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Identification and localization of cholinergic nerve endings and acetylcholine receptors in tissues of the immune system- animal and post-mortem human study

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Declaration by the candidate

The work described in this thesis was carried out by me under the supervision of,

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A report on this has not been submitted in whole or in part to any university or any other institution for another Degree or Diploma.

.....S Mythreye..... 26.08.2014.....

Mythreye Thayabaran Date

Declaration by the Supervisor

I certify that the above statement made by the candidate is true and that this thesis is suitable for submission to the University of Sri Jayewardenepura for the purpose of evaluation.

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I certify that the candidate has incorporated all corrections, additions and amendments recommended by the examiners.

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Ab	Antibody
ABC	Avidin biotin complex method
ACh	Acetylcholine
AChE	Acetylcholine esterase
AChR	Acetylcholine receptor
Ag	Antigen
ANS	Autonomic nervous system
APC	Antigen presenting cells
AR	Antigen retreival
BALT	Bronchial mucosa associated lymphoid tissue
BChE	Butyrylcholine esterase
BSA	Bovine serum albumin
cAMP	Cyclic AMP
CAP	Cholinergic anti- inflammatory pathway
CGRP	Calcitonin gene related peptide
ChAT	Choline acetyl transferase
CHT	Choline transporter
CNS	Central nervous system
DAB	Diamino benzedene
DBH	Dopamine betahydroxilase
ENS	Enteric nervous system
FDCs	Follicular dendritic cells
FITC	Fluorescein isothiocyanate
H & E	Heamatoxillin and Eosin
H_2O_2	Hydrogen peroxide

HEVs	High endothelial venules
HIER	Heat induced epitope retreival
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IF	Immunoflourescence
IL 2	Interleukin 2
INF-c	Inteferon c
IR	Immunoreactivity
LSAB	Labelled streptavidin biotin method
M2AChR	M2 subunit of muscarinic acetylcholine receptor
M3AChR	M3 subunit of muscarinic acetylcholine receptor
mAChR	muscarinic acetylcholine receptor
MALT	Mucosa associated lymphoid tissue
MHC	Major histocompatibility complex
MNLs	Mononuclear leukocytes
nAChR	nicotinic acetylcholine receptor
NALT	Nasopharynx associated lymphoid tissue
NPY	Neuropeptide Y
NSE	Neuron specific enolase
PAL	Peri-arteriolar lymphoid sheath
PBS	Phosphate buffered saline
PGP	Protein gene product
PL	Polymorpho leukocytes
PLC	Phospholipase C
PNS	Periphral nervous system
PP	Peyer's patches

PRV	Pseudorabies virus
SP	Substance P
TH	Tyrosine hydroxilase
TNF	Tumour necrosis factor
TRITC	Tetramethyl rhodamine isothiocyanate
VAChT	Vesicular acetylcholine transporter
VIP	Vasoactive intestinal polypeptide
WGA-HRP	Wheat germ agglutinin conjugated horseradish peroxidase
α1nAChR	α1 subunit of nicotinic acetylcholine receptor
α7nAChR	α7 subunit of nicotinic acetylcholine receptor
M2AChR	M2 subtype of muscarinic acetylcholine receptor
M3AChR	M3 subtype of muscarinic acetylcholine receptor

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The thesis is dedicated to my parents, my husband and my two children.

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ABSTRACT

The central nervous system is known to interact with the immune system through the autonomic nerves and modify the immune responses. Although sympathetic noradrenergic fibres has been considered as the predominant component controlling the functions of lymphoid tissues, recent researches point towards equally important role of cholinergic innervation. Our present study describes the cholinergic nerve supply of different lymphoid tissues by demonstrating the neural profiles, cholinergic nerve endings, acetylcholine receptors and enzymes of acetylcholine synthesis by immunohistochemistry and immunoflourescence techniques.

