissure

amyg – Amygdala

AP – area postrema

B9 – supralemniscal serotonergic nucleus

C – caudate nucleus

C1 – rostral ventrolateral medullary tegmental group

C2 – rostral dorsomedial medullary nucleus

C3 – rostral dorsal midline medullary nucleus

ca – cerebral aqueduct

cc – corpus callosum

cc – central canal

Cing ctx – cingulate cortex

cl – claustrum

CLi – caudal linear nucleus

CN – cerebellar nuclei

CVL – caudal ventrolateral serotonergic group

Diag.B – diagonal band of Broca

DR – dorsal raphe

DRc – dorsal raphe nucleus, caudal division

DRd – dorsal raphe nucleus, dorsal division

DRif – dorsal raphe nucleus, interfascicular division

DRl – dorsal raphe nucleus, lateral division

DRp – dorsal raphe nucleus, peripheral division

DRv – dorsal raphe nucleus, ventral division

DT – dorsal thalamus

EW – Edinger-Westphal nucleus

f - fornix

GC – periaqueductal grey matter

GP – globus pallidus

Hbm – medial habenular nucleus

Hbl – lateral hebenular nucleus

hyp – hypothalamus

hyp. d – hypothalamus, dorsal division

hyp. l – hypothalamus, lateral division

hyp. v – hypothalamus, ventral division

HIP – hippocampus

ic – internal capsule

IC – inferior colliculus

IP – interpeduncular nucleus

Is.Call. – Islands of Calleja

LHA – lateral hypothalamic area

LVHA – lateral ventral hypothalamic area

LDT –laterodorsal tegmental nucleus

LV – lateral ventricle

mtf – medullary tegmental field

MnR – median raphe nucleus

N.Acc – nucleus accumbens

N.Amb – nucleus ambiguus

N.Bas – nucleus basalis

NEO - neocortex

P – putamen

PFR – perifornical area

Pg – pineal gland

pVII – preganglionic motor neurons of the superior salivatory nucleus or facial nerve

pIX - preganglionic motor neurons of the inferior salivatory nucleus

PBg – parabigeminal nucleus

PC – cerebral peduncle

PIR – piriform cortex

PPT –pedunculopontine nucleus

PV – thalamic paraventricular nuclei

py – pyramidal tract

R – reticular nucleus of dorsal thalamus

RMg – raphe magnus nucleus

ROb – raphe obscurus nucleus

RPa – raphe pallidus nucleus

RVL – rostral ventrolateral serotonergic group

SC – superior colliculus

scp – superior cerebellar peduncle

Sep.M – medial septal nucleus
TOL – olfactory tubercle
vh – ventral horn
VPO – ventral pontine nucleus

3. Results

In both species of mole rat examined, immunohistochemically identifiable, morphologically homogenous, orexinergic (Orx+) cell bodies were limited to the hypothalamus, as previously reported in all other mammals studied to date. The terminal networks, while remaining similar in distribution between both species, are more strongly expressed in the Cape dune mole rat than in the highveld mole rat. The following descriptions of the Orx+ cell bodies and terminal networks, for both species of mole rat (unless otherwise specified), are provided in relation to the general anatomy of the brain, or to the neuronal groups of the cholinergic, catecholaminergic and serotonergic systems (as described for these particular species in Bhagwandin et al., 2008) where overlap occurs.

3.1. **Orexinergic Cell Body Distribution**

Both species of mole rat expressed Orx+ neurons only within the hypothalamus and were observed as sharing a common neuronal locality within the lateral hypothalamic area (LHA), perifornical region (PFR) and the lateral ventral hypothalamic supraoptic area (LVHA) (Figs. 1G-I, 2I-J). Within the LHA of both species a moderate density of Orx+ cells bodies were found to intermingle with the lateral hypothalamic cholinergic nucleus and the dopaminergic neurons of the A13 (zona incerta) nucleus. The Orx+ neurons of the PFR were observed to show a moderate density and did not overlap with any previously described cholinergic or dopaminergic neurons. In both species, the LVHA Orx+ neurons were found in the same region as the dopaminergic A15v (ventral division of the anterior hypothalamic group) nucleus, in the lateral and ventral portions of the hypothalamus, immediately dorsal to the greatly reduced optic tract (Fig. 3). No Orx+ neurons could be identified in the anterior hypothalamic paraventricular subnucleus in

either mole rat species. Thus, there appears to be three distinct clusters of Orx+ neurons in the hypothalamus of the mole rats studied, a large homogeneous cluster spanning the lateral and perifornical regions, a distinct cluster extending into the region of the zona incerta, and a final cluster in the ventral lateral hypothalamus adjacent to the optic tracts. Both mole rats exhibited neuronal cell bodies that were morphologically homogenous in all three clusters, and that were ovoid in shape, and a varying mixture of bi- and multi-polar types that showed no specific dendritic orientation (Fig. 3).

3.2. **Orexinergic Terminal Networks**

3.2.1 Telencephalon

Throughout the telencephalon of both species there was only one region of high-density Orx+ terminal networks, this being within the very anterior portion of the septal nuclear complex of the highveld mole rat (Fig. 2D). In both species a medium relative density of Orx + terminals were observed within the shell of the nucleus accumbens, the entire septal nuclear complex (overlapping with the cholinergic medial septal nucleus), the cholinergic diagonal band of Broca, and in small portions of the nucleus basalis and the olfactory tubercule (Figs. 1C-F, 2C-F). All remaining regions of the telencephalon (cerebral cortex, the dorsal striatopallidal complex, hippocampal complex, amygdalar complex) were observed to have a low-density terminal network in both species of mole rat.

3.2.2 Diencephalon

A medium density Orx+ terminal network characterized the entire hypothalamus in both species examined; however, high terminal network densities were noted in a region adjacent to the dorsal aspect of the third ventricle within the hypothalamus of both species of mole rat in the region where the dopaminergic neurons of the A15d (dorsal division of the anterior hypothalamic group) nucleus were located (Figs. 1H-J, 2H-I). A second region of high-density Orx+ terminals was found in the premammillary nuclei of both species; however, the mammillary nuclei themselves only exhibited a low density of Orx+ terminals. A low-density terminal network was observed through both the dorsal and ventral thalamus, except for the intralaminar central median nucleus of the dorsal

thalamus where a medium-density network of Orx+ terminals was observed. In contrast to this, the epithalamus evinced a high-density terminal network in the dorsal aspects of the paraventricular nuclei and a medium density terminal network in the the ventral midline part of the paraventricular thalamic nuclei and in the regions surrounding the habenular nuclei, especially the lateral habenular nucleus and dorsal most portions of the fasciculus retroflexus (Figs. 1H-I, 2H-I, 5).

3.2.3 Midbrain (Mesencephalon)

Within the midbrain of both species a high-density Orx+ terminal network was observed throughout the serotonergic dorsal raphe nuclear complex and the serotonergic median raphe nucleus (Figs. 1M-N, 2M-O, 6A). A second region of high-density Orx+ terminals was observed in the dorsomedial periaqueductal gray matter (DMPAG) in both species. The remainder of the periaqueductal gray matter was observed to contain a medium density Orx+ terminal network, which was also observed within the superior colliculus, parts of the ventral tegmental nuclear complex, specifically the A10dc, A10c and A10d nuclei, and in the serotonergic caudal linear nucleus (CLi) and supralemniscal serotonergic group (B9). The medium-density Orx+ terminal network within the midbrain of the highveld mole rat was more extensive than that seen in the cape dune mole rat and was observed in the A10 nucleus, the inferior colliculus, upper midbrain tegmentum and interpeduncular nucleus, whereas in the cape dune mole rat these regions only contained a low-density Orx+ terminal network, or in the case of the superior colliculus and interpeduncular nucleus appeared to be limited to a specific portion of these nuclei (Figs. 1J-N, 2K-N). All other regions of the midbrain evinced a low-density Orx+ terminal network in both species of mole rat.

3.2.4 Pontine region (Metencephalon)

Within the pons of both species, high-density Orx+ terminal networks were observed in the cholinergic laterodorsal tegmental nucleus (LDT), the noradrenergic diffuse nucleus of the locus coeruleus (A6d) and compact nucleus of the subcoeruleus (A7sc), and the serotonergic caudal nucleus of the dorsal raphe complex (DRc) (Figs. 1N-P, 2N-O, 4A, 6A). In the dorsal pontine tegmentum of both species, a medium-

density Orx+ terminal network was seen to overlap with the regions where the neurons of the cholinergic pedunculopontine tegmental nucleus (PPT) and noradrenergic diffuse nucleus of the subcoeruleus (A7d) were found. Other medium density Orx+ terminal networks were observed to overlap with the distribution of the noradrenergic neurons of the fifth arcuate nucleus (A5) and the pontine portion of the serotonergic median raphe nucleus (MnR) (Fig. 6B). All other portions of the pontine region were observed to contain a relatively low-density Orx+ terminal network.

