ORIGINAL ARTICLE

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LOCALIZATION PROFILE OF CATHEPSIN L IN THE BRAIN OF AFRICAN GIANT RAT (*Cricestomys gambianus*)

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ABSTRACT

Cathepsins, are members of the papain superfamily of mammalian lysosomal cysteine proteases. Among others there are two prominent members with broad substrate specificity, these are cathepsin B and cathepsin L that are known to be involved in the process of intra- and extra-cellular protein degradation and turnover. However, the *in vivo* targets of cathepsin L in nervous tissues are yet to be identified. We examined by immunofluorescence studies the distribution pattern of cathepsin L protein and determine the specific cell types synthesizing the enzyme in the brain of African giant rats (*Cricetomys gambianus*). Results showed that Cathepsin L protein was localized in various brain regions of the giant rats. In the telencephalon, immunoreactivity was identified in cerebral cortex and subcortical structures, hippocampus, amygdala and basal ganglia. Within the diencephalon high density of positive signals was observed in mediodorsal and lateral posterior thalamic nuclei and medial habenular nucleus. In the mesencephalon, cathepsin L was detected in the substantia nigra and cerebral peduncles. Strong labeling in the hypothalamus was present in the anterior commissure and median eminence while in the cerebellum cathepsin L was observed in the deep white matter, granule cell layer, stellate, and basket cells of cerebellar cortex and in the Purkinje neurons. The distribution pattern and functional implications of cathespin L in relation to spatial memory establishment, learning coordination and disease mechanisms is discussed.

Keywords: Cathepsin L, immunofluorescence, *Cricestomys*, brain

INTRODUCTION

Cathepsins, are members of the papain superfamily of lysosomal cysteine proteases. Various types of cathepsins have been identified including cathepsin B, D, H, L, S and P (Barrett and Kirschke, 1981; Maubach et al., 1997) which are distinguished by their structure and protein they cleave (Rawlings et al., 2014). Cathepsins are widely distributed in various biological tissues and fluids (Cowan et al., 2005). They play a major role in lysosomal protein degradation, and are considered to have several important functions, including bone protein turnover, antigen presentation and disease related tissue remodelling (Mohamed et al., 1996; Turk et al., 2012). Cathepsins are expressed as inactive precursor proteins with N-terminal propeptides (Mach et al., 1994; Ménard et al., 1998). In the processing of these proteases, the precursor proteins become active as mature enzymes by releasing the propeptides at low pH in lysosomes (Mach et al., 1994; Katutuma and Kominami, 1995). The propeptides are known to exhibit specific inhibition to their cognate cathepsins (Fox et al., 1992; Carmona et al., 1996)

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It has also been shown that the propeptides purified from a given cathepsin can inhibit the activity of that enzyme *in vitro* (Delaria et al., 1994; Coulombe et al., 1996). Structurally, Cytotoxic T-lymphocyte antigen-2 alpha (CTLA-2α) discovered in mouse activated T-lymphocytes (Denizot et al., 1989) is homologous to the pro-region of the cathepsin L and exhibits selective inhibition of cathepsin L (Kurata et al., 2001, Desha et al., 2010). Simultaneous inhibition of multiple cathepsins in hippocampus was found to block long-term spatial memory in mouse (Dash et al., 2000), while the sharp modulation in the expression of crammer (CTLA-2α analogy) correlated well with the establishment of long-term memory (Comas et al., 2004). Prolonged activation of these cathepsins is reported to be associated with neuronal degeneration in Alzheimer's disease (Nixon, 2000). Regions that contained high density of amyloid precursor proteins in the brain were also found to express high level concentrations of cathepsin B and L mRNA (Callahan et al., 1998). Similarly, mice lacking cathepsins B and L showed neuronal loss and brain atrophy (Bednarski et al., 1997; Felbor et al., 2002). However, the cellular localization and physiological function of cathepsin L in the brain is not well understood.

We developed interest to use giant rats to study the distribution pattern of cathepsin L in the brain because giant rats have great ability to learn and remember, well developed sense of smell and can be trained easily (Verhagen et al., 2003; Bart et al., 2010). The ability to sniff in the rats has also been linked to adult neurogenesis in the olfactory bulb (Olude et al 2014). In this context, the giant rat appears to provide an excellent model for the study of the distribution pattern and functional implications of cathepsin L in the brain in relation to memory establishment and learning coordination.

MATERIALS AND METHODS

Animals

Giant rats (*Cricestomys gambianus*) which are native to sub-Saharan Africa are nocturnal and omnivorous members of the Nesomyidae family within the Muroidea superfamily. They are large colony-dwelling rodents, with adult body lengths of 25 to 45 cm and tail lengths of 35 to 45 cm. Adult weigh 1 to 1.5 kg. Both sexes reach reproductive maturity at 7 to 8 months. Pregnant females give birth to 1 to 5 pups following a gestation period of 27 to 36 days; in the wild and in captivity, several litters can be produced each year. The rats live up to 8 years in captivity. They are agricultural pests in the wild and an invasive species in Florida (USA). In Africa, they are sometimes hunted and eaten.

