The Effects of Ageing on the Spinal Neurones Controlling Micturition and Continence

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The candidate confirms that the work submitted is his/her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

In humans, the prevalence of urinary incontinence increases with old age. This condition has a major impact on the quality of life of elderly people, and is associated with anxiety, depression, social embarrassment, interrupted sleep and financial burden. The main goal of the research presented in this thesis was to examine potential changes in neuronal circuitry underlying urinary incontinence to increase our understanding of the pathophysiology of urinary incontinence in ageing, using the mouse as a model. Measuring the micturition characteristics of 3, 24 and 32 month old mice revealed that increasing age increases the frequency of micturition, total volume of urine produced and volume/void in night and daytime experiments. In addition, 32 month old mice had a shorter interval between voids than the younger animals. To better understand the spinal cord circuitry involved in regulating micturition, the location and structure of the dorsolateral nucleus (innervating the external urethral sphincter) in mouse spinal cord were determined using retrograde labelling techniques and immunohistochemistry for a marker for cholinergic neurones, choline acetyltransferase (ChAT). The DLN was found to be localised in L6-S1 of mouse spinal cord. The nature of the inputs onto retrogradely labelled (by injection of Fluorogold) dorsolateral nucleus neurones and autonomic neurones in the spinal cord which control micturition were also determined using immunohistochemistry to detect excitatory (glutamatergic) and inhibitory (GABA and glycinergic) terminals in 3, 24 and 32 month old mice. This revealed that during ageing there is a net decrease of excitatory and increase in inhibitory inputs onto DLN, sympathetic and parasympathetic preganglionic neurones. The shift in the balance of excitatory-inhibitory presynaptic inputs to these neurones provides new insight to the mechanism of urinary incontinence in ageing.

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List of Abbreviations

ABC	Avidin-biotin peroxidase
AG	Aged mice
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-metyl-4-isoxazole-propanoic acid
ATP	Adenosine triphosphate
CGRP	Calcitonin-gene related peptide
ChAT	Choline acetyltransferase
CNS	Central nervous system
CTB	Cholera toxin B subunit
DLN	Dorsolateral nucleus
DRG	Dorsal root ganglia
EA	Extreme aged mice
EUS	External urethral sphincter
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
FG	FluoroGold
H&E	Hemotoxylin and eosin
HRP	Horseradish peroxidase
IML	Intermediolateral cell column
i.p	Intraperitoneal
IR	Immunoreactive
LUT	Lower urinary tract
M	Molar
m.o	Month old
NMDA	N-metyl-D-aspartate
PAG	Periaqueductal gray
PFA	Paraformaldehyde

PMC	Pontine micturition centre
PRV	Pseudorabies virus
RDLN	Retrodorsolateral nucleus
SNB	Spinal nucleus of bulbospongiosus
VAChT	Vesicular acetylcholine transporter
VGLUT1	Vesicular glutamate transporter 1
VGLUT2	Vesicular glutamate transporter 2
YO	Young adult mice
VSOP	Voided stain on paper
WGA-HRP	Wheat-germ agglutinin-horseradish peroxidase
5-HT	5-hydroxytryptamine (serotonin)
µm	Micrometer

Chapter 1

General Introduction

1.1 Ageing and Urinary Incontinence

The ageing population in humans is increasing, owing to the advancement of modern health care system. In the UK for example, the national statistics of life expectancy rate in 2014 was 78.3 years for males and 82.8 years for women (http://www.ons.gov.uk). According to the World Health Organisation (WHO), in 2050, the life expectancy worldwide is projected to increase to 80.7 years for men and 86.1 years for women. The global trend of population ageing is likely to influence patterns of future health care services and development of effective health response to ageing populations.

In individuals, ageing is one of the most complex biological process. Ageing generally refers to the process of growing older. Technically, it is defined as the progressive physiological changes in an organism that lead to senescence. Ageing is a biological process that takes place in a cell, an organ or a whole organism over a passage of time, over an entire adult lifespan of any living things (Finch et al., 1990). Many definitions of ageing commonly associate it with its deleterious effects and time-dependant functional decline (López-Otín et al., 2013). The ageing process is associated with increasing chances of morbidity. A number of pathologies also increase with age including type 2 diabetes, heart disease, cancer, arthritis, kidney function and urinary incontinence. Of these, urinary incontinence is one of the most common problems in the ageing population.

In the healthy human, emptying of the bladder and disposal of urine is carried out at an appropriate time and place deemed as socially suitable. However, the ability to retain urine is significantly reduced in the elderly population and this phenomenon is known as urinary incontinence.

Urinary incontinence is defined by the International Continence Society as complaint of any involuntary loss of urine. It may be a small occasional leak or a total loss of bladder control. There are 3 main types of incontinence, although many other variance of incontinence have been described. The 3 main types are stress incontinence, urge incontinence and mixed incontinence. Involuntary loss of urine in stress incontinence occurs on physical exertion such as sneezing or coughing. Urge incontinence is associated with urgency, a sudden compelling desire to pass urine. Mixed incontinence happens as a result of both stress and urgency. Urinary incontinence in the elderly is predominantly of urge and mixed type. They frequently experience bladder storage symptoms of urinary incontinence including increased daytime frequency, nocturia, urge and urgency, and in extreme cases involves persistent leakage of urine. The main complaint in the ageing population is being unable to withhold urine when there is an urge to void (urge incontinence). Leakage is often described as wetting of pants before being able to get to the toilet. In extreme cases, free flow of urine ensues continuously, indicating persistent leakage of urine. Nocturia is another common symptom of incontinence in the elderly. It is defined as the need to wake and pass urine at night with more than 2 episodes per night. Nocturia is the most prevalent urinary incontinence symptoms complained about by the ageing people (Irwin et al., 2006).

Prevalence of urinary incontinence increases with increasing age. The prevalence of this condition has been shown to increase with age in both sexes with prevalence rate of urinary incontinence in the 65-80 years old age group may be up to 11.6% (Collerton et al., 2009). At 85 and above, the prevalence rate is 35% (Wehrberger et al., 2012). Women are more likely than men to be incontinent. Nearly one third of the female population aged 65 and above experienced urinary incontinence and the prevalence of urinary incontinence in elderly women above 85 is between 26.6 – 35% (Collerton et al., 2009, Wehrberger et al., 2012) while in men is between 12.6 – 24% (Collerton et al., 2009, Wehrberger et al., 2012). Pregnancy and childbirth, menopause and the structure of the female urinary tract account for this difference. Apart from that, prevalence of urinary incontinence is also higher among older people who live in care homes. Xu and Kane (2013) reported that institutionalised elderly above 85 in nursing home have greater risk of urinary incontinence at 69%. It occurs in 24% of aged women and 21% in men. Urge incontinence was also reported in 11.8% in both sexes and increased with ageing (Irwin et al., 2006).

This condition has a major impact on the quality of life of the elderly. Psychologically, it is described as a very distressing and embarrassing experience in the elderly. Urinary incontinence has been linked to symptoms of anxiety and depression (Coyne et al., 2012). Patients who suffer from urinary incontinence developed fear and anxiety that they might wet themselves in public areas and during social events. They described leakage as socially embarrassing (Ness, 2012). Socially, it limits the normal social activities, keeps the patient at home and disrupts the daily undertakings of the sufferer. Over time incontinence leads to avoidance of social activities, decrease self-esteem and promotes social isolation (Yip et al., 2013). There is generally lack of appropriate support and general silence around this problem, which is still considered a taboo.

Frequent nocturnal voiding in the elderly is associated with interrupted sleep, distress and anxiety. Nocturia is associated with waking at night and then not being able to get back to sleep. This symptom is bothersome, increases sleepiness during daytime and affects the general quality of life. Many patients impose drinks restriction on themselves by reducing their fluid intake to reduce the volume of urine produced. But this can increase the concentration of urine and cause further discomfort. Financially, managing urinary incontinence also carries significant financial burden. As the population ages, the number of older people with urinary incontinence will significantly increase, as will the associate cost of their care. At national level, the total cost of overactive bladder with urge urinary incontinence in the UK is to be £454 million per year. The average cost for a UK patient of £403 per annum for prescription, medication, anti-anxiety/depressant medication and incontinence pad (Irwin et al., 2011).