3.2.5 Medulla oblongata (Myelencephalon) and Cerebellum

Within the medulla there was only one region that contained a high-density Orx+ terminal network and this was the area postrema (AP) of the Cape dune mole rat (Fig. 1T). Interestingly, in the highveld mole rat there was only a low-density Orx+ terminal network in this structure. Medium-density Orx+ terminal networks were observed in all the regions where serotonergic neurons were located, which include the raphe magnus nucleus (RMg) and its continuation in the rostral and caudal ventrolateral serotonergic columns (RVL and CVL), the raphe pallidus nucleus (RPa) and the raphe obscurus nucleus (ROb) (Fig. 6C). The remaining regions of the medulla that showed medium-density Orx+ terminal networks were coincident with the catecholaminergic nuclei previously reported, including the rostral ventrolateral medullary tegmental group (C1) (Fig. 4C), rostral dorsomedial medullary nucleus (C2), caudal ventrolateral medullary tegmental group (A1) and the caudal dorsomedial medullary nucleus (A2). The extent of this medium-density network around the C2 and A2 nuclei was quite expansive and appeared to include the majority of the nuclei of the solitary tract. A global low-density Orx+ terminal network was observed throughout the remaining regions of the medulla oblongata. No specific species differences were observed in the medulla. A low-density Orx+ terminal network was observed throughout all regions of the cerebellum, both cortical and nuclear, for both species.

4. Discussion

In the current study, it was observed that both species of mole rat displayed three clusters of orexinergic immunoreactive neurons within the hypothalamus, thereby maintaining significant congruency with previous studies in other mammals (see references listed in Introduction). In addition, it was noted that no immunoreactive orexinergic (Orx+) cell bodies, in either species of mole rat, could be identified within the anterior hypothalamic paraventricular organ as was previously reported in some Murid rodents (Nixon and Smale, 2007). In both species of mole rat the orexinergic terminal network distribution, for the most part, maintained similarity to that observed in other mammals; however, a few differences were identified between both species of mole rat and collectively in all other mammals studied to date.

4.1 **Comparison to other rodents and other mammals**

4.1.1 Distribution of orexinergic cell bodies

The distribution of Orx+ cell bodies, in both species of mole rat, was both similar and different to those previously reported in other rodents. Both species of mole rat expressed Orx+ neurons within the perifornical region (PFR) and the lateral hypothalamic area (LHA), comprising the main cluster of orexinergic neurons, which is a common feature of the orexinergic system shared by all rodents studied to date; however, neither species of mole rat, as with the Syrian hamster and degu, displayed Orx+ neurons within the anterior hypothalamic paraventricular subnucleus as seen in the Long-Evans rat and grass rat (Nixon and Smale, 2007; but see Novak and Albers, 2002 who do not report these neurons in the Nile grass rat but they used an Orexin B antibody). In this case we appear to have a difference in the nuclear organization of the orexinergic system within the rodents, with the two Murid rodents studied (Long-Evans rat and Nile grass rat, both closely related) showing a difference to the closely related Syrian hamster (a member of the Cricetidae) and the more distantly related Ctenohystrica (Octodon and the bathyergid mole rats) (Nixon and Smale, 2007; Blanga-Kanfi et al., 2009). This is not the first time such Murid *vs* non-Murid differences have been observed for such systems in the rodents (Bhagwandin et al., 2006). In the mole rats, as with all other rodents, two additional, but smaller, clusters of orexinergic neurons were located, one in the

dorsolateral region of the hypothalamus intermingling with the region of the zona incerta, and a second cluster in the ventrolateral region of the hypothalamus near the optic tract (that is immunoreactive for both orexin-A and orexin-B, Nixon and Smale, 2007), although the optic tract is greatly reduced in the mole rats. Thus, within the rodents, while many similarities in neural systems occur across all species, there are at least two differences reported to date (orexinergic anterior hypothalamic paraventricular neurons, Nixon and Smale, 2007; and cortical cholinergic neurons, Bhagwandin et al., 2006). These differences would be of interest to study further and delineate potential phylogenetic boundaries for these features and the possible behavioural correlates of these differences.

An important aspect of the parcellation of the orexinergic system needs to be highlighted at this point. In the current study we have lumped the orexinergic neurons of the PFR and LHA into a single cluster that we have termed the main cluster (in this study and in a previous study on microchiropterans, Kruger et al., 2010). This was done as there appears to be no anatomical distinction in the aggregation of the orexinergic neurons across this large cluster, being organized in what can be loosely termed a diffuse nucleus; however, a previous study of the connectivity of these regions of the main cluster (PFR and LHA) would suggest that these two regions likely represent distinct nuclei (Yoshida et al., 2006). Yoshida et al. (2006) showed that, while the PFR and LHA orexinergic neurons project to many of the same regions, the intensity of the projection differs substantially between several of these terminal territories. In addition, they showed that the medial, or PFR, portion of what we term the main orexinergic cluster innervates the anterior and ventromedial hypothalamus plus the suprachiasmatic nucleus, whereas the LHA orexinergic neurons do not. They also showed that the LHA orexinergic neurons innervate the dorsal raphe, whereas the PFR orexinergic neurons do not (Yoshida et al., 2006). These differences in projection fields appear to warrant a distinction of what we term the main orexinergic cluster into medial or PFR and LHA orexinergic nuclei. It would be of importance to study such differences across species to determine whether such differential projection territories exist in other mammals and warrant cross species parcellation of the orexinergic system in a similar manner.

In the mole rats and other rodents the location of the main cluster of orexinergic (Orx+) cell bodies (PFR and LHA), is similar to that seen in all other mammals studied to date (Iqbal et al., 2001; Moore et al., 2001; Zhang et al., 2001, 2002; Yamamoto et al., 2006; Kruger et al., 2010). Interestingly, all mammalian species appear to have orexinergic neurons overlapping with the medial most portion of the zona incerta. Of less similarity is the appearance of orexinergic neurons in the ventrolateral hypothalamus, near the optic tract, which while present in most mammals (and appears greatly expanded in the kangaroo, Yamamoto et al., 2006), are lacking in the microchiropterans (Kruger et al., 2010) and the two species of hamster studied to date (McGranaghan and Piggens, 2001; Mintz et al., 2001; Khorooshi and Klingenspor, 2005; Vidal et al., 2005; Nixon and Smale, 2007). Further contrasts in the location of orexinergic neurons amongst mammals include: (1) the presence of Orx+ neurons in the dorsomedial hypothalamic region in the sheep, minipig and kangaroo (Iqbal et al., 2001; Yamamoto et al., 2006; Ettrup et al., 2010); and (2) Orx+ neurons in the supraoptic and paraventricular magnocellular nucleus of the minipig (Ettrup et al., 2010). Thus, while for the most part the nuclear organization of the orexinergic system appears to quite conserved across mammalian species, there are definitely some points of departure that may be interesting in both a functional and phylogenetic aspect, and warrants the investigation of orexinergic cellular location in a broader range of mammalian species.

4.1.2 Distribution of orexinergic terminal networks

Previous studies of the terminal networks of the orexinergic neurons have demonstrated that in mammals the majority of the brain is either in receipt of very minor, or no, orexinergic innervation. In this sense the mole rats studied herein are no exception. These regions of minor to no orexinergic innervation include the cerebral neocortex, the dorsal striatopallidal complex, the dorsal thalamus, cerebellum and most of the brainstem.

Despite this, there are areas of the brain that consistently show medium to dense orexinergic innervation across many of the mammalian species studied to date. Within the telencephalon of the mole rats studied, medium to high-density orexinergic terminal networks were observed in the septal nuclei, the shell of the nucleus accumbens and the

cholinergic basal forebrain. This telencephalic distribution is similar across all rodents and all mammals for which these projections have been described to date. Similarly, in the mole rats and all mammals studied, the paraventricular nuclei of the epithalamus exhibited a high-density orexinergic terminal network in the dorsal division. This specific network extended around the habenular nuclear complex in the mole rats, again being similar to that seen in other mammals. A medium density network was observed throughout the mole rat hypothalamus as seen in all other mammals. Interestingly, in several mammals studied, a clear medium-density orexinergic terminal network is observed in the intergeniculate leaflet, but this was not observed in the mole rats. This is likely due to the major reduction in size of the visual system in these species (Nemec et al., 2004). Previously, Vidal et al. (2005) demonstrated that the orexinergic projection to the intergeniculate leaflet arises from the orexinergic neurons located near the zona incerta. The mole rats possess these zona incerta orexinergic neurons, but not the projection to the intergeniculate leaflet. This indicates that these Orx+ zona incerta neurons mostly likely have projections to other structures in addition to the intergeniculate leaflet, and the existence of these additional, but currently unknown projections, are the possible reason for the maintenance of this cell group in the mole rats.