Housing of the rats

Sokoine University of Agriculture through APOPO (Anti-Persoonsmijnen Ontmijnende Product Ontwikkeling) has giant rats that are trained to detect landmines (Bart et al., 2010). APOPO personnel established breeding colony in which wild-caught males and females were housed to live under conditions as close as possible to their natural environment. Pups were taken from their parents at various ages and handled extensively in an effort to produce gentle, social and easily trained rats that are more tractable. The rats were weaned at 4 weeks of age and tagged for identification. The rats ate a varied diet of fruits, vegetables, grains, and commercial rodent chow. A veterinarian regularly examined the rats and provided health care as needed.

Tissue preparation

All experiments conformed to the law concerning the protection and control of animals (guidelines for animal experimentation) of Sokoine University of Agriculture. Five adult male and five female African giant rats after completion of two months training for landmines detection were taken and prepared for this study. The rats were anesthetized with sodium pentobarbital

(70mg/kg) by intraperitoneal injection and transcardiac perfusion with 0.01M phosphate-buffered saline (PBS; pH 7.4), followed by 4%paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO) in 0.1 phosphate buffer (PB; pH 7.4). Brain tissues were dissected and postfixed in 4% PFA for 2 hours at 4°C before processing to paraffin wax and sectioning.

Antibody generation

Recombinant cathepsin L was purified using methods described previously with minor modifications (Kurata et al., 2003) Affinity-purified rabbit anti-cathepsin L Immunoglobulins G were obtained as described previously (Camenisch et al., 1999;). In brief, egg yolk immunoglobulin fractions were prepared from eggs laid by hens immunized against recombinant cathepsin L. Chicken anti-cathepsin L Immunoglobulins Y were affinity-purified through columns with recombinant protein-conjugated resins. The specificity of the purified antibody was characterized by Western blot as shown in our previous report (Bui et al., 2014).

Immunofluorescence analysis

Sections were deparaffinized in xylene and then rehydrated through a descending ethanol series to phosphate-buffered saline (0.01M PBS-pH7.4). Tissue sections were immersed in a solution of 0.3%v/v hydrogen peroxide in distilled water for 30min at room temperature (RT) to inhibit endogenous peroxidase activity and then washed (3x5 min) in PBS. Sections were incubated with 10% goat normal serum for 30 min at RT to block non-specific binding. The sections were incubated with the cathepsin L antibody diluted at 1:500 in PBS, for 24 h in a dark, humid chamber at 4°C. For negative control, PBS was applied in place of primary antibody. Sections were then washed (3X5min) in PBS followed by incubation with Alexa Fluor® 488-conjugated chicken anti-rabbit IgG (FITC) at a dilution of 1:100 (Molecular Probes) for 1hour at RT. At the end of incubation, the sections were washed (3X5min) in PBS and mounted. Immunolabeling was analyzed using Olympus BH-2 microscope fitted with Olympus camera. Morphological structures refer to the neuron-anatomical atlas from Paxinos and Franklin (2001).

RESULTS

Detection of cathepsin L in cerebral cortex and subcortical structures

In the cortices, labelling for cathepsin L was observed in all layers of the retrosplenial agranular cortex and in the fibres of cingulum (Fig. 1A).

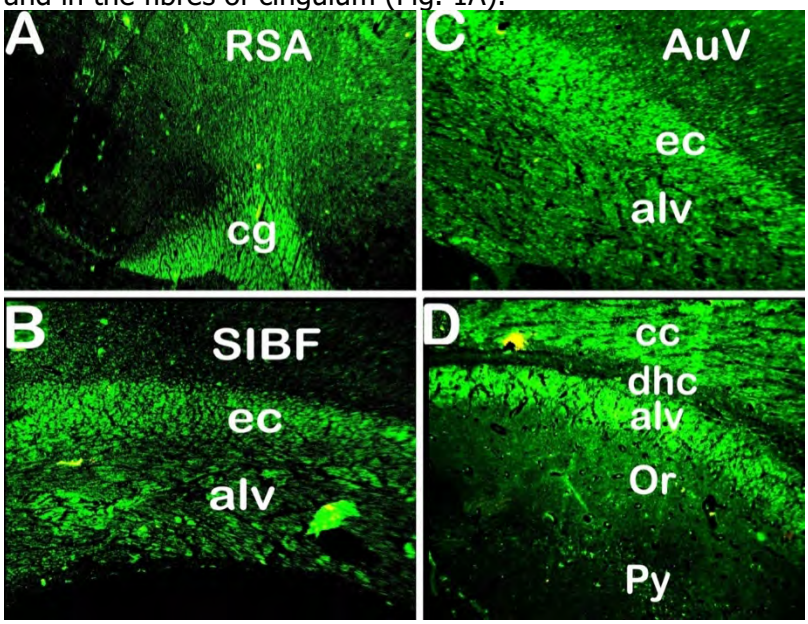

Fig. 1: Immunofluorescence localization of cathepsin L protein in coronal sections of the cerebral cortex, subcortical structures and hippocampus. Strong immunolabeling is seen in **(A)** retrosplenial agranular cortex (RSA) and cingulum (cg), **(B,C and D)** External capsule (ec); corpus callosum (cc) and alveus of the hippocampus (alv) and moderately in secondary auditory cortex ventral area (AuV), primary somatosensory cortex (SIBF), pyramidal neurons (py) and stratum oriens (Or) and none in dorsal hippocampal commissure (dhc).