Management of bladder dysfunction in the elderly is currently far from ideal. Many drugs such as anticholinergics have been tried but with rather disappointing results. This is either due to poor efficacy of the drugs or the intolerable side effects. Amongst the side effects reported include nausea, dry mouth, constipation, insomnia and fatigue. This problem creates major challenges in clinical management. While new pharmacological drugs are slowly developing, a more common approach to patients of urinary incontinence is to provide behavioural and symptomatic treatments. These conservative managements include lifestyle modification, pelvic floor muscle training, scheduled voiding regimens and sanitary advice. There is a need to emphasis that urinary incontinence is not inevitable and can be treated and managed effectively. Elderly patients need to be assured that problems with urinary continence are treatable and are not merely an abnormal part of growing old.

1.2 Anatomy of Lower Urinary Tract

Urinary incontinence is a defect in the storage mechanism of the lower urinary tract (LUT). The LUT consists of the urinary bladder and urethra in male and female. In male, however, there is an addition of the prostate gland and the penile urethra. The general functions of LUT are mainly to store the urine which it receives from the kidneys via the ureters, and the expulsion of urine from the body. In this section, the anatomy of the urinary bladder, urethra and urethral sphincter are described together with their specific functions, histological features, vascular supplies and neural innervations.

1.2.1 Urinary Bladder

The current knowledge on the anatomy of bladder has been reviewed extensively by Mangera et al. (2013). The urinary bladder is a hollow and muscular pelvic organ. When empty, it adopts a tetrahedral (three-sided pyramid) shape and lies in the true pelvis, posterior to the symphysis pubis. The urinary bladder is a distensible structure, thus the position of the bladder may vary according to the amount of urine it contains. The position may also differ in infant and adult. In infancy, the bladder is mainly an abdominal organ owing to the small pelvic size, but as the body grows, the bladder lies in the pelvic area (Mangera et al., 2013). The urinary bladder consists of an apex, body and base. The base of the bladder is located posteroinferiorly, triangular in shape and is alternatively known as the trigone area. While other parts of the bladder mucosa are wrinkled, the area in the trigone is smooth and lacks trabeculation. The two superolateral angle of the ureters and the anteroinferior angle of the internal urethral orifice form the boundary of the triangular base (Figure1.1).

Superolaterally, the bladder is connected to right and left kidneys by the two ureters and inferiorly connected to the urethra. The bladder is separated from the symphysis pubis by the fat in the retropubic space of Retzius. The bladder has a superior surface and two inferolateral surfaces. The superior surface of the bladder is covered by peritoneum. The peritoneum is reflected posteriorly to the rectovesicular pouch in male and vesicouterine pouch of Douglas in female.

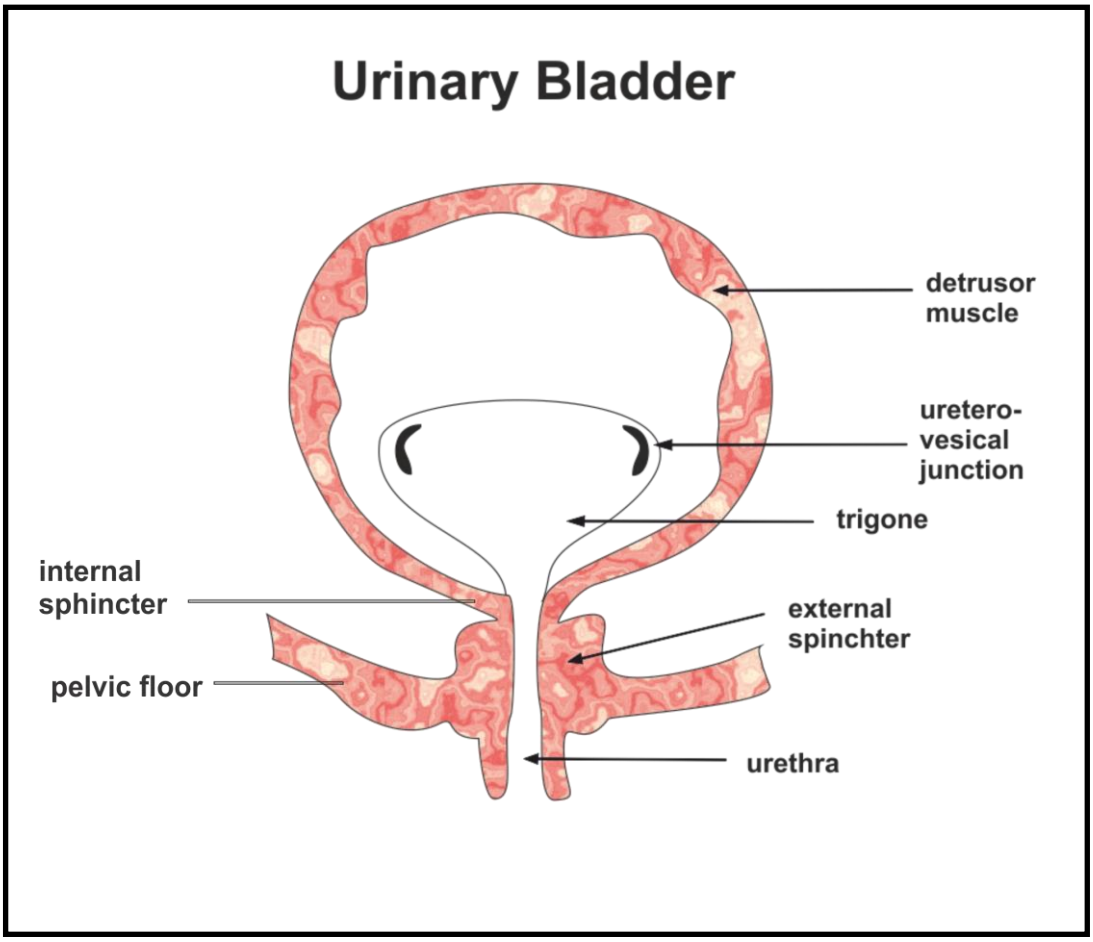

Figure 1.1 The diagram illustrates the morphology of urinary bladder and external urethral sphincter which are highly involve in the control of continence and micturition.

The bladder consist of smooth muscle with an inner lining of specialised and functionally important epithelial cells, with additional cell types that include nervous and vascular supplies, connective tissue cells, interstitial cells and immune system cells. Histologically, the urinary bladder consists of three main layers. They are the mucosal layer, muscularis propria and adventitia. The mucosal layer consists of the innermost urothelium, a basal membrane and lamina propria. Bladder mucosa is lined with urothelium which is a transitional stratified epithelium that line the urinary tract including the upper urethra and prostate gland (Birder, 2013). The urothelium consist of basal cell layer, intermediate layer and apical layer of 'umbrella' cells. This structural arrangement allow the bladder to effectively function as a barrier for infection, release of signalling molecules and in relaying sensory stimuli after detection of physiological and chemical stimuli (Birder and de Groat, 2007). Numerous studies have documented the secretory property of the urothelium, among other it is known to secrete transmitters or mediators such as acetylcholine (Kullmann et al., 2008), ATP (Wang et al., 2005), cytokines (Wood et al., 2012), nitric oxide (Birder et al., 1998) and prostaglandins (Downie and Karmazyn, 1984). However, the mechanism underlying the release of these chemical mediators is currently a subject of rigorous investigations.

Apart from the mucosa, the urinary bladder wall is made up of the smooth muscle known as the detrusor muscle. The detrusor muscle's fibers are arranged in spiral, longitudinal and circular bundles. These structural arrangements of detrusor muscle are less obvious throughout the body of the bladder, but become more distinct towards the bladder neck. In males the detrusor muscle is thicker than in females as it needs greater pressure to overcome the prostate and the lengthier urethra. Arterial blood supply to the bladder is mainly derived from the superior, middle and inferior arteries which arise from hypogastric trunk of the internal iliac artery. Minor contribution arise from obturator and inferior gluteal arteries, with addition of uterine

and vaginal arteries in female. Venous drainage is provided by plexus of veins around the bladder which eventually drains into the hypogastric vein. Lymphatic drainage occurs into the paravesical, hypogastric, external iliac and common iliac lymph nodes (Mangera et al., 2013).