As in all species where the orexinergic terminal networks have been described, the mole rats studied herein have high to moderate density projections to all the serotonergic nuclei, the pontine cholinergic nuclei, and the locus coeruleus complex (Bhagwandin et al., 2008). In addition, both mole rats expressed a high to medium-density Orx+ terminal network within the periaqueductal grey matter and the ventral tegmental area (VTA), similar to that previously reported for other rodents and other mammals. Interestingly though, while a low to absent density of Orx+ terminal networks has been reported for the superior colliculus in other rodent species, a medium-density Orx+ terminal network was observed in this study despite the reduced size of the superior colliculus previously noted for mole rats (Nemec et al., 2004; Da Silva, 2006). Variations in terminal densities within the inferior colliculus (IC) and interperduncular (IP) nulei, amongst rodent species, were similarly noted in both species of mole rat. The highveld mole rat expressed a medium-density Orx+ terminal network in both the IC and IP, similar to the hamster, grass rat, degu and wistar rat; whereas the Cape dune mole rat

demonstrated a low-density Orx+ terminal network within the IC and IP, consistent with the Long-Evans and Wistar rats; however, these terminal networks also appear to vary significantly across the IP and IC of other mammals studied to date.

Interestingly no other rodent species, except for the Cape dune mole rat of the current study, exhibited a high-density Orx+ terminal network within area postrema (AP) with medium-density Orx+ terminal networks reported within AP for the degu and wistar rat, whereas the highveld mole rat demonstrates a low-density Orx+ terminal network for this homologous region. The catecholaminergic medullary nuclei (C1, C2, A1 and A2) expressed a medium-density Orx+ terminal network in both species of mole rat and this is congruent with previous rodent species however it must be noted that there is no clarity with regard to the distribution of Orx+ terminal networks within C2 amongst other rodent and mammalian species studied to date.

References:

Adkins R.M., Walton A.H, Honetcutt, R.L., 2003. Higher-level systematics of rodents and divergence time estimates based on two congruent nuclear genes. Mol. Phylogenet. Evol. 26, 409-420.

Baldo B.A., Daniel R.A., Berridge C.W., Kelley A.E., 2003. Overlapping distributions of orexin/hypocretin- and dopamine-ß-hydroxylase immunoreactive fibers in rat brain. Regions mediating arousal, motivation, and stress. J. Comp. Neurol. 464, 220–237.

Berthoud, H.R., Patterson, L.M., Sutton, G.M., Morrison, C., Zheng, H., 2005. Orexin inputs to caudal raphe neurons involved in thermal, cardiovascular, and gastrointestinal regulation. Histochem. Cell Biol. 123, 147-156.

Bhagwandin, A., Fuxe, K., Manger, P.R., 2006. Choline acetyltransferase immunoreactive cortical interneurons do not occur in all rodents: a study of the phylogenetic occurrence of this neural characteristic. J. Chem. Neuroanat. 32, 208-216.

Bhagwandin, A., Fuxe, K., Bennett, N.C., Manger, P.R., 2008. Nuclear organization and morphology of cholinergic, putative catecholaminergic and serotonergic neurons in the brains of two species of African mole-rat. J. Chem. Neuroanat. 35, 371-387.

Blanga-Kanfi, S., Miranda, H., Penn, O., Pupko, T., DeBry, R.W., Huchon, D., 2009. Rodent phylogeny revisited: analysis of six nuclear genes from all major rodent clades. BMC Evol. Biol. 9: 71.

Bourgin, P., Huitron-Resendiz, S., Spier, A.D., Fabre, V., Morte, B., Criado, J.R., Sutcliffe, J.G., Henriksen, S.J., de Lecea, L., 2000. Hypocretin-1 modulates rapid eye movement sleep through activation of locus coeruleus neurons. J. Neurosci. 20, 7760–7765.

Broberger, C., de Lecea, L., Sutcliffe, J.G., Hokfelt ,T., 1998. Hypocretin/orexin and melanin-concentrating hormone-expressing cells form distinct populations in the rodent lateral hypothalamus: relationship to the neuropeptide Y and agouti gene-related protein systems. J. Comp. Neurol. 402, 460–74.

Bux, F., Bhagwandin, A., Fuxe, K., Manger, P.R., 2010. Organization of cholinergic, putative catecholaminergic and serotonergic nuclei in the diencephalon, midbrain and pons of sub-adult male giraffes. J. Chem. Neuroanat. 39, 189-203.

Cernuda-Cernuda, R., Garcia-Fernandez, J.M., Gordijin, M.C.M., Bovee-Geurts, P.H.M., DeGrip, W.J., 2003. The eye of the African mole-rat *Cryptomys anselli*: to see or not to see? Eur. J. Neurosci. 17, 709-720.

Chen, C.T., Dun, S.L., Kwok, E.H., Dun, N.J., Chang, J.K., 1999. Orexin A-like immunoreactivity in the rat brain. Neurosci. Lett. 260, 161-164.

Chemelli, R.M., Willie, J.T., Sinton, C.M., Elmquist, J.K., Scammell, T., Lee, C., Richardson, J.A., Williams, S.C., Xiong, Y., Kisanuki, Y., Fitch, T.E., Nakazato, M., Hammer, R.E., Saper, C.B., Yanagisawa, M., 1999. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell 98, 437-451.

Chou, T.C., Rotman, S.R., Saper, C.B., 2004. Lateral hypothalamic acetylcholinesterase-immunoreactive neurons co-express either orexin or melanin concentrating hormone Neurosci. Lett. 370, 123–126.

Cutler, D.J., Morris, R., Sheridhar, V., Wattam, T.A.K., Kolmes, S., Patel, S., Atch, J.R.S., Wilson, S., Buckingham, R.E., Evans, M.L., Leslie, R.A., Williams, G., 1999. Differential distribution of orexin-A and orexin-b immunoreactivity in the rat brain and spinal cord. Peptides 20, 1455-1470.

Date, Y., Ueta, Y., Yamashita, H., Yamaguchi, H., Matsukura, S., Kangawa, K., Sakurai, T., Yanagisawa, M., Nakazato, M., 1999. Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. Proc. Natl. Acad. Sci. USA 96, 748-753.

Da Silva, J.N., Fuxe, K., Manger, P.R., 2006. Nuclear parcellation of certain immunohistochemically identifiable neuronal systems in the midbrain and pons of the Highveld mole-rat (*Cryptomys hottentotus*). J. Chem. Neuroanat. 31, 37-50.

Dwarika, S., Maseko, B.C., Ihunwo, A.O., Fuxe, K., Manger, P.R., 2008. Distribution and morphology of putative catecholaminergic and serotonergic neurons in the brain of the greater canerat, *Thryonomys swinderianus*. J. Chem. Neuroanat. 35, 108-122.

Edwards, C.M.B., Abusnana, S., Sunter, D., Murphy, K.G., Ghatei, M.A., Bloom, S.R., 1999. The effect of the orexins on food intake: comparison with neuropeptide Y, melanin-concentrating hormone and galanin. J. Endocrinol. 160, R7–12.

Espana, R.A., Reis, K.M., Valentino, R.J., Berridge, C.W., 2005. Organization of hypocretin/orexin efferents to locus coeruleus and basal forebrain arousal-related structures. J. Comp. Neurol. 481, 160-178.

Estabrooke, I., McCarthy, M.T., Ko, E., Chou, T.C., Chemelli, R.M., Yanagisawa, M., 2001. Fos expression in orexin neurons varies with behavioral state. J. Neurosci. 21, 1656-1662.

Ettrup, K.S., Sørensen, J.S., Bjarkam, C.R., 2010. The anatomy of the Göttingen minipig hypothalamus. J. Chem. Neuroanat. 39, 151-165.

Gallyas, F., 1979. Silver staining of myelin by means of physical development. Neurolog. Res. 1, 203–209.

Gutjahr, G.H., van Rensburg, L.J., Malpaux, B., Richter, T.A., Bennett, N.C., 2004. The endogenous rhythm of plasma melatonin and its regulation by light in the highveld mole-rat (*Cryptomys hottentotus pretoriae*): a microphthalmic, seasonally breeding rodent. J. Pineal Res. 37, 185-192.

Gravett, N., Bhagwandin, A., Fuxe, K., Manger, P.R., 2009. Nuclear organization and morphology of cholinergic, putative catecholaminergic and serotonergic neurons in the brain of the rock hyrax, *Procavia capensis*. J. Chem. Neuroanat. 38, 57-74.

Hagan, J.J., Leslie, R.A., Patel, S., Evans, M.L., Wattam, T.A., Holmes, S., 1999. Orexin A activates locus coeruleus cell firing and increases arousal in the rat. Proc. Natl. Acad. Sci. USA 96, 10911–10916.

Hungs, M., Fan, J., Lin, L., Lin, X., Maki, R.A., Mignot, E., 2001. Identification and functional analysis of mutations in the hypocretin (orexin) genes of narcoleptic canines. Genome Res. 11, 531-539.

Iqbal, J., Pompolo, S., Sakurai, T., Clarke, I.J., 2001. Evidence that orexin-containing neurones provide direct input to gonadotropin-releasing hormone neurones in the ovine hypothalamus. J Neuroendocrinol. 13, 1033-1041.

Khorooshi, R.M., Klingenspor, M., 2005. Neuronal distribution of melanin-concentrating hormone, cocaine- and amphetamineregulated transcript and orexin B in the brain of the Djungarian hamster (*Phodopus sungorus*). J. Chem. Neuroanat. 29, 137-148.

Kilduff, T.S., Peyron, C., 2000. The hypocretin/orexin ligand-receptor system: implications for sleep and sleep disorders. Trends Neurosci. 23, 359-365.