The cingulum contains prominent medial and dorsal prefrontal connections, including those of the anterior cingulate cortex. All these were densely labeled for cathepsin L. Numerous positive signals were also observed in the external capsule, corpus callosum and alveus of the hippocampus while moderately appeared in ventral area of secondary auditory cortex and primary somatosensory cortex (Fig. 1B, C and D).

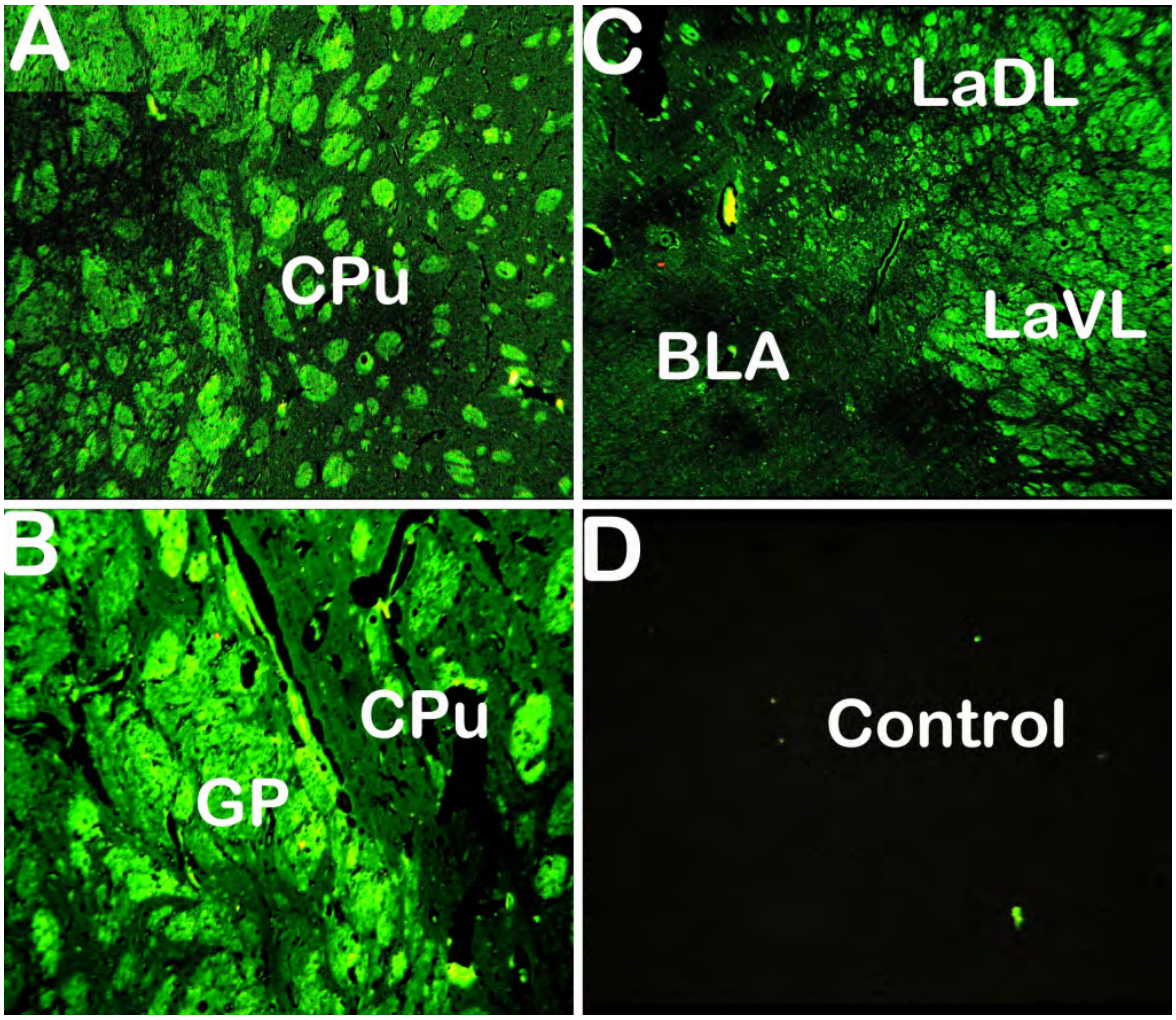

Fig. 2: Immunofluorescence labeling of cathepsin L protein in coronal sections of basal ganglia and amygdala. Intense labeling is observed in **(A)** caudate putamen (CPu), **(B)** Globus pallidus(GP), and in **(C)** in lateral amygdaloid dorsal-dorsolateral part (LaDL) and lateral amygdaloid dorsal-ventrolateral part (LaVL) but is not observed in basolateral amygdaloid nuclei (BLA) and in **(D)** the control section.