The bladder receives innervation from the autonomic nervous system. In humans, sympathetic preganglionic nerves to the bladder commence from T11-L2 spinal level, and travels in the hypogastric nerve. They cause the detrusor smooth muscle to relax while contracting the smooth muscle at the bladder neck during storage phase. Parasympathetic nerves commence as preganglionic fibres from the S2-S4 level, and travel through the pelvic splanchnic nerve. They cause the detrusor muscle contraction while relaxing the smooth muscle of the bladder neck during voiding. Both sympathetic and parasympathetic afferent pathways supply sensory innervation for the perception of fullness and noxious stimuli from the bladder (Fowler et al., 2010). The neural control of bladder and urethral sphincter will be discussed in more detail in this chapter section 1.3.

The urinary bladder has two principle functions. First, it acts as a low pressure reservoir for the storage of urine. Urine is produced from the kidneys, passes through the ureters, collected and stored in the bladder. The bladder can accommodate a large volume of urine without significant increase in pressure. This unique property of bladder compliance is important as it allows the bladder to receive increasing volume of urine with little change to bladder pressure until it reaches the capacity of bladder. Without maintenance of the low pressure, two inevitable consequences will be incontinence and kidney damage. The highly important prevention of high intravesical pressure is attributed to the passive characteristics of the connective-tissue elements of the bladder and the active

properties of the smooth muscle in the bladder (M. Levin, 1999). The secondly function of the bladder is to generate sufficient pressure to aid expulsion of urine. Urine is eliminated via the urethra through the micturition reflex (Lukacz et al., 2011).

1.2.2. Urethra

The urethra is the urinary outlet that allows urine to passes through during voiding. In male, the total length of the urethra is about 20cm. It is divided into three parts – the prostatic, membranous and penile urethra. The prostatic urethra is about 4 cm in length and passes through the prostate. The urethra emerges from the prostate as membranous urethra and pierces the urogenital diaphragm. This part of the urethra is surrounded by the external urethral sphincter. At the root of the penis, the urethra continues as penile urethra. It pass through the corpus spongiosum and opens to the environment at the external urethral meatus at the tip of the glans penis. The urethra receives arterial blood supply from adjacent vessels and internal pudendal arteries and venous drainage into internal pudendal vein. Lymphatic drainage occurs into the internal and common iliac lymph nodes. Histologically, like the bladder, the urethra is lined by transitional epithelium. The exception occurs at the distal glans penis which is lined by stratified squamous epithelium.

The female urethra is much shorter and usually about 4 cm in length (Ashton-Miller and DeLancey, 2007). The urethra passes down anterior to the vaginal wall. It opens to the environment at the external urethral meatus on the vestibule, below the clitoris. The female urethra receives arterial supply from inferior vesical, vaginal and internal pudendal arteries. It drains into the vesical plexus of veins into the internal pudendal veins. Lympathic drainage of deep part of urethra drains into the

internal iliac nodes, while a superficial part drains into the inguinal and subinguinal lymph nodes. The proximal urethra is lined by transitional epithelium but distal urethra is lined by non-keratinised stratified squamous epithelium.

1.2.3. Urethral sphincters

There are two sphincter mechanisms involved in the maintenance of continence. They are the internal urethral sphincter and the external urethral sphincter. The internal urethral sphincter is formed by a continuation of the longitudinal layer of smooth muscle which blends with the thicker circular smooth muscle fibers from the detrusor. It is located at the level of the bladder neck and proximal urethra. The internal urethral sphincter action is involuntary and is under autonomic control (Mangera et al., 2013).

The external urethral sphincter (EUS) is a striated skeletal muscle of the pelvic floor which surrounds the urethra. In males, the EUS covers inferior to the prostate and is located at the level of membranous urethra (Jung et al., 2012). The fibres are oriented in a horse-shoe shape and are not fixed to the levator ani muscle. This implies that the closure of urethra in males is independent of levator ani muscle (Yucel and Baskin, 2004). This striated muscles also do not attached to any bony structure and and thus act as true sphincters. Its contraction do not produces any movement except for constriction of the urethral lumen (Ashton-Miller and DeLancey, 2007). In the females, the sphincter begins at the inferior end of the bladder and closely associated with compressor urethral muscle and the urethrovaginal sphincter (Jung et al., 2012). All these muscles serve to contract the proximal part of the urethra. Sebe et al. (2005) demonstrated that the striated muscle component of the external urethral sphincter is most thick in the middle third

of the urethra where it forms the true annular sphincter surrounding the urethra. This finding was supported by imaging study using magnetic resonance imaging (Macura and Genadry, 2007).

The EUS is a unique muscle in terms of its action. On one hand, it can be contracted voluntarily under somatic motor control, but on the other hand it must be precisely coordinated with bladder activity, which is a function of the autonomic system. In humans, the external urethral sphincter is innervated by the pudendal nerves which originated from Onuf's nucleus at the level of S2-S4.

1.3 Neural control of micturition and continence

The LUT is innervated by both peripheral autonomic and somatic nerves. The three sets of nerves involved are (1) parasympathetic sacral pathways via the pelvic nerve, (2) sympathetic thoracolumbar pathways via the hypogastric nerve and (3) sacral somatic motor neurones via the pudendal nerve (Fowler et al., 2008). These nerves are controlled by a complex set of neural pathways in the spinal cord, brainstem and brain which coordinate different part of the LUT to maintain proper urinary function. In this section, the central and peripheral neural pathways controlling micturition and continence will be discussed.

1.3.1. Onuf's nucleus and its homologue dorsolateral nucleus

1.3.1.1. Neuroanatomy of Onuf's nucleus

The control of continence is highly dependent on the function of the external urethral sphincter (Thor and de Groat, 2010). The EUS is an intrinsic urethral muscle and is alternatively known as the rhabdosphincter muscle. In man, the EUS is a thin ring of striated muscle surrounding the urethra at the base of the urethra. It is innervated by pudendal nerve branches which derive from motor neurones located in the Onuf's nucleus of the spinal cord. In human, Onuf's nucleus is a distinct cell group of motor neurones located at spinal sacral segment S2-S3. Through serial section histological staining and light microscopy, Onufrowicz (1899) was the first to examine the arrangement of cell groups in the sacral region of spinal cord in human and described the presence of this spinal nucleus, which he called group X. Although typically found in the second sacral segment, this group may extend slightly into the distal part of the first sacral and proximal part of third sacral. He noted that cells in group X are multipolar, but in contrast with other cell groups in the lumbosacral segment, they are characterised by their small size and densely packed arrangement. He postulated that group X is possibly the centre for innervation of striated muscles contributing to erection and ejaculation. Since Onufrowicz first pointed it out, very few studies had focused on this group of neurones for many years. However, the significance of this nucleus re-emerged after Mannen et al. (1977) described it's relation to bladder-rectal function.

Mannen et al. (1977) performed a histopathological examination on five spinal cords from amyotrophic lateral sclerosis (ALS) patients. They stained the sections with Kluver-Barrera, Hematoxylin & Eosin (H&E) and Nissl stains and compared them with normal spinal cords. This study showed that, while other large motor

neurones were completely destroyed in the ventral horn in ALS, neurones in the group X that Onuf described were remarkably well-preserved with no sign of degeneration in all five cases. These neurones were confined in the second sacral cord, medium in size and located in a ventrolateral position in the ventral horn. They concluded their study by making a deduction on the function of the Onuf's nucleus. Since bladder-rectal function remains intact in early and middle stage of ALS, and the finding that Onuf's nucleus was also well-preserved in ALS, they proposed that the motor neurones in Onuf's nucleus are responsible for the innervation and direct control of the external urethral and anal sphincter.

By light microscopic examination of counterstained sections from 59 human spinal cords, Onuf's nucleus was found to occupy the ventrolateral position in the ventral horn in man in the first, second and third sacral segments (Schrøder, 1981). Since this pattern strongly resembled that seen in experimental animals, Schroder concluded that the pool of specialised motor neurones residing in here, controls not only the EUS, but also the external anal sphincter and two perineal muscles, namely the bulbospongiosus and ischiocavernosus. Since the perineal muscles are involved in penile erection and ejaculation in male, Onuf's nucleus serves as a very important group of motor neurones for micturition, defecation, urinary and faecal continence and sexual purpose.