Kirouac, G.J., Parsons, M.P., Li, S., 2005. Orexin (hypocretin) innervation of the paraventricular nucleus of the thalamus. Brain Res. 1059, 179-188.

Kotz, C.M., Teske, J.A., Levine, J.A., Wang, C., 2002. Feeding and activity induced by orexin A in the lateral hypothalamus in rats. Regul. Pept. 104, 27-32.

Kunii, K., Yamanaka, A., Nambu, T., Matsuzaki, I., Goto, K., Sakurai, T., 1999. Orexins/hypocretins regulate drinking behaviour. Brain Res. 842, 256-261.

Kruger, J-L., Dell, L-A., Pettigrew, J.D., Manger, P.R., 2010. Cellular location and major terminal networks of the orexinergic system in the brains of five microchiropteran species. J Chem Neuroanat. 40, 256-262.

Limacher, A.M., Bhagwandin, A., Fuxe, K., Manger, P.R., 2008. Nuclear organization and morphology of cholinergic, putative catecholaminergic and serotonergic neurons in the brain of the Cape porcupine (*Hystrix afri*caeaustralis): Increased brain size does not lead to increased organizational complexity. J. Chem. Neuroanat. 36, 33-52.

Lovegrove, B.G., Muir, A., 1996. Circadian body temperature rhythms of the solitary Cape mole rat, *Georychus capensis* (Bathyergidae). Physiol. Behav. 60, 991-998.

Lovegrove, B.G., Papenfus, M.E., 1995. Circadian activity rhythms in the solitary Cape mole rat (*Georychus capensis*:Bathyergidae) with some evidence of splitting. Physiol Behav. 58, 679-685.

Lubkin, M., Stricker-Krongrad, A., 1998. Independent feeding and metabolic actions of orexins in mice. Biochem. Biophys. Res. Commun. 25, 241-245.

Manger, P.R., 2005. Establishing order at the systems level in mammalian brain evolution. Brain Res. Bull. 66, 282-289.

Maseko, B.C., Bourne, J.A., Manger, P.R., 2007. Distribution and morphology of cholinergic, putative catecholaminergic and serotonergic neurons in the brain of the Egyptian Rousette flying fox, *Rousettus aegyptiacus*. J. Chem. Neuroanat. 34, 108-127.

McGranaghan, P.A., Piggins, H.D., 2001. Orexin A-like immunoreactivity in the hypothalamus and thalamus of the Syrian hamster (*Mesocricetus auratus*) and Siberian hamster (*Phodopus sungorus*), with special reference to circadian structures. Brain Res. 904, 234-244.

Mintz, E.M., van Den Pol, A.N., Casano, A.A., Albers, H.E., 2001. Distribution of hypocretin-(orexin) immunoreactivity in the central nervous system of Syrian hamsters (*Mesocricetus auratus*). J. Chem. Neuroanat. 21, 225–238.

Mondal, M.S., Nakazato, M., Date, Y., Murakami, N., Hanada, R., Sakata, T., Matsukura, S., 1999. Characterization of orexin-A and orexin-B in the microdissected rat brain nuclei and their contents in two obese rat models. Neurosci. Lett. 273, 45-48.

Moore, R.Y., Abrahamson, E.A., Van Den Pol, A., 2001. The hypocretin neuron system: an arousal system in the human brain. Arch. Ital. Biol. 139, 195-205.

Nambu, T., Sakurai, T., Mizukami, K., Hosoya, Y., Yanagisawa, M., Goto, K., 1999. Distribution of orexin neurons in the adult rat brain. Brain Res. 827, 243-260.

Negroni, J., Bennett, N.C., Cooper, H.M., 2003. Organization of the circadian system in the subterranean mole rat, *Cryptomys hottentotus* (Bathyergidae). Brain Res. 967, 48-62.

Nemec, P., Burda, H., Peichl, L., 2004. Subcortical visual systems of the African mole-rat *Cryptomys anselli*: to see or not to see? Eur. J. Neurosci. 20, 757-768.

Nixon, J.P., Smale, L., 2007. A comparative analysis of the distribution of immunoreactive orexin A and B in the brains of nocturnal and diurnal rodents. Behav. Brain Funct. 13, 28.

Novak, C.M., Albers, H.E., 2002. Localization of hypocretin-like immunoreactivity in the brain of the diurnal rodent, *Arvicanthis niloticus*. J. Chem. Neuroanat. 23, 49-58.

Oelschlager, H.H.A., Nakamura, M., Herzog., Burda, H., 2000. Visual system labelled by c-Fos immunohistochemistry after light exposure in the ‘blind’ subterranean Zambian mole-rat (*Cryptomys anselli*). Brain Behav. Evol. 55, 209-220.

Oosthuizen, M.K., Cooper, H.M., Bennett, N.C., 2003. Circadian rhythms of locomotor activity in solitary and social species of African mole rats (Family: Bathyergidae). J. Biol. Rhythms 18, 481-490.

Peyron, C., Tighe, D.K., van den Pol, A.N., de Lecea, L., Heller, H.C., Sutcliffe, J.G., Kilduff, T.S., 1998. Neurons containing hypocretin (orexin) project to multiple neuronal systems. J. Neurosci. 18, 9996-10015.

Pieters, R.P., Gravett, N., Fuxe, K., Manger, P.R., 2010. Nuclear organization of cholinergic, putative catecholaminergic and serotonergic nuclei in the brain of the eastern rock elephant shrew, *Elephantulus myurus*. J. Chem. Neuroanat. 39, 175-188.

Piper, D.C., Upton, N., Smith, M.I., Hunter, A.J., 2000. The novel brain neuropeptide, orexin-A, modulates the sleep-wake cycle of rats. Eur. J. Neurosci.12, 726–730.

Pu, S., Jain, M.R., Kalra, P.S., Kalra, S.P., 1998. Orexins, a novel family of hypothalamic neuropeptides, modulate pituitary luteinizing hormone secretion in an ovarian steroid dependent manner. Reg. Peptides 78, 133–136.

Raghanti, M,A., Stimpson, C.D., Marcinkiewicz, J.L., Erwin, J.M., Hof, P.R., Sherwood, C.C., 2008. Cholinergic innervation of the frontal cortex: differences among humans, chimpanzees, and macaque monkeys. J. Comp. Neurol. 506, 409-424.

Raghanti, M.A., Stimpson, C.D., Marcinkiewicz, J.L., Erwin, J.M., Hof, P.R., Sherwood, C.C., 2008. Differences in cortical serotonergic innervation among humans, chimpanzees, and macaque monkeys: a comparative study. Cereb. Cortex 18, 584-597.

Risold, P.Y., Griffond, B., Kilduff, T.S., Sutcliffe, J.G., Fellmann, D., 1999. Preprohypocretin (orexin) and prolactin-like immunoreactivity are coexpressed by neurons of the rat lateral hypothalamic area. Neurosci. Lett. 259, 153–156.

Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R.M., Tanaka, H., Williams, S.C., Richardson, J.A., Kozlowski, G.P., Wilson, S., Arch, J.R.S., Buckingham, R.E., Haynes, A.C., Carr, S.A., Annan, R.S., McNulty, D.E., Liu, W.S., Terrett, J.A., Elshourbagy, N.A., Bergsma, D.J., Yanagisawa, M., 1998. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 92, 573-585.

Siegel, J,M., 1999. Narcolepsy: a key role for hypocretins (orexins). Cell 98, 409-412.

Takahashi, N., Okumura, T., Yamada, H., Kohgo, Y., 1999. Stimulation of gastric acid secretion by centrally administered orexin A in conscious rats. Biochem. Biophys. Res. Commun. 254, 623–627.

Thannickal, T.C., Moore, R.Y., Nienhuis, R., Ramanathan, L., Gulyani, S., Aldrich, M., Cornford, M., Siegel, J.M., 2000. Reduced number of hypocretin neurons in human narcolepsy. Neuron 27, 469–474.

Vidal, L., Blanchard, J., Morin, L.P., 2005. Hypothalamic and zona incerta neurons expressing hypocretin, but not melanin concentrating hormone, project to the hamster intergeniculate leaflet. Neuroscience 134, 1081-1090.

van den Pol, A.N., 2000. Narcolepsy: a neurodegenerative disease of the hypocretin system? Neuron 27, 415–418.

Yamamoto, Y., McKinley, M.J., Nakazato, M., Yamashita, H., Shirahata, A., Ueta,Y., 2006. Postnatal development of orexin-A and orexin-B like immunoreactivities in the Eastern grey kangaroo (*Macropus giganteus*) hypothalamus. Neurosci. Lett. 392, 124-128.

Yoshida, K., McCormack, S., Espana, R.A., Crocker, A., Scammell, T.E., 2006. Afferents to the orexin neurons of the rat brain. J. Comp. Neurol. 494, 845-861.

Yoshimichi, G., Yoshimatsu, H., Masaki, T., Sakata, T., 2001. Orexin-A regulates body temperature in coordination with arousal status. Exp. Biol. Med. 226, 468-476.