Cathespin L immunoreactivity in the basal ganglia

Immunoreactivity was extensively distributed in fibres of globus pallidus, in rostral and caudal areas of dorsal striatum {caudate-putamen} (Fig. 2A and B). Strong labelling was also observed in the amygdala including the dorsolateral and ventrolateral parts of lateral amygdaloid dorsal nuclei but was not detected in basolateral amygdaloid nuclei and in the control sections (Fig. 2 C and D).

Hippocampus and habenular bodies

The highest level of immunoreactivity for cathepsin L in the hippocampus was detected in

fimbria of hippocampus and moderately in the axonal fibres in stratum lucidum where mossy fibres from all parts of the granule cell layer of the dentate gyrus terminate at pyramidal neurons and interneurons in subfields of Cornu Amonis 3 (CA3) region (Fig. 3A-D). Immunofluorescent signals were also detected in the medial habenular nucleus, a chief relay nucleus of the descending dorsal diencephalic conduction system (Fig. 3B). Lateral to the fimbria of hippocampus is the internal capsule connected dorsally to the external capsule.

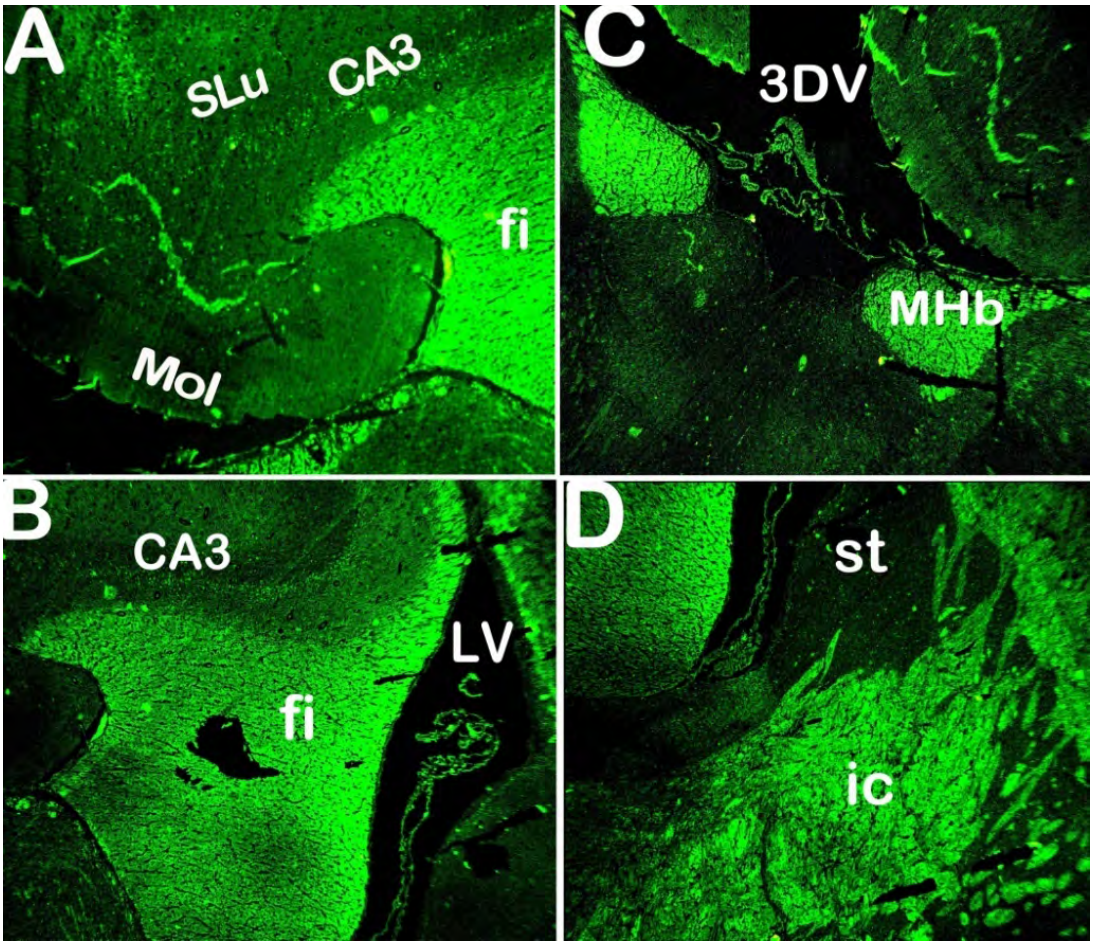

Fig. 3: Immunofluorescence localization of cathepsin L protein in coronal sections of the hippocampus and habenular bodies. Strong labeling is shown in **(A** and **B)** fimbria of the hippocampus (fi) and moderately in ventral hippocampus in stratum lucidum (SLu) in mossy fibres that project to CA3 pyramidal neurons. Labeling is not seen in the molecular layer (Mol). Positive signals were also present in **(C)** Medial habenular bodies (MHb) and **(D)** in the internal capsule (ic) but were not observed in stria terminalis (st).