1.3.1.2. Unique features of Onuf's nucleus

The Onuf's motor neurones innervating the EUS have unique characteristics as compared to other somatic spinal motor neurones. Firstly, as initially determined in counterstained histological sections of cat spinal cord (Dekker et al., 1973) and later by Sasaki (1991), Onuf's motor neurones are uniformly smaller in size (diameter

between 19.7-50μm) compared to most other large somatic motor neurones of skeletal muscle. This observation concurs with earlier descriptions by Onufrowicz (1899) that these neurones are relatively small in size and closely packed together. Besides that, (Dekker et al., 1973) further noted that the motor neurones supplying pelvic muscles via the pudendal nerve have more distinctive and pronounced bundles of dendrites projecting rostrocaudally. Similarly, in an Epon-embedded semithin sections of electron microscopy study on the Onuf's nucleus in cat, (Konishi et al., 1978) observed as many as 800 to 1200 densely packed dendrites running chiefly in the rostrocaudal direction. In sagittal sections, extensive dendritic bundles were arranged longitudinally and tangled with each other, while transversely oriented small bundles of dendrites were seen in the areas adjacent to the longitudinally oriented large dendrite bundles. Similar organisation and size differences have also been observed in another study on Onuf's nucleus in cat (Sasaki, 1994).

In addition to that, their retrograde HRP study in cat, Sasaki (1994) revealed the presence of recurrent axon collaterals which were observed in about half of the motor neurones within Onuf's nucleus. However, in an intracellular recording of the Onuf's motor neurones in cat, (Mackel, 1979) has earlier shown that there was no evidence of any recurrent inhibitory effects on the motor neurones innervating the EUS and anal sphincter. The presence of recurrent axon collaterals in the absence of recurrent inhibition, suggests a recurrent facilitation within the motor neurones pool to reinforce simultaneous activation. This is to ensure sustained innervation to the motor units for tonic contraction and efficient closure of the sphincter.

1.3.1.3. Onuf's nucleus is sexually dimorphic

Another unique feature of Onuf's nucleus is that it is sexually dimorphic. Sexual dimorphism is a structural or morphological difference between sexes which may arise during normal course of development. Breedlove and Arnold (1980) investigated the Onuf's motor neurones and reported the presence of a sexually dimorphic nucleus in rat L5-L6 spinal cord particularly on the spinal nucleus of the bulbospongiosus (SNB), being larger in size in male than in female. They further performed hormonal manipulation studies and reported that the number of neurones in the SNB of either male or female rats is not altered by adult gonadectomy or hormonal treatment with testosterone propionate(Breedlove and Arnold, 1981). However, the size of individual SNB neurones is increased in the presence of androgen in either sex. Similar observation was mentioned in another study in rat in which the Onuf's nucleus appears as a sexually dimorphic nucleus and is influenced by the level of testosterone hormone (Jordan et al., 1982). In their study, they tested a single dose of testosterone on day two of life to the female rat and noted a masculinized Onuf's nucleus, which highlighted the importance of the interaction of androgens with their receptors early in development. By performing injection of various tracers into perineal muscles and retrograde labelling, similar pattern of sexual dimorphism were reported in perineal motor neurones of Mongolian gerbil (Ulibarri et al., 1995) and spotted hyenas (Forger et al., 1996).

1.3.1.4. Tracing studies of Onuf's nucleus

Various anatomical studies have located the origin of the pudendal nerve in different species. One strategy has been to retrogradely label spinal neurones by injection of tracers into the EUS. In mammals, the motor neurones innervating the EUS were localised in the ventrolateral part of the ventral horn in L5-S2 in

Mongolian gerbil (Ulibarri et al., 1995), S1-S2 in hamster (Gerrits et al., 1997), S1 in guinea pig (Kuipers et al., 2004), S1-S2 in cat (Sato et al., 1978), S1-S2 in dog (Kuzuhara et al., 1980), and L7-S1 in South American monkey (Saimiri) (Nakagawa, 1980). Similarity has been suggested between the functions of Onuf's nucleus in animals and humans. However in laboratory rodents, there is a separation of neuronal clusters controlling urinary and faecal continence. The Onuf's nucleus homologue is located in two separate columns – the dorsolateral (DLN) and SNB groups. In rat, the neurones in the DLN innervate the EUS while the motor innervation to the external anal sphincter is provided by the SNB. The DLN is identifiable by its ventrolateral position in the ventral horn of caudal lumbar and rostral sacral segment (McKenna and Nadelhaft, 1986). The ischiocavernosus and bulbospongiosus muscles, which are involved in facilitating penile erection and ejaculation, are innervated by the DL and SNB, respectively (McKenna and Nadelhaft, 1986). Another prominent pool of motor neurones in the ventral horn of L6-S1 segment is the retrodorsolateral nucleus (RDLN). The RDLN accommodates motor neurones innervating the flexor digitorum brevis muscles of the rodent's foot.

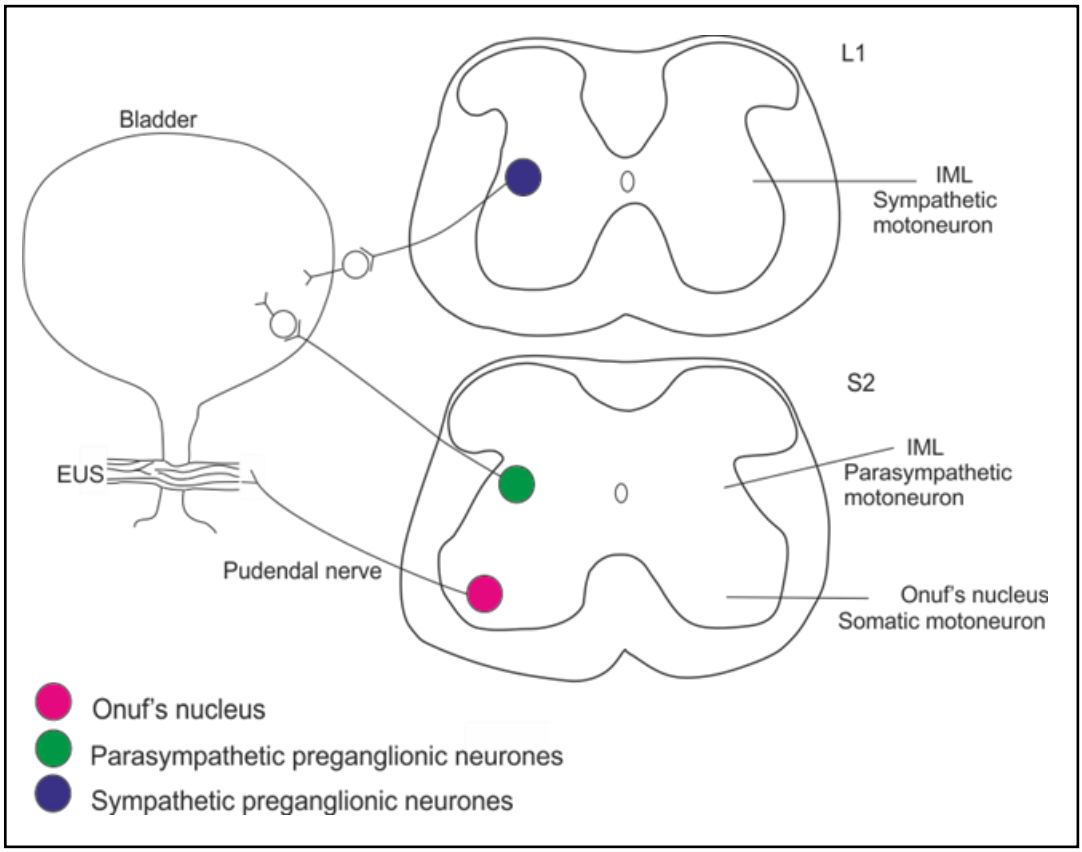

Figure 1.2: Innervation from the lumbosacral spinal cord to the bladder and the EUS. The EUS is innervated by the pudendal nerves which originated from the Onuf's nucleus in the sacral cord in human. The bladder is innervated by sympathetic and sacral parasympathetic nerves providing autonomic control

1.3.2. Sympathetic preganglionic neurones innervating the bladder

Sympathetic and parasympathetic motor neurones belong to the group of autonomic nervous control. The autonomic nervous system provides highly coordinated functions that are carried out without voluntarily control. Almost every part of the body is innervated by autonomic nervous system including the bladder and urethra. The physiological influence of sympathetic and parasympathetic division is generally antagonistic to each other.