Zhang, J.H., Sampogna, S., Morales, F.R., Chase, M.H., 2001. Orexin (hypocretin)-like immunoreactivity in the cat hypothlamus: a light and electron microscopic study. Sleep 24, 67–76.

Zhang, J.H., Sampogna, S., Morales, F.R., Chase, M.H., 2002. Co-localization of hypocretin-1 and hypocretin-2 in the cat hypothalamus and brainstem. Peptides 23, 1479–1483.

Figure legends

Figure 1: Drawings of sections through one half of the brain of the Cape-dune mole rat (*Bathyergus suillus*) depicting the distribution of orexinergic terminal network densities (an absence of shading represents low density, grey shaded areas represents medium density and black shaded areas represents high density) and Orx immunoreactive neurons (each black dot represents a single neuron) relative to the nuclear organization of the cholinergic system, catecholiminergic system and serotonergic system [previously described in Bhagwandin et al. (2008) for this species]. Absence of shading indicates either a minor terminal network or no terminal network (see text for details). See list for abbreviations.

Figure 2: Drawings of sections through one half of the brain of the Highveld mole rat (*Cryptomys hottentotus*) depicting the distribution of orexinergic terminal network densities (an absence of shading represents low density, grey shaded areas represents medium density and black shaded areas represents high density) and Orx immunoreactive neurons (each black dot represents a single neuron) relative to the nuclear organization of the cholinergic system, catecholaminergic system and serotonergic system [previously described in Bhagwandin et al. (2008) for this species]. Absence of shading indicates either a minor terminal network or no terminal network (see text for details).See list for abbreviations.

Figure 3: **A**. Photomicrographic montage showing the Orx immunoreactive neurons within the hypothalamus of *Bathyergus suillus*. Scale = 500um. **B**. and **C**. High power photomicrographs showing the morphology of Orx immunoreactive neurons within the hypothalamus of *Bathyergus suillus*. Scale = 50um.

Figure 4: High power photomicrographs showing varying orexinergic terminal network densities in the brains of *Cryptomys hottentotus* (**A**-**D**) and *Bathyergus suillus* (**E**). **A**. High density terminal network within the A6d neuronal group. **B**. Low density terminal network within the cingulate cortex. **C**. Medium density terminal network in the region of

the C1 nucleus. **D**. Medium density terminal network within the cholinergic medial septal nucleus. **E**. Medium density terminal networks surrounding the central canal. Scale = 100 µm and applies to all. See list for abbreviations.

Figure 5: Photomicrographs illustrating the difference in expression of high density orexinergic terminal networks within the paraventricular nucleus of: **A**. *Bathyergus suillus*, **B**. *Cryptomys hottentotus*. Scale = 500um and applies to both. See list for abbreviations.

Figure 6: High power photomicrograph showing varying terminal network densities within the brain of *Cryptomys hottentotus*. **A**. high density orexinergic terminal networks within the serotonergic dorsal raphe nuclear complex (**DR**). **B**. high density orexinergic terminal networks in the region of the serotonergic median raphe nucleus (**MnR**). **C**. medium density oerxinergic terminal networks within the serotonergic raphe obscurus (**ROb**) nucleus, Scale = 50um and appiles to all. See list for abbreviations.

Figure 1A

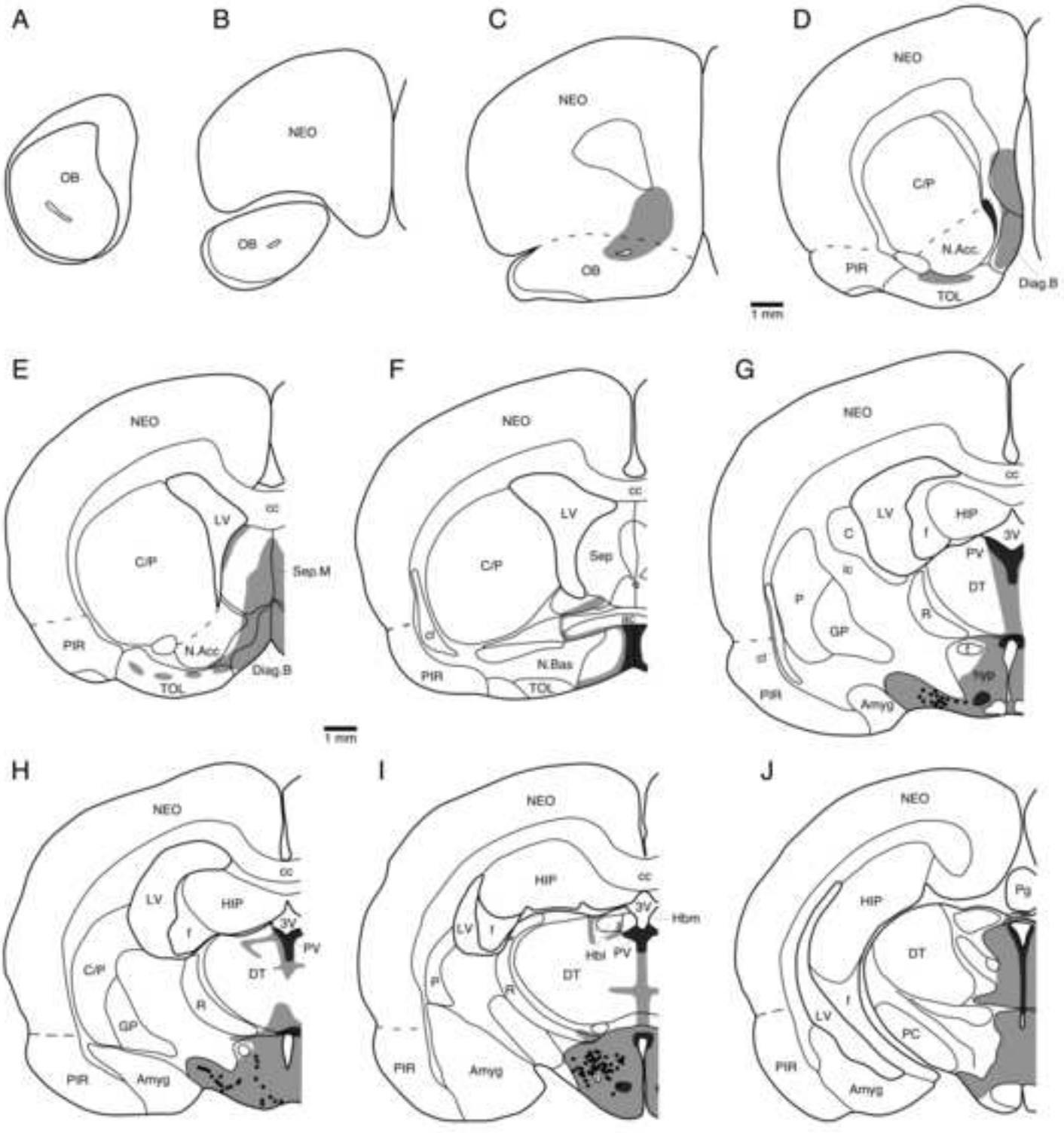

A
B
C
D
OB
NEO
OB
NEO
OB
C/P
N.Acc
PIR
TOL
Diag.B
1 mm
E
F
G
NEO
LV
cc
C/P
Sep.M
PIR
N.Acc
TOL
Diag.B
Sep
N.Bas
HIP
3V
PV
DT
P
GP
R
Hyp
Amyg
1 mm
H
I
J
HIP
f
Hbm
Hbl
Pg
PC