Fresh immune tissues such as spleen, liver, lymph nodes, thymus and terminal ileum of small intestine were retrieved from Balb/C mice, Wistar rats and humans and were processed to examine the microscopic ultra structural variations in Haematoxylin & Eosin stained slides. Primary antibodies raised in rabbit and rat against α1 & α7subunits of nAChRs, M2 & M3 subunits of mAChRs, VAChT, ChAT, and Neuron Specific Enolase (Sigma Aldrich, USA) were used to label the 4 μm thin tissue sections. Biotinylated anti-rat and anti-rabbit IgG were used as secondary antibodies. Labelled Streptavidin Biotin (LSAB) technique was used; with DAB as chromogen and counterstained with Mayer's haematoxylin. Skeletal muscle and cerebral cortex were processed as positive controls. Similarly, immunoflourescence technique was done by labelling the tissues with the above mentioned primary antibodies and FITC conjugated secondary IgGs. Immunohistochemistry slides were observed and digitalized using

Dino capture (Taiwan), while the immunoflourescence slides observed through advanced fluorescence microscope (Olympus FSX100). An inflammatory response was induced in Balb/C mice by injecting irritant sterile turpentine in their hind limbs and the histopathological changes examined in tissues presented with sterile abscess and local lymph nodes using H & E staining. Further using anti-α1nAChR and anti-VAChT in those tissues cholinergic innervation was investigated.

The immunohistochemistry slides were evaluated and the intensity of immunostaining was determined based upon a score of 0 (No staining), 1+ (focal staining/ less than 30% of IR cells), 2+ (focal to diffuse/moderate staining, 30% - 60% cells), 3+ (Strong staining, 61 - 100% of cells) (Schaulder et al, 2008).

Immunoreactivity of α1 & α7nAChRs and the M2AChRs were clearly identified in capsule, interlobar septa, corticomedullary junction and the cortex in thymus of Balb/C mice and Wistar rats. Similarly, VAChT and ChAT were localized at the proximities of those sites. In spleen the distribution of α1 & α7nAChRs and the M2 & M3 mAChRs were identified in the capsule, trabeculae and sinusoids of red pulp in the Balb/C mice, Wistar rats and humans and the VAChT, ChAT and NSE were also localized towards their vicinity. The distribution of α1 & α7nAChRs and the M2 & M3AChRs and the localization of VAChT and ChAT were mainly found in the capsule subcapsular sinus, medullary cords and paracortex of lymph nodes of the three species. Peyer's patches, liver and the lymphoid aggregations of posterior part of tongue did not express immunoreactivity towards anti-VAChTs, and ChAT. The distribution of α7nAChRs and the M2 & M3 mAChRs were not identified in those tissues. The expression of NSE at peri-portal triad of liver shows the nerve control of this tissue. It is apparent that the AChRs specifically localize in the sites rich in T cells. Further α7nAChRs were found mainly in the subcellular compartments distributed with tissue macrophages. The strong

IR of neuron specific enolase in capsules of spleen and lymph nodes provides evidence that the innervation from autonomic nerves traverse through their supporting framework reinforced by reticulin fibres to the parenchyma. The expression of α1nAChR in the polymorpho nuclear leukocyte and macrophages and the localization of VAChT in the similar sites confirm that parasympathetic cholinergic innervation in those sites and suggest that the inflammatory response may be regulated through α1nAChRs. Localization of VAChT and ChAT confirms that the presence of cholinergic neural control of immune tissues is predominantly through the α1 & α7nAChRs and M2 & M3 AChRs.

Knowledge gained regarding the in-situ localization and expression of cholinergic receptors could be used as a consistent anatomical background to understand the functional interaction between nervous and immune system leading to intervention of new therapeutic applications. The parasympathetic receptor distribution suggests cholinergic control of immune responses and by regulation of excess inflammatory response by the inflammatory reflex. A complete understanding of parasympathetic innervation of immune tissues is essential for designing more specific molecular based drug treatment in autoimmune and inflammatory diseases.