Retrograde labelling from the bladder has revealed that the sympathetic postganglionic neural innervation arises predominantly from neurones located in the

inferior mesenteric and major pelvic ganglia (Vera and Nadelhaft, 1992). These postganglionic neurones in turn are innervated by preganglionic neurones in located in the intermediolateral cell group of the thoracolumbar level of the spinal cord, as revealed by injections of the transneuronal tracing pseudorabies virus into the bladder of male rats (Nadelhaft et al., 1992). Application of HRP to the hypogastric nerve of the cat revealed that sympathetic preganglionic neurones influencing the bladder were located in the intermediolateral cell column (IML) and intercalated nucleus of L2-L5 (Morgan et al., 1986). In dog, following the injection of HRP into the bladder, it was suggested that sympathetic preganglionic neurones projecting to the intramural ganglia of the bladder were located in the spinal segments of L1-L4 mainly in intercalated nucleus and IML (Petras and Cummings, 1978). However, sympathetic preganglionic neurones typically synapse in ganglia in the sympathetic chain and since no other study has reported similar patterns, it is possible that these were parasympathetic preganglionic neurones or indeed labelled as a result of spillage of tracer during the injections.

Sympathetic innervation has inhibitory effects on detrusor muscle causing relaxation of bladder wall, and excitatory effects on smooth muscle of the bladder neck and urethra causing constriction of internal urethral sphincter and urethra (Keast et al., 1990). Keast et al. (1990) performed a pharmacological study in cat showing that sympathetic hypogastric nerve stimulation or exogenous catecholamines produce β-adrenergic receptor mediated inhibition of the body and α-adrenergic receptor mediated contraction of the base, dome and urethra. The postganglionic sympathetic fibres innervate the two parts of the bladder to promote storage of urine. Surrounding the bladder outlet, sympathetic postganglionic fibres release norepinephrine to activate α1-adrenergic receptors which constrict the smooth muscle of bladder neck and internal urethral sphincter. In the body of the bladder, β3-adrenergic receptors of the detrusor muscle receive postganglionic

sympathetic innervation and stimulation of these receptors causes relaxation of the detrusor smooth muscle. Thus, the sympathetic bladder innervation allows bladder filling via constriction of the bladder outlet and inhibition of bladder wall contraction.

1.3.3. Parasympathetic preganglionic neurones innervating the bladder

The autonomic parasympathetic system innervates the visceral organs, smooth muscle and secretory glands and is important for the maintenance of homeostasis. Parasympathetic outflow originates from parasympathetic preganglionic neurones which serve as an important site for the integration, organisation and modulation of the outflow. Each preganglionic neurone synapses with its postganglionic neurone in the peripheral ganglion, typically located near or within the target organ. They respond to the external environment as well as internal physiological changes, and work in concert with the sympathetic outflow to control and regulate functions including heart rates, gastrointestinal motility and micturition.

The origin and distribution of the parasympathetic innervation of the urinary tract was revealed by retrograde transport of HRP from the cut pelvic nerve of the cat (Nadelhaft et al., 1980). The majority of labelled cells were localised in the intermediate gray matter in S2, but could extend into adjacent laminae of the spinal cord. The parasympathetic preganglionic cells were noted to be medium in size, oval or spindle-shaped and lie in transverse orientation. They also observed an organotopic organisation of the sacral parasympathetic nuclei, in which the bladder neurones are located on the lateral edge, while the colonic neurones are located more dorsally. Their findings were consistent with earlier findings by electrophysiological study in cat by de Groat and Ryall (1968) and an HRP study in cat by (Morgan et al., 1979), which indicated separation in the location of

preganglionic neurones innervating the bladder in the lateral band and neurones innervating the colon and rectum in the dorsal band.

In the dog, following the injection of HRP into the bladder trigone and proximal urethra, parasympathetic preganglionic neurones projecting to the bladder were located in the spinal segments of S2-S3 which lies mainly in the intermediolateral cell column and intercalated nucleus (Petras and Cummings, 1978). Similarly, by using HRP retrograde tracer on the cut end of individual ventral roots of the sacral cord, (Yamamoto et al., 1978) studied the distribution of parasympathetic preganglionic neurones in cats. They observed the location of the labelled projecting neurones to originate from the intermediate region of spinal segments S2-S3.

However, in rat the parasympathetic preganglionic neurones appear to occupy a position more rostral in the spinal cord compared to other mammals. In male Sprague Dawley rats, preganglionic neurones labelled by the retrograde transport of HRP in the pelvic nerve were present in spinal segments L6-S1 (Hancock and Peveto, 1979). In transverse sections, these cells were observed to aggregate and form a cluster in the middle and along the lateral border of the IML. The sacral autonomic neurones were investigated in an autopsy study in Shy-Drager Syndrome patients by Konno et al. (1986). They described the intermediolateral cell nucleus as distributed throughout the S3-S4 cord segments in human, extending from the lateral aspect of intermediolateral gray to the dorsolateral border of ventral horn. Comparing their observation with studies in other species, they concluded that the cytoarchitecture of sacral autonomic neurones is universal among mammals.

Morgan et al. (1993) further analysed the morphology of parasympathetic neurones by injecting neurobiotin or horseradish peroxidase into single preganglionic neurons in the lateral sacral parasympathetic nucleus of the cat in vivo. By using light microscopy, they observed that these neurones have multiple branching of dendrites and the dendrites had major transverse and longitudinal orientations. The transversely orientated dendrites are long, and could reach up to the lateral edge of the dorsal horn, lateral funiculus, ventral horn as well as towards the midline and into dorsal gray commissure.

Parasympathetic action facilitates contraction of bladder to expel the urine during micturition. The preganglionic parasympathetic neurone fibres travel along the pelvic nerve to the intramural ganglia and synapses with the postganglionic neurones by releasing acetylcholine. Parasympathetic excitatory transmission in the bladder is mediated by acetylcholine acting on post-junctional muscarinic M3 receptors and evoked contraction of the bladder. When the muscarinic agonist carbachol is applied to bladder strips in organ bath, addition of the non-selective antagonist atropine and selective muscarinic antagonists revealed that the contraction of the human bladder occurs mainly, if not exclusively, via the M3 receptor (Fetscher et al., 2002). Similar contractile response to the muscarinic agonist predominantly via M3 receptors have also been observed in rats (Longhurst et al., 1995) and mice (Choppin and Eglen, 2001). Furthermore, muscarinic receptor knockout mice revealed that the M3 subtype is the principal receptor involved in excitatory transmission, supported by the observation that M3 receptor knock-out mice exhibit bladder distension and develop urinary retention (Matsui et al., 2000).

In addition to that, adenosine triphosphate (ATP) is also an excitatory transmitter mediating noncholinergic contractions (Burnstock et al., 1972). In bladders of guinea pig, application of ATP mimicked the nerve mediated contraction, which is rapid but brief. Application of the ATP antagonist quinidine was shown to block bladder contractions both to nerve stimulation and to exogenous application of ATP, but not to acetylcholine. Later studies have supported the finding of purinergic transmission in the bladder of rat (Igawa et al., 1993). ATP excites the bladder smooth muscle by acting on purinergic P2X receptors which are ligand gated ion channels, in which P2X1 receptor mRNA is the major subtype expressed in human urinary bladder (Valera et al., 1995).

1.3.4. CNS regions controlling bladder neurones and micturition pathways

During the period of storage, the filling of urine into the bladder occurs. For storage of urine to occur, the muscle of the bladder is relaxed and both the internal urethral sphincter and the external urethral sphincter are contracted. This synergistic actions result in the holding of urine in the bladder, closure of bladder outlet and prevention of leakage. As filling occurs, the bladder wall is stretched to accommodate the urine until it reached its micturition threshold. Once the bladder is full, mechanoreceptor signals pass to the central nervous system to facilitate micturition.