Figure 1B

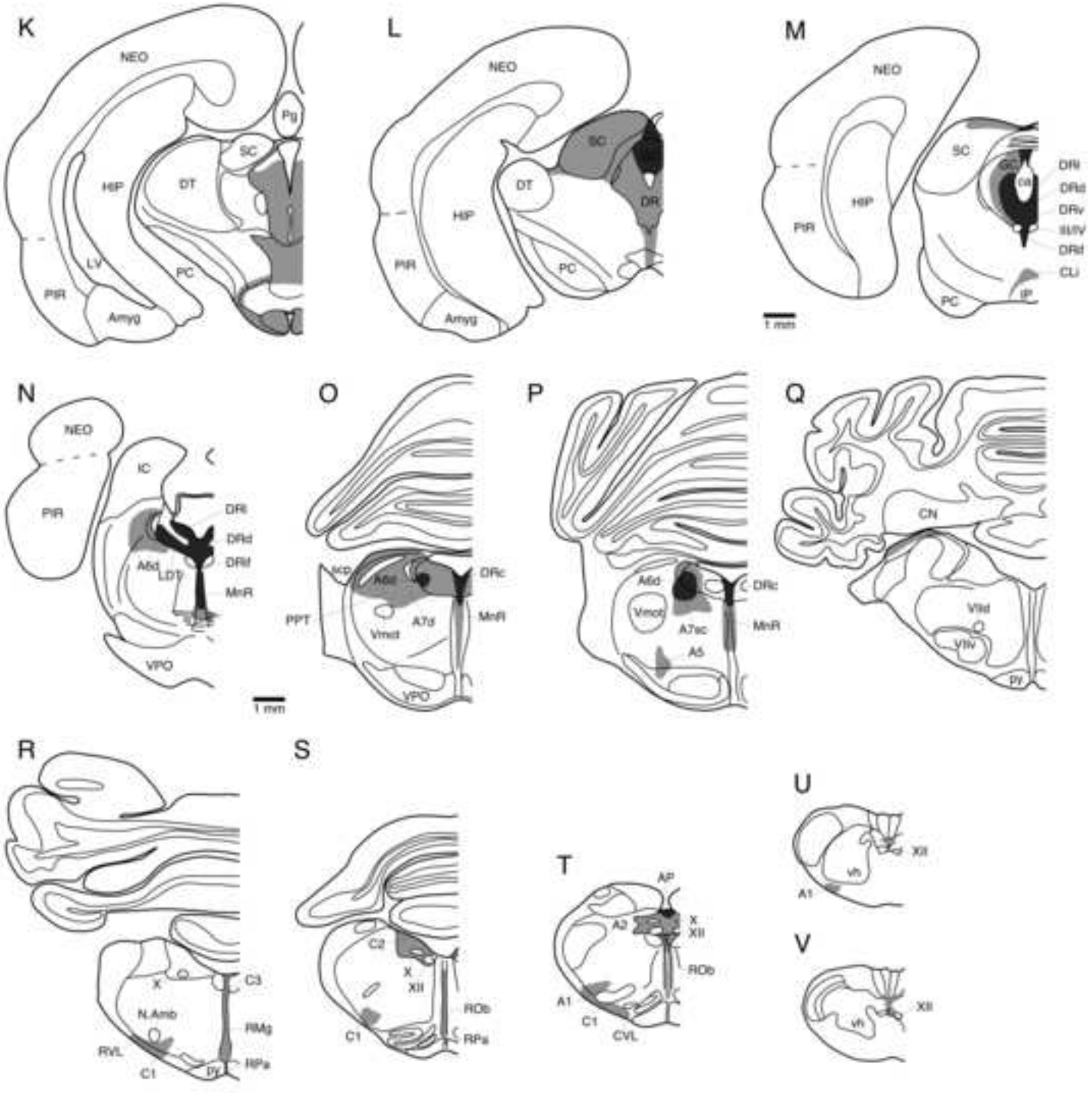

K
NEO
Pg
SC
HIP
DT
LV
PIR
PC
Amyg
L
NEO
SC
DT
DR
HIP
PIR
PC
Amyg
M
NEO
SC
HIP
PIR
PC
DRI
DRd
DRv
ll/lV
DRd
CLi
IP
1 mm
N
NEO
IC
PIR
DRI
DRd
DRlf
A6d
LDT
MnR
VPO
1 mm
O
DRc
MnR
PPT
A7d
Vmot
VPO
P
A6d
DRc
Vmot
A7sc
MnR
A5
Q
CN
Vlld
Vllv
py
R
C3
X
N.Amb
RMg
RVL
C1
py
RPa
S
C2
X
XII
ROb
C1
RPa
T
AP
X
XII
A2
ROb
A1
C1
CVL
U
XII
vh
A1
V
XII
vh

Figure 2A

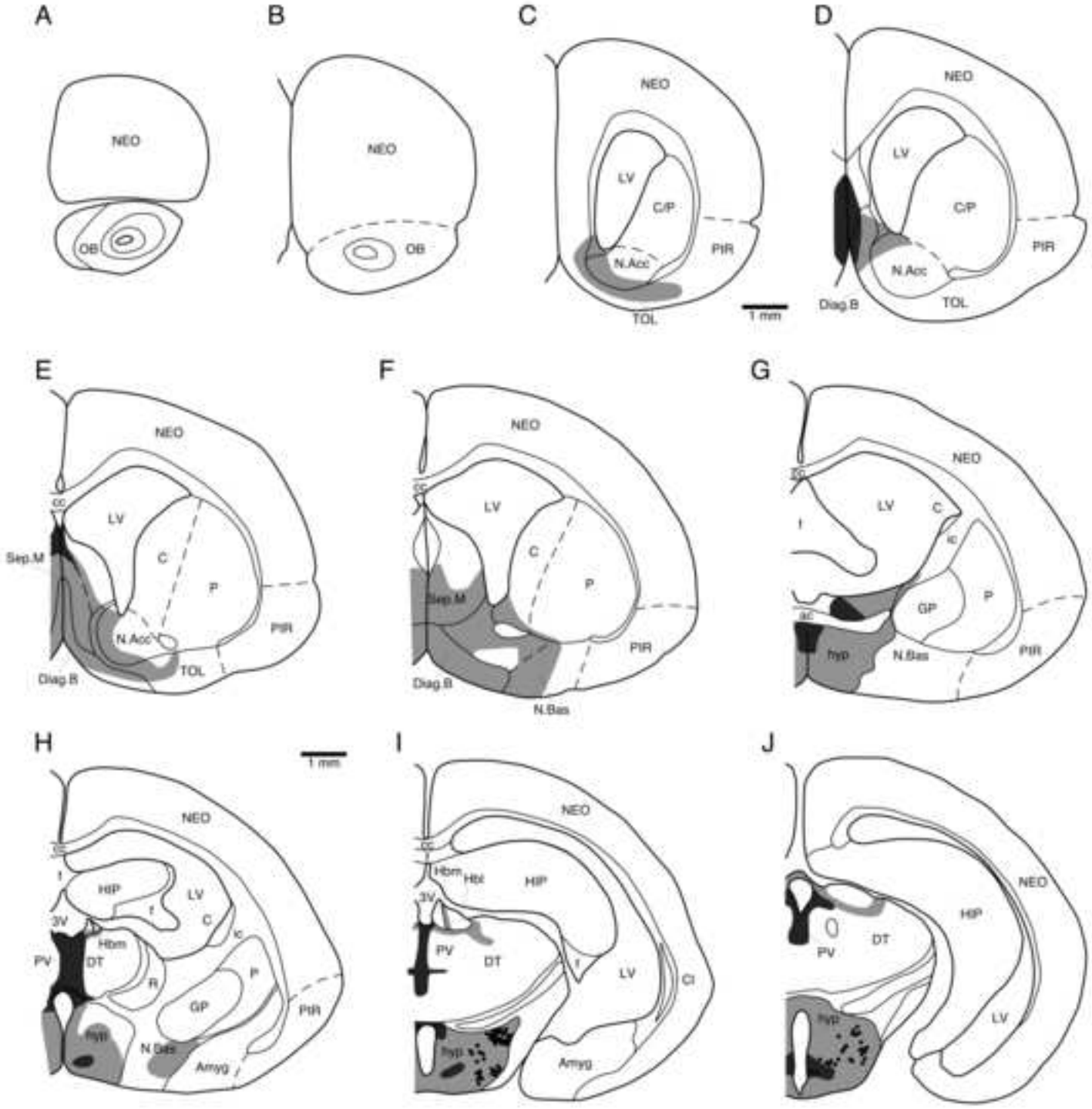

A
B
C
D
NEO
OB
LV
C/P
N.Acc
PIR
TOL
Diag.B
1 mm
E
F
G
Sep.M
P
GP
hyp
N.Bas
H
I
J
HIP
Hbm
Hbl
3V
PV
DT
R
Amyg
CI