All these structures were densely labelled for cathepsin L (Fig. 3 D). The internal capsule conveys information from primary and supplementary motor areas, frontopontine and thalamic peduncles to the brain stem and cerebellar regions and from thalamus to prefrontal cortex. Various thalamic nuclei also showed strong labelling for cathepsin L.

Mesencephalon and Ventricular system

Choroid plexus located in the ventricular system is important in maintaining generation and flow

of cerebrospinal fluid (CSF). The plexus displayed high level of cathepsin L immunoreactivity within epindymal cells (Fig. 4A). Within the mesencephalon, immunoreactivity was observed in the cerebral peduncle that forms a continuation of the internal capsule of the cerebral hemispheres. Cells of the pallidal portion of the basal ganglia, which are found interspersed in the internal capsule, are also found in the cerebral peduncles where they are known as the reticular portion of the substantia nigra that play important role in motor control and reward-based learning (Fig. 4B). All these structures, cerebral peduncles, reticular portion of the subtantia nigra, internal capsule and basal ganglia are regions that were strongly labelled for cathepsin L.

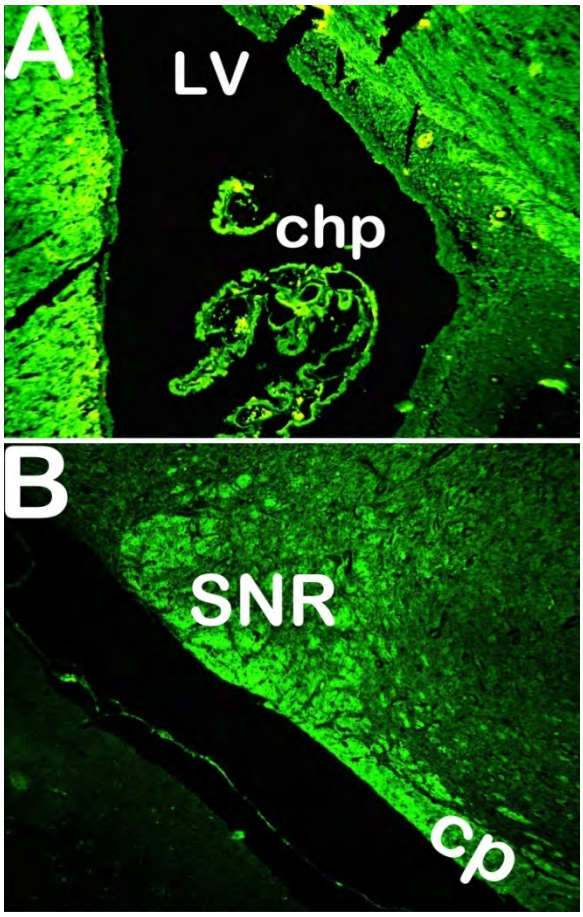

Fig. 4: Immunofluorescence labeling of cathepsin L protein in coronal section of ventricular systems and mesencephalon. **(A)** Intense immunoreactivity is present in the epindymal cells of choroid plexus (chp) and **(B)** in basal part of cerebral peduncle (cp) and reticular part of substantia nigra (SNR).

Thalamus, septum and hypothalamus

The thalamus constitutes the dorsal portion of the diencephalons and coordinates information flow to the cerebral cortex. In the thalamus, intense labelling was detected in the ventrolateral thalamic nucleus, ventral posterolateral thalamic nucleus, ventral posteromedial thalamic nucleus and in ventromedial thalamic nucleus but at moderate level in paracentral thalamic nucleus. Other thalamic regions with positive signals included the lateral posterior thalamic nucleus and the external medullary lamina. In the hypothalamus, strong immunoreactivity was confined to the anterior part of the anterior commissure and median eminence. (Fig. 6A and B).

Cerebellum

The cerebellum is composed of cerebellar cortex, internal white matter and three pairs of deep nuclei, the fastigial, the interposed and the dentate. Intense immunoreactivity for cathepsin L protein was identified in the internal white matter and the deep cerebellar nuclei (Fig. 7A). Moderate labelling was noted in granule cell layer, in randomly distributed cells that were considered to represent Golgi cells and /or granule cells. In the Purkinje neurons positive signals were detected in the cell bodies and in the molecular layer in the stellate and the basket cells, that are inhibitory interneurons, dispersed among the excitatory axons of granule cells and the dendrites of the inhibitory Purkinje cells. (Fig. 7B).