Sensation from the bladder is transmitted to the CNS via general visceral afferent fibers. The afferent fibers on the superior part of bladder follow the course of the sympathetic efferent nerve back to CNS, while afferent fibers on the inferior portion of the bladder follow the course of the parasympathetic efferent (Thor and de Groat, 2010). Sensory information on bladder filling is carried by A-δ fibers from the

bladder to a group of neurones in dorsal horn of S1-S2 spinal cord in cat (Sasaki, 1994). Injections of cholera toxin B subunit (CTB) into the urethral in cat produced labeling of the spinal terminals of primary afferent neurones in lateral and medial lamina I, the intermediate gray matter, and the dorsal commissure gray matter (de Groat WC, 2001). These spinal neurones relay ascending projections to the periaqueductal gray (PAG) within the tegmentum of the midbrain (Holstege and Mouton, 2003). The PAG plays an important role as regulator of LUT, receiving information from the bladder concerning the rate of bladder filling, processing the information and determining whether to activate the pontine micturition centre (PMC) to initiate voiding (Blok et al., 1995). In an electron microscopic study in cats, Blok et al. (1995) investigated projections from lumbosacral neurones to the brainstem by injecting wheat-germ agglutinin-horseradish peroxidase (WGA-HRP) into the lumbosacral cord. They showed that the sacral cord projections to the brainstem terminate mainly in the PAG. Many labeled terminals, which were filled with mainly round, dense core vesicles and with asymmetrical synapses, were found on dendrites in the lateral part of the PAG. Previously, Blok and Holstege (1994), demonstrated in an anterograde transport study in cat, stereotaxic injection of WGA-HRP and tritiated leucine in the PAG showed that this area contains neurones projecting specifically to the PMC and serve as a link between the sacral cord and the PMC (Figure 1.3).

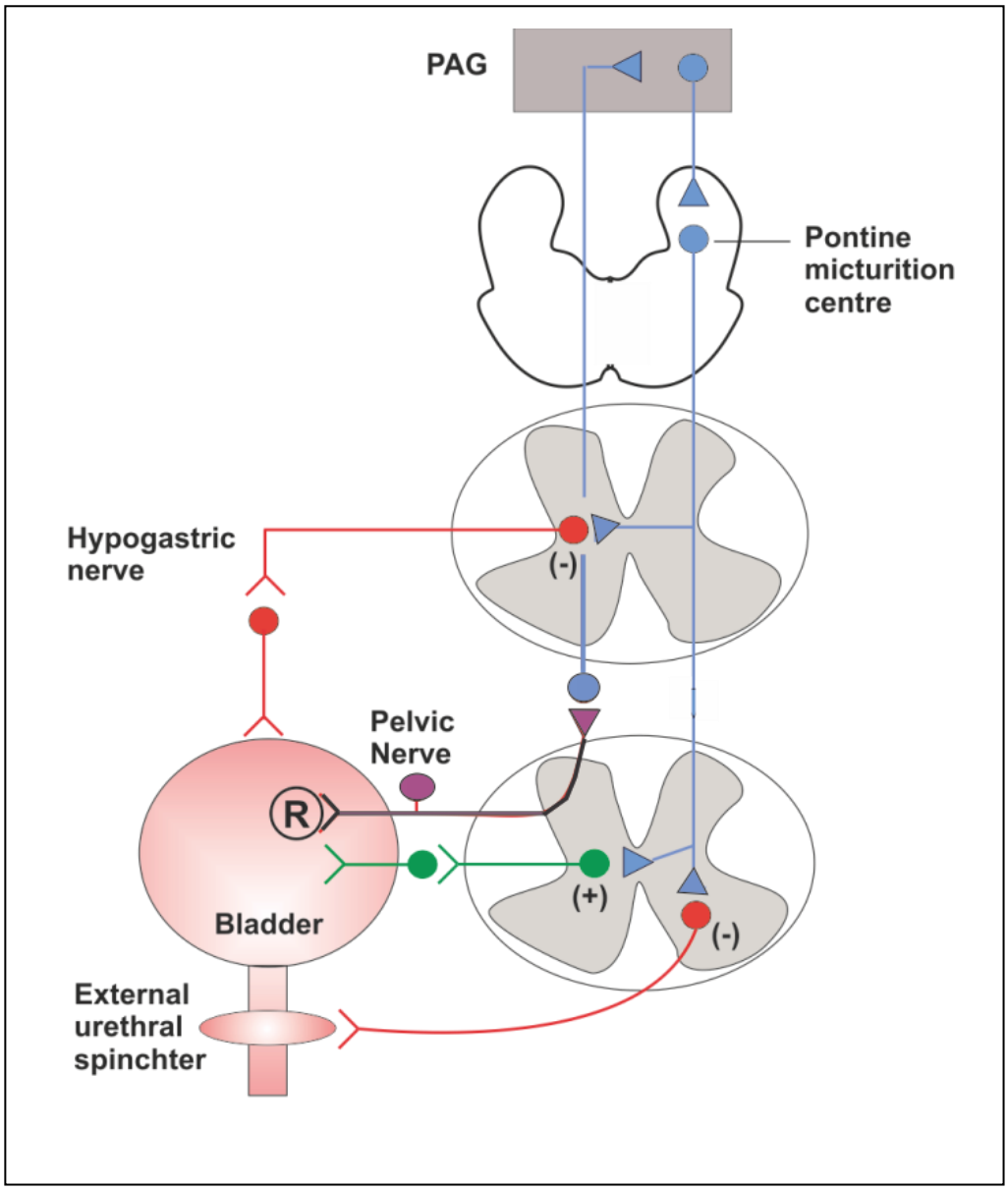

Figure 1.3 Micturition reflex pathway.

When bladder reaches its micturition threshold, the receptors on afferent nerve terminals (R) conveys signals in the form of intense afferent firing via the pelvic nerve to the CNS (purple) and activates the spinobulbospinal reflex pathways (blue). Ascending afferent input from the spinal cord pass through relay neurones in the PAG before reaching the PMC. PMC activates the parasympathetic outflow to the bladder (green) to cause detrusor muscle contraction and inhibits the sympathetic and pudendal outflow to the bladder neck and EUS (red), respectively, to allow passage of urine. Note that open triangles represent axon terminals in the peripheral nervous system, while filled triangles represent axon terminals in the CNS . Figure modified from Fowler et al. (2008).

The PMC is the control nucleus of voiding which receives afferent inputs from the PAG. PMC is a group of cells located dorsally in the pontine medial tegmentum, and is alternatively known as the M-region or Barrington's nucleus, named after Barrington, who in 1925 was the first to describe this pontine control centre for micturition in the cat (Barrington, 1925). He described that destruction of a small and specific part of the brainstem ventral to the internal edge of the superior cerebellar peduncle is followed by a permanent inability to empty the urinary bladder if the lesion is bilateral, but not if it is unilateral. It serves as the coordinator of the motor neurones that innervates the urinary bladder and external urethral sphincter, both located in the sacral spinal cord. The PMC acts like an on-off switch, activation of which initiates micturition or vice versa. In an acute decerebrate cat, Sugaya et al. (1987) demonstrated that electrical stimulation and carbachol injection into the PMC causes the contraction of the detrusor muscle producing an increase in the intravesical pressure, and relaxation of the urethra which produced a sharp decrease in the urethral pressure. These observations mimic normal physiology of micturition.

Neurones in the PMC send long descending fibres directly to the parasympathetic preganglionic neurones in the IML of sacral spinal cord. By using light microscopy and anterograde [3H] leucine tracing study in cat, (Holstege et al., 1979) provided evidence that the PMC projects to the area of the intermediolateral cell column of the sacral spinal cord. To confirm that the projection was specific to parasympathetic preganglionic neurones, Blok et al. (1997) injected in the PMC with WGA-HRP and injected the bladder wall with CTB. Electron microscopy revealed that anterogradely labeled fibres from the PMC were found to terminate on somata and dendrites of the retrogradely labeled preganglionic bladder motor neurones. The terminals contained round vesicles and an asymmetric synapse

which strongly suggest that the PMC has an excitatory effect on parasympathetic bladder neurones.