Figure 2B

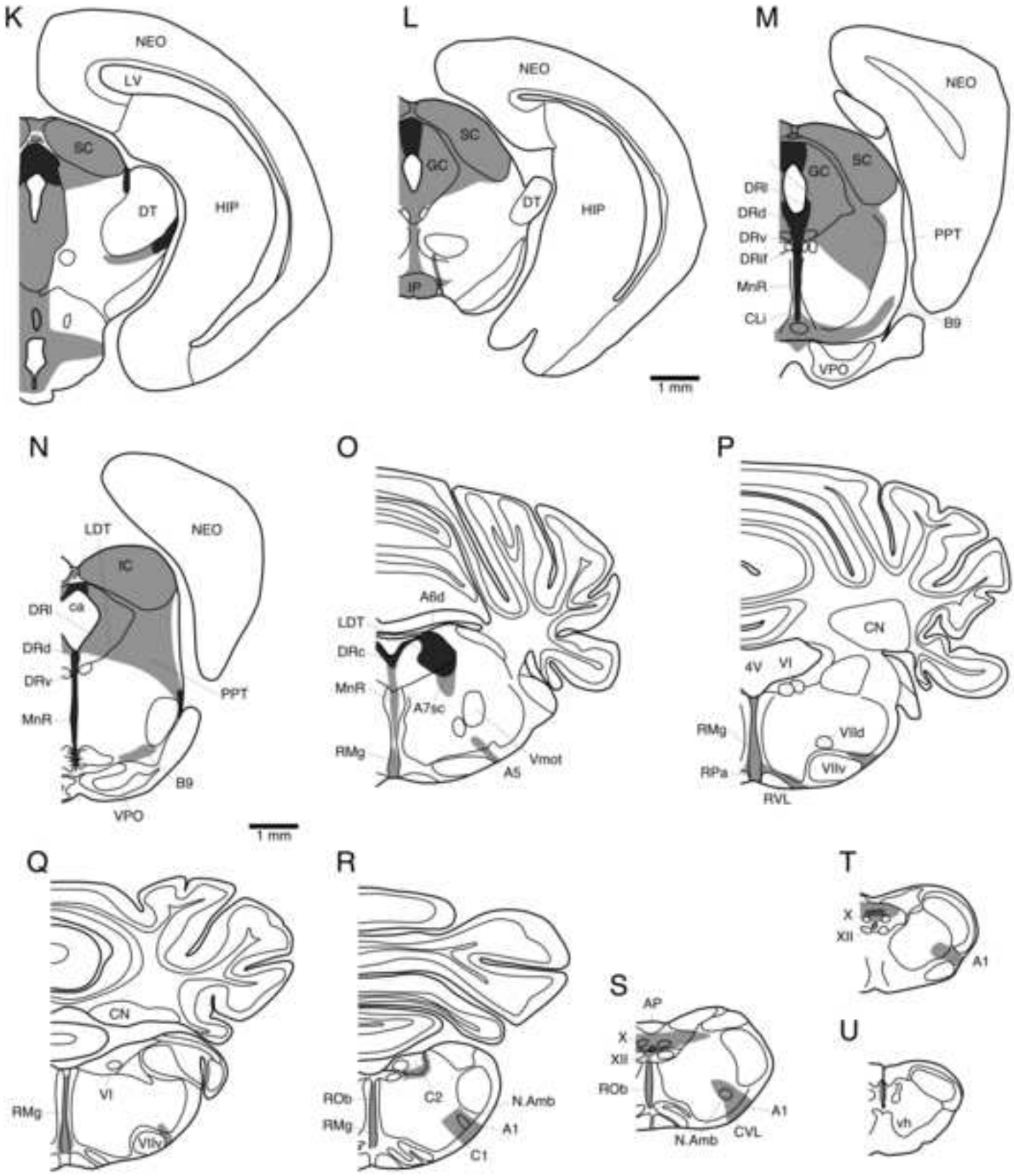

K
NEO
LV
SC
DT
HIP
L
NEO
SC
GC
DT
HIP
IP
1 mm
M
NEO
SC
GC
DR
DRd
DRv
DRlf
MnR
CLi
PPT
B9
VPO
N
LDT
NEO
IC
DRl
ca
DRd
DRv
MnR
PPT
B9
VPO
1 mm
O
A6d
LDT
DRc
MnR
A7sc
RMg
Vmot
A5
P
CN
4V
VI
RMg
VIId
RPa
VIIv
RVL
Q
CN
RMg
VI
VIIv
R
RObRMg
C2
N.Amb
A1
C1
S
AP
X
XII
RObRMg
A1
N.Amb
CVL
T
X
XII
A1
U
vh