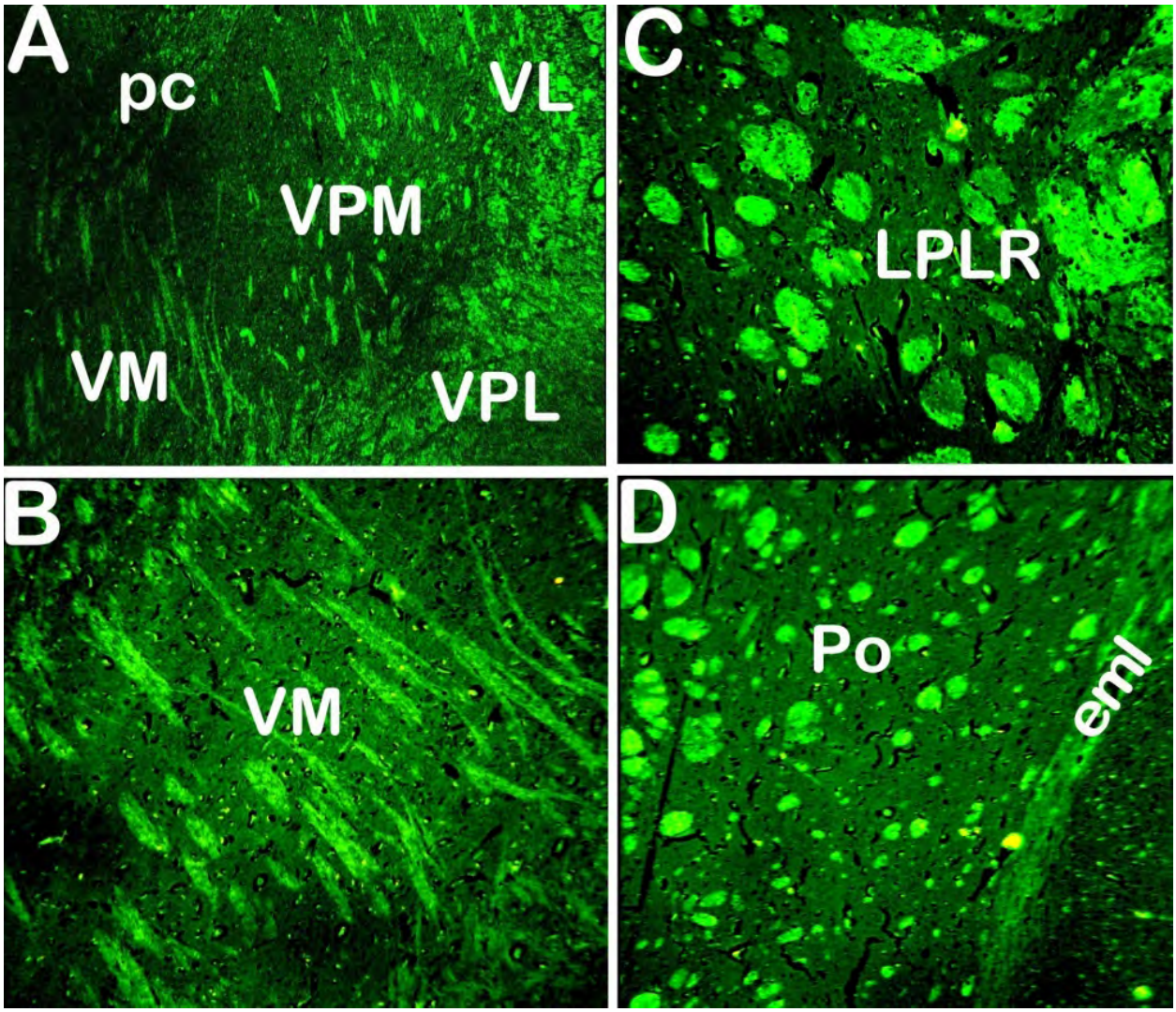

Fig. 5: Immunofluorescence localization of cathepsin L protein in coronal sections of thalamus. Strong labeling is seen in **(A** and **B)** the ventrolateral thalamic nucleus (VL), ventral posterolateral thalamic nucleus (VPL), ventral posteromedial thalamic nucleus (VPM) and in Ventromedial thalamic nuclei (VM) but weak in paracentral thalamic nucleus (pc). Positive signals are also present in **(C)** laterorostral part of lateral posterior thalamic nucleus (LPLR) and in **(D)** posterior thalamic nuclear (Po) and external medullary lamina (eml).