Activation of the PMC also causes relaxation of the EUS through indirect innervation to Onuf's nucleus via the inhibitory GABAergic and glycinergic interneurones located in the dorsal gray commissure of the sacral spinal cord. Blok et al. (1997) described an indirect GABAergic pathway inhibition from PMC to Onuf's nucleus. In the study, they injected the PMC with WGA-HRP and demonstrated anterograde projections from the PMC in the brainstem to the dorsal gray commissure of the sacral cord in cat. More than half of the terminal inputs made contact with GABA-immunoreactive interneurones. Thus they postulated that during micturition, the PMC directly stimulates the parasympathetic preganglionic neurones to contract the bladder and indirectly inhibit the motor neurones in Onuf's nucleus via GABA interneurones to relax the sphincter. Similarly, in another study to investigate the influence of glycine-IR interneurones in cats by (Sie et al., 2001), the PMC was injected with the anterograde tracer WGA-HRP, serially sectioned and the sacral cord segments were processed for light and electron microscopic to detect anterograde labeling, as well as for glycine and GABA, using post-embedding immunogold labeling with antibodies. About 30% of labeled PMC terminals made contact with glycine-IR dendrites in the dorsal gray commissure. The GABA and glycine-IR interneurones are thought to be the major inhibitory transmitters regulating the EUS motor neurones activity during micturition.

1.3.5. Other segmental and supraspinal inputs to Onuf's nucleus

Apart from direct innervation from the dorsal gray commissure GABA and glycine interneurons as mentioned above, Onuf's nucleus also receives polysynaptic inputs from bladder and urethral afferents. Electrophysiological studies show that stimulation of either pelvic nerve or pudendal nerve afferent fibers can activate polysynaptic spinal segmental reflexes. For example, Fedirchuk et al. (1992) examines the short-latency synaptic input from a variety of cutaneous perineal afferents to the motor neurones innervating these muscles. When electrical stimulation was applied to ipsilateral and contralateral peripheral afferents in decerebrate or chloralose anesthetized cats, intracellular recordings from antidromically identified EUS motor neurones showed excitatory postsynaptic potentials (EPSPs). They also observed that the shortest central latencies were 1.5 ms, which suggest that there are disynaptic excitatory, in addition to tri- and oligosynaptic connections within these reflex pathways. The segmental reflex is considered polysynaptic based on the central delay. Besides that, following pseudorabies virus injection into the bladder (Sugaya et al., 1997) and urethra (Vizzard et al., 1995) in rats, neurones containing PRV immunoreactivity were located in the superficial and deeper laminae of the dorsal horn, in the region of the sacral parasympathetic nucleus and in the dorsal commissure. The dorsal horn interneurones are likely to participate in the segmental reflex activation of sphincter motor neurones, while the dorsal gray commissure interneurons are likely to be inhibitory.

While being somatic and under voluntary control, Onuf's nucleus also receives direct inputs from brain region that specifically control autonomic neurones. It is unique among somatic motor neurones as it receives inputs from the

paraventricular hypothalamic nucleus (Holstege and Tan, 1987). However, the function of this input remains speculative. This finding has raised the question whether Onuf's motor neurones belong to the somatic or to the autonomic motor system. Furthermore, it was noted that Onuf's nucleus does not degenerate in patients suffering from ALS, a fatal illness destroying all somatic motor neurones (Mannen et al., 1977). On top of that, post-mortem histopathological study conducted on patients who suffered a pronounced autonomic failure condition known as Shy-Drager syndrome, surprisingly revealed extensive destruction of Onuf's nucleus (Chalmers and Swash, 1987). Thus, Onuf's motor neurones occupy a position between somatic and autonomic motor neurones. These unique features of the Onuf's motor neurones are reflected by the peculiar behaviour of the EUS. While on one hand, it can be contracted voluntarily; on the other hand it must be precisely coordinated with bladder activity, which is a function of the autonomic motor system.

Onuf's nucleus also receives dense serotoninergic and adrenergic inputs (Thor et al., 1993). On a study in baboons, Rajaofetra et al. (1992) performed immunohistochemistry to detect serotonin (5-HT) immunoreactivity and electron microscopy to identify synapses in the Onuf's nucleus in the intact and transected spinal cord at thoracic level. In baboons, the Onuf's nucleus consists of the ventrolateral and dorsomedial compartment in a single spinal nucleus, similar to human. In transected spinal cord at thoracic level, the innervation of serotonin to the whole Onuf's nucleus was diminished on the ventral half but was completely absent in the dorsal half. They concluded the supraspinal serotonin innervates both the ventral and dorsal part of Onuf's nucleus, whilst intraspinal serotonin inputs innervate only the ventral half of the nucleus. The sphincter motor neurones may receive brainstem projection from 5-HT-containing neurons in the raphe nucleus while the intraspinal innervation may come from spinal 5-HT-IR cells that have been

described by (Newton et al., 1986). Rajaofetra et al. (1992) has also demonstrated noradrenaline innervation to Onuf's nucleus was completely lost in transected spinal cord indicating its supraspinal origin. On the other hand, innervation by substance-P and calcitonin-gene related peptide (CGRP) containing terminals were not affected by transection of spinal cord suggesting that their origins are within in the spinal cord.

1.4 Synaptic changes in ageing

In the CNS, ageing is mainly associated with a decline in cognitive function and neurodegenerative diseases. Extensive studies were done to determine the cause and the underlying mechanisms, especially in age-related disorders such as Alzheimer's disease and Parkinson's disease. Several explanations have been sought regarding neurochemical, structural and functional change in the CNS during ageing. In the past, these age-related brain pathologies were commonly attributed to neuronal loss and shrinkage of brain tissues. However, recent investigations on the number of neurones in advanced age suggest that a profound loss of neurons does not significantly contribute to age-related cognitive impairment. For example, (Peters and Sethares, 2002) performed a quantitative study on the number of neurones in layer 1 of the cortex in rhesus monkeys and demonstrated no significant age-related loss of neurons. They ruled out neuronal loss as a cause of age-dependent cognitive decline.

The current trend on studies on human and animals suggest that age-related cognitive decline is more likely to be associated with alteration in synaptic connectivity than neuronal loss. New data are pointing towards subtle alterations in the synaptic components, alteration in the plasticity in aged animals and disruption

of synaptic communication network that lead to behavioural impairment. In the brain, age-related cognitive deficits correlate with cortical synaptic loss (Wong et al., 1998). They demonstrated that these losses are more pronounced in deeper cortical layers. The loss of synapses in ageing may be associated with decrease in the dendritic arborisation and spines of layer V pyramidal neurones (Wong et al., 2000, Duan et al., 2003). Other studies have also shown the loss of dendritic branches and reduction in the numbers of dendritic spines in aged rats (Wong et al., 1998), monkeys (Cupp and Uemura, 1980) and humans (Masliah et al., 1993). In addition to that, in an electron microscopy study, (Peters et al., 2008) demonstrated that in layer 2/3 of the frontal cortex of ageing monkeys, there is a 30% reduction in the density of asymmetric and symmetric synapses as a function of age. The asymmetric synapse represents excitatory synapse while symmetric synapse corresponds to inhibitory synapse. While both types of synapses are decreased by similar amounts, only the loss of asymmetric excitatory synapses is strongly correlated with cognitive impairment in ageing monkeys.

1.4.1. Synaptic changes to spinal neurones controlling bladder and urethral spincter

Degenerative changes linked to ageing have previously been shown to have marked effects on the structure and function of neuronal circuitry in the areas involved in bladder function. In a series of investigations by (Santer et al., 2002) and (Ranson et al., 2003a, Ranson et al., 2005, Ranson et al., 2007a, Ranson et al., 2007b) in young (3 month) and aged (24 month) rats, the distribution of neurotransmitter-specific innervations to the lumbosacral spinal cord have been described, providing valuable information on the age-related changes to the neurones controlling pelvic functions, including micturition and continence control. (Santer et al., 2002) analysed age-related changes in the preganglionic neurones in

the lumbosacral spinal cord, by injecting retrograde tracers into the major pelvic ganglion of young adult and aged male rats followed by electron microscopy study. The study revealed significant reductions in the synaptic apposition length made by glutamate-IR boutons onto the dendrites of sympathetic, but no changes were found in parasympathetic preganglionic neurones. Synaptic apposition length from boutons immunoreactive for glycine or gamma-aminobutyric acid (GABA) was unchanged. 5-HT-IR terminals are closely associated with preganglionic autonomic neurones, and these are reduced in number in sympathetic, but not parasympathetic, spinal nuclei of aged rats. Besides that, the percentage distribution density of TRH-IR terminals are also significantly reduced in number in regions containing preganglionic sympathetic, but not parasympathetic, neurones.