Figure 3

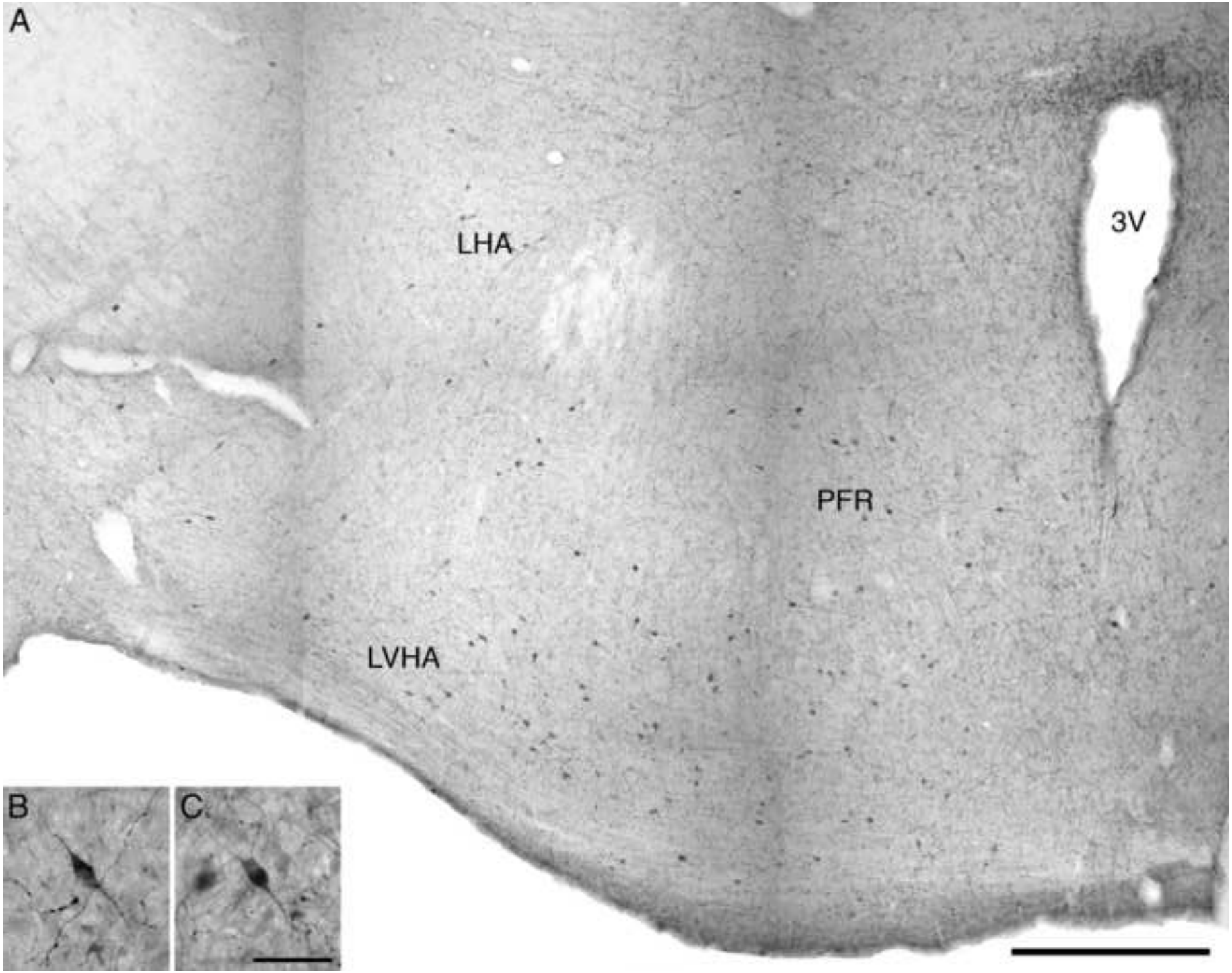


Figure 4

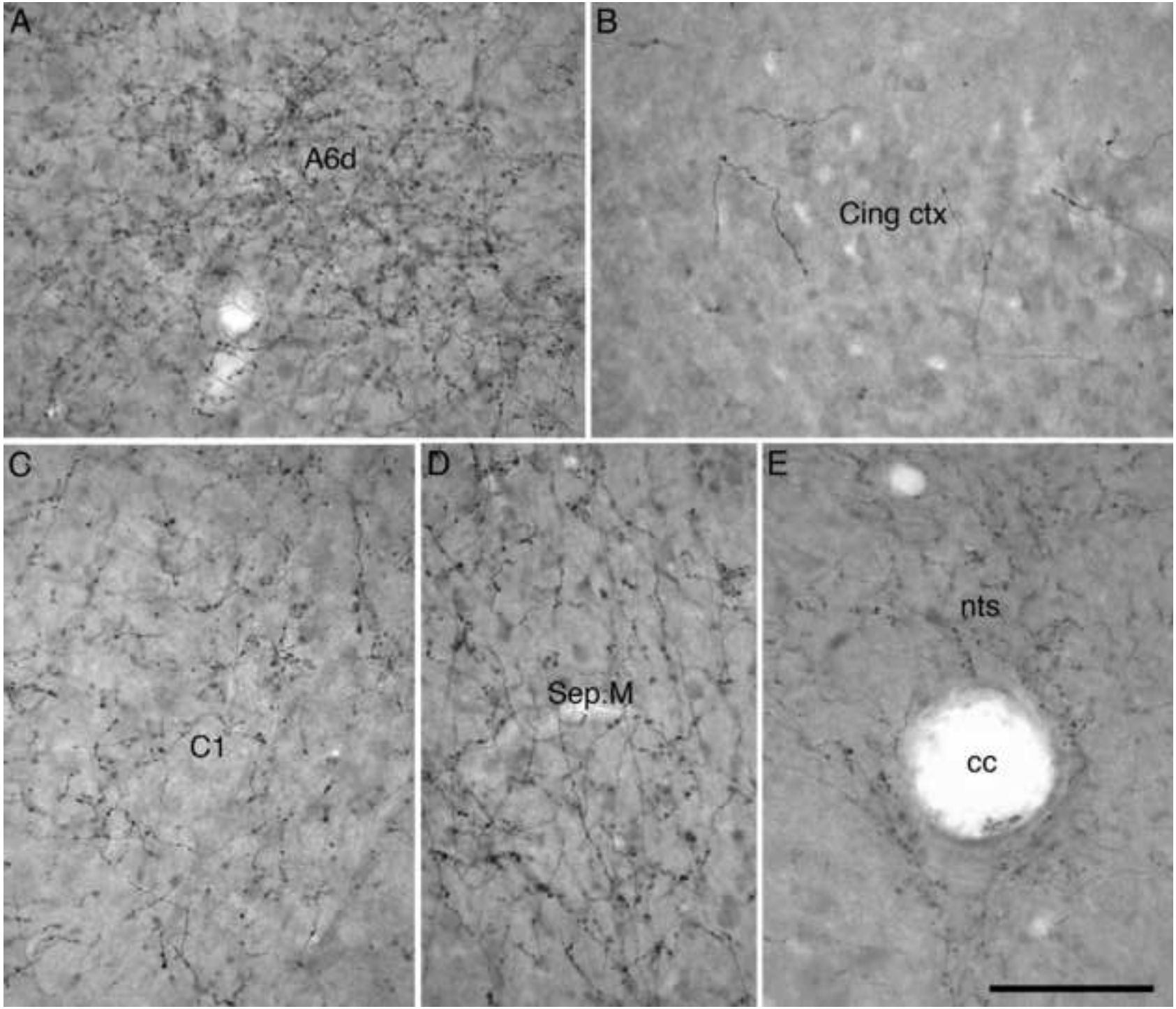


Figure 5

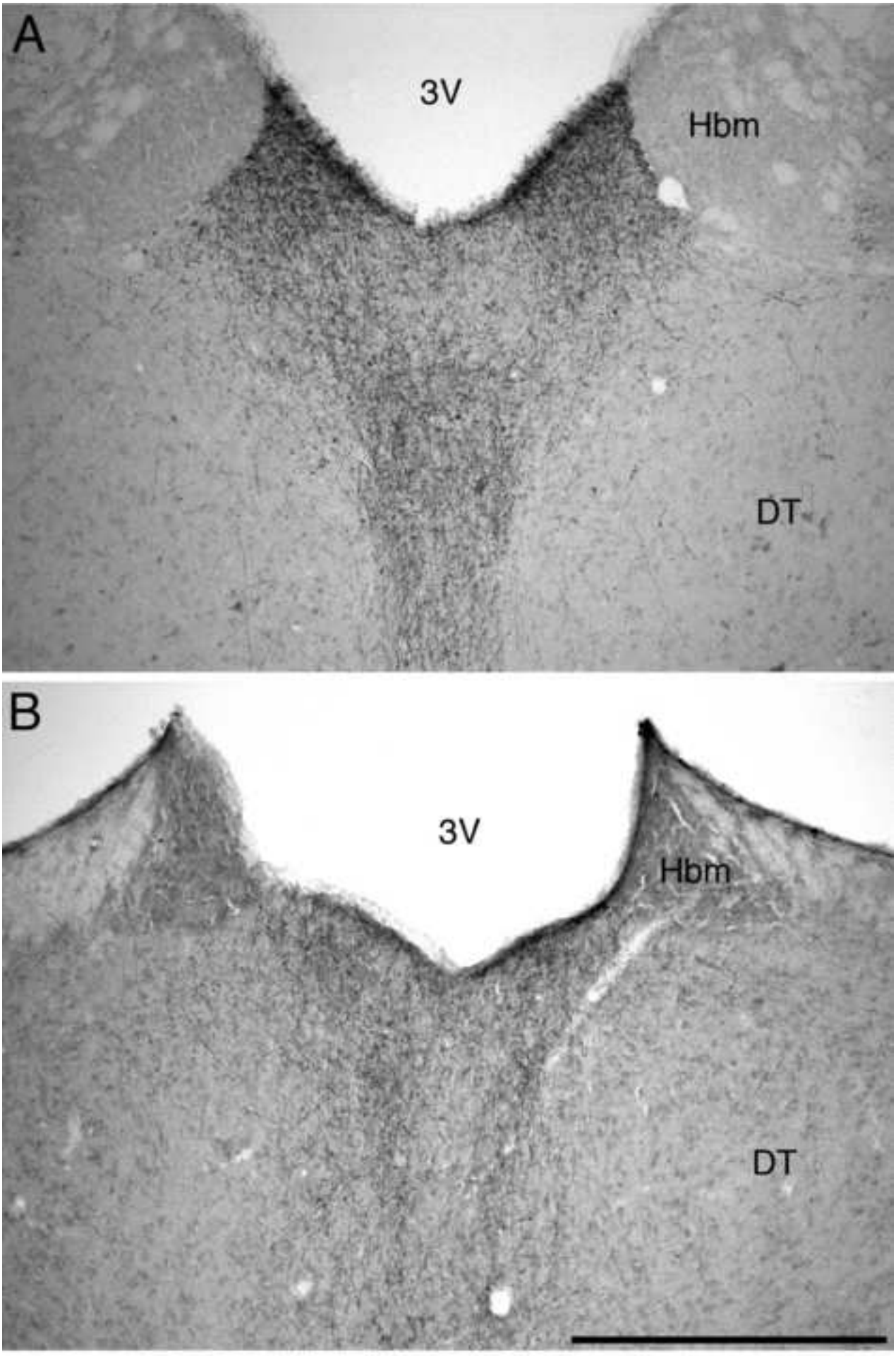
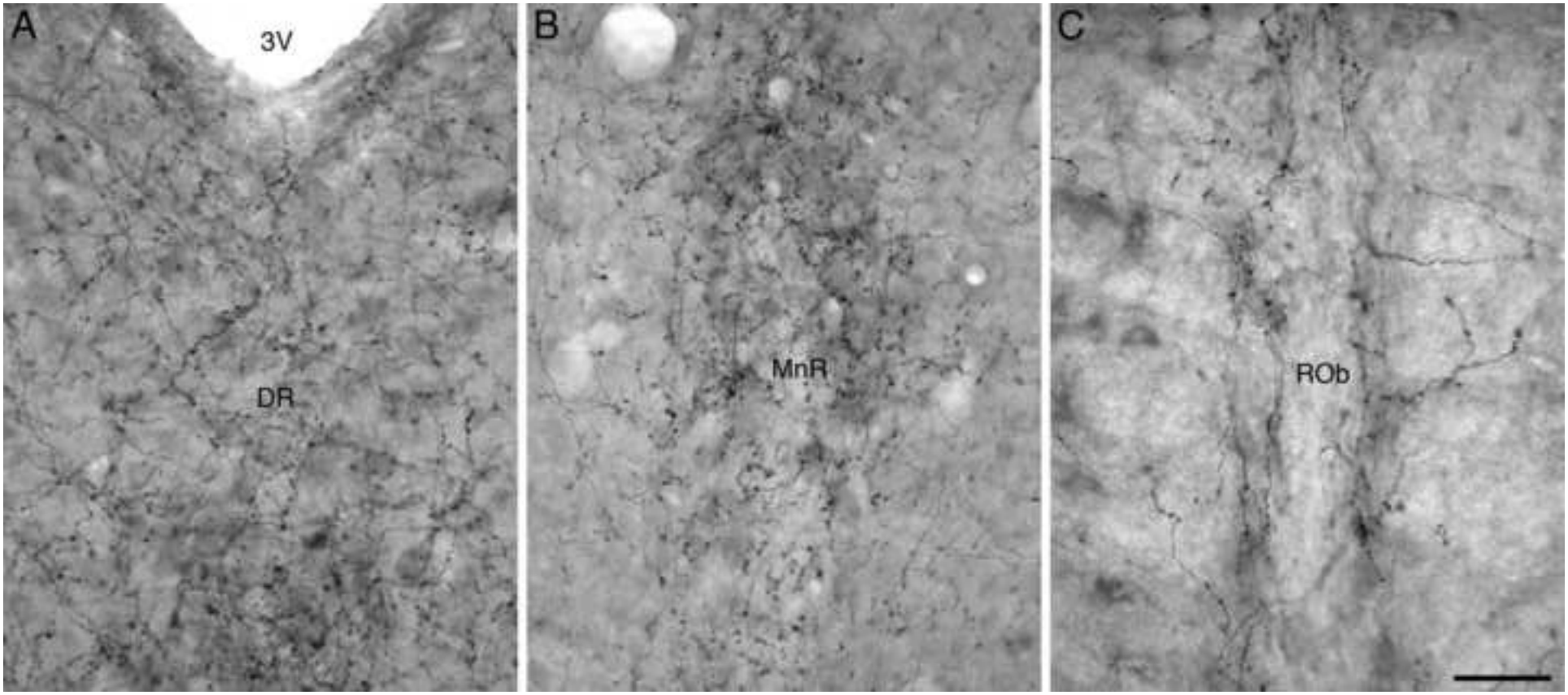


Figure 6



***Ethical Statement**

The mole rats used in the present study were caught from wild populations in South Africa under permission and supervision from the appropriate wildlife directorates. All animals were treated and used according to the guidelines of the University of the Witwatersrand Animal Ethics Committee, which parallel those of the NIH for the care and use of animals in scientific experimentation.