Table 1 Morphological structures refer to the neuron-anatomical atlas from Paxinos and Franklin (2001)

BRAIN REGION	DENSITY OF POSITIVE CELLS
CEREBRAL CORTEX AND SUBCORTICAL REGIONS	
Primary somatosensory cortex	++
Secondary somatosensory cortex	++
Retrosplenial cortex	+++
Secondary motor cortex	++
Cingulated cortex	+++
Corpus callosum	++
External capsule	+++
SEPTUM REGION	
Septal hippocampal nucleus	++
Septal fibrial nucleus	++
Nucleus of the anterior commissure	+++
BASAL GANGLIA	
Caudate putamen	+++
Globus pallidus, lateral part	+++
Globus pallidus, vental part	+++
Substantia nigra pars reticulate	+++
Internal capsule	+++
AMYGDALA	
Basolateral amygdaloid nucleus	-
Lateral amygdaloid nucleus dorsal-dorsolateral part	+++
Lateral amygdaloid dorsal-ventrolateral part	+++
HIPPOCAMPUS	
Alveus of hippocampus	+++
Fimbria of hippocampus	+++
Stratum oriens	-
Stratum pyramidale	++
Stratum lacunosum moleculare	-
Stratum lucidum	-
Dentate gyrus	-
Molecular cell layer of Dentate gyrus	-
THALAMUS	
Ventrolateral thalamic nuclei	++
Ventral posterolateral thalamic nuclei	+++
Ventral posteromedial thalamic nuclei	+++
Ventromedial thalamic nuclei	++
Paracentral Thalamic nuclei	+++
Lateral posterior thalamic nuclei	+++
External medullary lamina	++
Medial habenular bodies	+++
Choroid plexus	+++
HYPOTHALAMUS	
Median eminence	+++
Nucleus of the optic tract	++
CEREBELLUM	
Molecular layer	+
Purkinje cell layer	+++
Granule cell layer	++
White matter	+++

The intensity of CTLA-2α mRNA and protein expression was classified as follows: negative (-), moderate (++), high (+++), For Immunofluorescence evaluation, we used fimbria of the hippocampus and Medial habenular bodies (+++), stratum lucidum of the hippocampus and secondary auditory cortex ventral area (++), Pyramidal neurons (+) and stratum oriens and dorsal hippocampal commissure (-).

DISCUSSION

This study presents a detailed demonstration of cathepsin L protein distribution in various regions of the brain of African giant rats. Significant localization was confined to nerve fibres bundles than in nerve cells. In the cerebral cortex and subcortical structures, cathepsin L was densely detected in restrosplenial agranular cortex and in the cingulum. The restrosplenial agranular cortex is a major nodal point for the integration and subsequent distribution of information to and from the hippocampal formation, the midline limbic, visual cortices and the thalamus (Purves et. al., 2001). Similarly, the cingulum is a prominent white matter tract that extends longitudinally above the corpus callosum and is implicated in many cognitive functions. At its rostral limit it curves around the front of the genu of the corpus callosum while caudally it curves behind the splenium allowing for communication between components of the limbic system. Identification of cathepsin L protein in these structures suggests its role in learning that involves spatial stimuli and navigation and in simple learning such as classical conditioning. Similarly, immunoreactivity in the corpus callosum may suggest involvement of cathepsin L in many biological processes in the central nervous system that are yet to be identified taking into consideration that corpus callosum consists of contralateral axon projections connecting right and left hemispheres. Most communications between regions in different halves of the brain are carried over the corpus callosum (Purves et al., 2001).

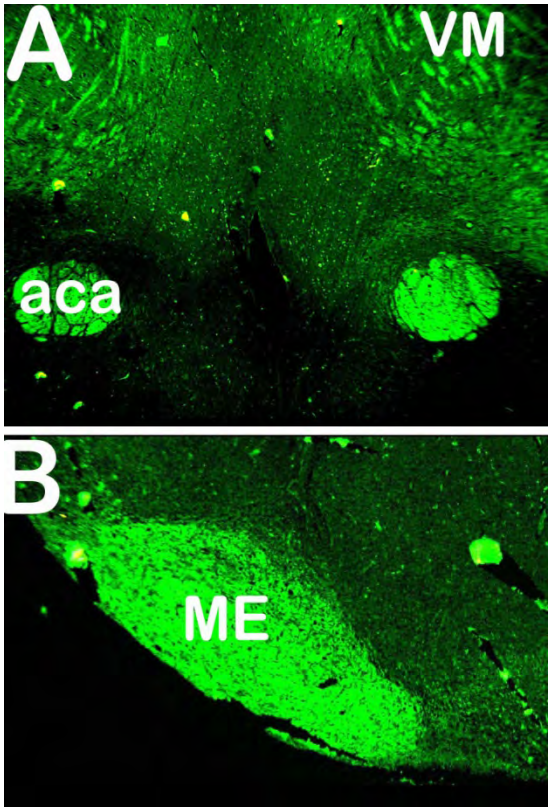

Fig. 6: Immunoreactivity of cathepsin L protein in coronal sections of the anterior commissure and Median eminence. Strong labeling is shown in **(A)** anterior part of anterior commissure (aca) and **(B)** in median eminence (ME).

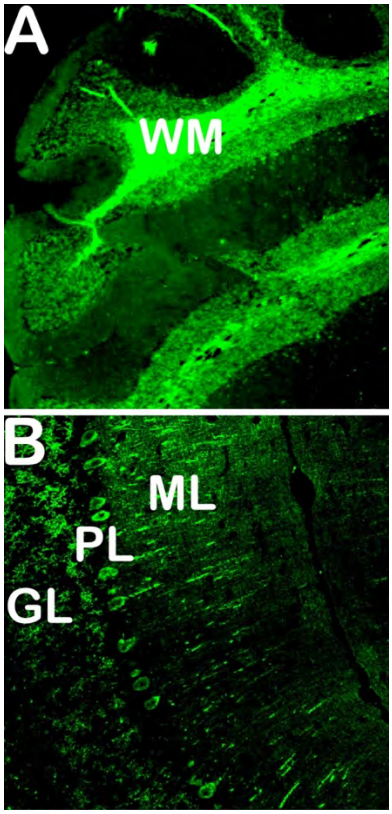

Fig. 7: Immunofluorescence localization of cathepsin L protein in coronal sections of the cerebellum. Intense labeling is shown in **(A)** deep white matter (wm) and moderately in **(B)** granular layer (GL), Purkinje neurons (PL) and in the molecular layer (ML) of the cerebellum.

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Localization of cathepsin L in the external and internal capsules also presents an interesting discussion regarding the function of this enzyme. The external capsule is a series of white matter fibre tracts called cortical association fibres in the brain that run between the most lateral segment of the lentiform nucleus and the claustrum. The fibres are responsible for connecting the cerebral cortex to other cortical areas and join the internal capsule around the lentiform nucleus. The internal capsule is also a white matter structure containing both ascending and descending axons going to and coming from the cerebral cortex. It is situated in the inferomedial part of each cerebral hemisphere of brain carrying information past the basal ganglia, separating the caudate nucleus and the thalamus from the putamen and the globus pallidus (Bryan, 1977). All these structures were intensely labelled for cathepsin L. Whether the localization of cathepsin L to these structures is related to their role in action of selection and initiation, through the integration of sensorimotor, cognitive and motivational information within dorsal striatum (caudate-putamen), social behaviour and learning of spatial reversals or to a novel function is a question that remains to be resolved because these structures are components of the same functional system.

The alveus of the hippocampus also showed high degree of cathespin L labelling. The alveus is composed of the white myelinated fibres that arise from cell bodies of subculum and hippocampus and eventually merges with the fimbria of the hippocampus that goes on to become the fornix. The fimbria was also strongly labelled for cathespin L. These structures are part of the limbic system including the hippocampus, amygdala, thalamus, mammilary body, habenular and some mid brain areas. All these components were strongly labelled for cathepsin L. The presence of cathepsin L in these structures is suggestive of its role in the regulation of limbic system functions that include motivation and reward, emotion, learning, and memory establishment (Marc and Sergio, 2014). In conjunction with the localization of cathepsin L in amygdala, concerns are also raised regarding the function of cathepsin L in information, processing and consolidation of memories associated with emotional events and decision-making. Recent studies suggest that the amygdala regulates memory consolidation in other brain regions (Amunts et al., 2005; Blair, 2001). Following any learning event, the long-term memory for the event is not formed instantaneously. Rather, the information of the event is slowly assimilated into long-term storage over time. In case of amygdala damage, long term memory establishment and emotional events are impaired (Amunts et al., 2005; Maren, 1999). The localization of cathepsin L in the amygdala extends these observations regarding the relationship between cathepsins and memory formation.

Localization of cathepsin L in choroid plexus correlated well with our previous studies (Luziga et al., 2008) on the distribution of CTLA-2α in these structures. One of the most prominent labelling structures for both cathepsin L and CTLA-2α was the choroid plexus. This observation suggests that the fine equilibrium between the synthesis and secretion of cathespin L and CTLA-2α is part of the brain processes to maintain normal growth and development. In addition to cerebrospinal fluid production, the choroid plexus act as a filtration system, removing metabolic wastes, foreign substances, and excess neurotransmitters from the cerebrospinal fluid. In this way the choroid plexus has a very important role in maintaining the delicate extracellular environment required by the brain to function optimally.

Intense labelling for cathepsin L was also detected in the white matter within the cerebellum in which the deep nuclei including the dentate, interposed and fastigial are embedded into it. This finding suggests that cathepsin L has a role in the integration of sensory perception and motor output of cerebellum, taking into consideration that these nuclei receive inhibitory (GABAergic) inputs from Purkinje cells and excitatory (Glutamatergic) inputs from the mossy fibre pathway (Acsady et a., 1998) and most of output fibres of cerebellum originate from these nuclei (Purves et al., 2001). In addition, cathepsin L was also found in the two inhibitory interneurons of the molecular layer, the stellate and basket cells. These cells also form GABAergic synapses onto Purkinje cell dendrites that were also labelled for cathepsin L.

In conclusion, this study shows that the localization of cathepsin L in the brain of African giant rat is predominant in the cerebral cortex, subcortical structures, hippocampus, amygdala, basal ganglia, thalamic nuclei, hypothalamus and the cerebellum. Many of these structures are involved in long term memory formation and storage of memory traces for spatial information, olfaction, emotion, behavior and motivation. These findings are suggestive of a specialized function of cathepsin L in relation to learning formation and memory establishment and open ways to new studies on the functional implications of cathepsin L in the central nervous system.

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