The effects of ageing on the innervation patterns of 5-HT and tyrosine hydroxylase (TH) to the lumbosacral spinal nuclei have also been investigated (Ranson et al., 2003a). Quantitative image analysis revealed significant age-associated declines in the innervation of most regions including the IML, sacral parasympathetic nucleus, dorsal grey commissure and in the DLN. However, no changes were observed in the 5-HT innervation of the sacral parasympathetic nucleus, and no changes in TH-like immunoreactivity to the DLN. (Ranson et al., 2005) performed quantitative image analysis was used to determine age-related changes in the substance P-containing innervation of autonomic and somatic nuclei in the lumbosacral spinal cord, which are associated with the control of micturition and sexual reflexes. Substance P-IR innervation of the DLN remained robust in aged animals and was not significantly different from young adults, although significant age-related decline was observed in the innervation to sympathetic and sacral parasympathetic nucleus.

Ranson et al. (2007b) investigated the distribution and localisation of vesicular glutamate transporters, VGLUT1 and VGLUT2-IR axon terminals innervating pelvic motor neurones in rats. VGLUT1 terminals were sparsely distributed within the DLN dorsolateral nucleus and the rest of pelvic motor nuclei. However, there was a robust innervation of VGLUT-IR terminals in the DLN. Quantification of VGLUT2 immunoreactive boutons in close association with these dendrites of motor neurones innervating levator ani mucle was carried out in young and aged animals using light microscopy. This revealed a significant decline in the numbers of VGLUT2 immunoreactive boutons on the more distal dendrites of motor neurones in aged rats. Age-related changes in the number and size of large cholinergic terminals immunoreactive for vesicular acetylcholine transporter (VAChT), were documented in male rats (Ranson et al., 2007a). The most significant changes were a large increase in the number and size of cholinergic terminals within the DLN of aged animals (24 month old), suggesting they remain robust in ageing. The summary of the findings is provided below (Table1.1)

From these studies, it is clear that different nuclei possess different susceptibility to changes in the innervations in ageing, which may explain the poor bladder control in late age. But to get a better picture and to increase our understanding of CNS regulation in ageing, several issues need to be addressed. For instance, the distribution of strong and fast-acting excitatory glutamate and inhibitory GABA and glycine-IR terminals, and the impact of ageing on these axon terminals has not been thoroughly investigated in the DLN and autonomic neurones and warrant further investigation.

Table 1.1: Age-associated changes of neurotransmitter-specific axon terminals on spinal neurones controlling micturition

Paper (Year)	Neurotransmitter- IR terminals	DLN	Sympathetic	Parasympathetic
Santer et.al (2002)	Glutamate	-	↓	NC
	GABA	-	NC	NC
	Glycine	-	NC	NC
	5HT	-	↓	NC
	TRH	-	↓	NC
Ranson et. al (2003)	5-HT	↓	↓	NC
	TH	NC	↓	↓
Ranson et. al (2005)	Substance-P	NC	↓	↓
Ranson et. al (2007)	VGLUT2	- (↓ in LA motor neurones, but no age-related comparison was done in DLN)	-	-
Ranson et. al (2007)	VAChT	↑	-	-

NC: no changes

1.5. Research question, hypothesis, aim and objectives

There is relatively little published work on morphological changes in synapse during normal ageing in the spinal cord, particularly to the autonomic and somatic motor neurones that regulate bladder function. The study of age-associated changes to these neurons may be key to understand neurogenic forms of bladder and sphincter dysfunction in the elderly. In the past two decades, new information on the neural control of micturition mainly derives from studies conducted by Holstege, de Groat, Thor and Santer groups. While Holstege and de Groat focused their studies on and contributed heavily to the circuitry and supraspinal control of the micturition pathways, Santer's groups devoted their research on the spinal control of pelvic function, including micturition and continence. Their study focused on the lumbosacral spinal cord including the ventral horn motor neurones that innervate the urethral sphincter. Their studies also attempted to investigate the effects of ageing on the neurones that involves in modulating the function of lower urinary tracts, by comparing the spinal nervous system of the young and aged animals as described previously in section 1.4.2. Much of the understanding on the effects of ageing on the spinal neurones comes from these studies. However, many more vital areas to understand the role of neural control of continence are yet to be explored, especially in the growing field of gerontology. Building up from the previous studies, the current study explore the possibility of alteration or even possible reversal of the excitatory/inhibitory inputs and ratio in the innervations to the neurones controlling micturition and continence.

The subject of excitatory/inhibitory innervation imbalance and its effects on system function in ageing have recently been proposed and debated in neurogerontology. New researches were conducted and new findings emerged as to demonstrate the changes in the balance of excitatory/inhibitory influence to the neurones in the

central nervous system, mainly in the brain. In the cerebral cortex, Majdi et al. (2007) investigated the density of excitatory and inhibitory presynaptic inputs across the cerebral cortex in young and aged male Fisher 344 rats using immunohistochemistry to label the presynaptic boutons, confocal microscopy and quantitative analysis. They analysed the distribution of glutamatergic and GABAergic presynaptic terminals on the pyramidal neurones in area 4 of cortex. They found gradual decline in the density of excitatory and inhibitory presynaptic boutons, however the ratio of excitatory-inhibitory density was not significantly altered. Another study in the brain cortical area, Casu et al. (2002) showed significant age-related decrease in population of cholinergic boutons in opposition to proximal and distal dendrites of layer V pyramidal neurones. Besides that, Palomba et al. (2008) studied the effects of ageing on the hypothalamic suprachiasmatic nucleus, the circadian pacemaker that regulate sleep-wake cycles. They analysed the GABAergic presynaptic terminals. The findings reveal that the number and areas of GABAergic presynaptic boutons are significantly decreased in old versus young mice. These findings show marked reduction of the GABAergic terminals synaptic network of the ageing biological clock. Besides that, pathological disease such as ALS has also been shown to be cause by altered balance, mainly hyperexcitation of the somatic motor neurones (Chang and Martin, 2009).

If the balance and neuronal homeostasis would disrupt the functioning of a neurones and summation of outcome is seen as altered normal bodily function, one wonders whether this is also the case in the uncontrolled micturition and poor continence control as observed in the elderly. Given the high dependency of the LUT organs on its innervation, it is reasonable to postulate that a slight alteration of the command centers in the spinal cord will have a direct effect on the behaviour of bladder and its closely associated counterpart - the EUS.

1.5.1 Hypothesis

By looking at the changes in the pattern of innervations elsewhere in the brain and lumbosacral spinal cord in ageing, it was postulated that the mechanism of urinary incontinence observed in aged mouse may be explained by the alteration of balance of the excitatory/inhibitory innervation to neurones that innervates the lower urinary tract. It was hypothesised that urge incontinence which is highly associated with bladder overactivity may be due to hyperexcitability of the autonomic neurones innervation the bladder wall, while the poor continence control and urinal leakage may be due to hypoexcitability of the Onuf's nucleus that innervates the EUS.

1.5.2 Aim and thesis outline

To test this hypothesis, the pattern of innervations was investigated of both excitatory and inhibitory inputs to the neurones innervating the bladder and EUS in female mice. In chapter 2, the description of micturition behavior and the fact that aged female mice do experience increase in the frequency of micturition as seen in elderly humans was established. This physiological and behavioural study is important as it serves as the basis for further correlation study on the changes of neurobiology in ageing. In chapter 3, the investigation proceed to establish the exact location of the motor neurones innervating the EUS in mice by performing surgery, injection and neuronal retrograde labelling to the mice EUS. Although the location of Onuf's nucleus and its homologue - the DLN have extensively been studied in numerous species, detailed study and its documentation have not been conducted in mouse. Since mouse is used throughout this study and is a very good model to be used in future research on incontinence, the DLN motor neurones in mouse was described in detail.

In chapter 4, to study the pattern of innervation and its associated changes in ageing, quantitative and comparative study on the presynaptic terminals inputs of excitatory glutamate-containing terminals and inhibitory GABA and glycine-containing terminals that made close apposition with the DLN motor neurones, and onto autonomic parasympathetic and sympathetic neurones innervating the bladder was performed. Through immunohistochemistry and confocal imaging, the inputs were measured and the ratio of excitatory to inhibitory influence to these neurones was demonstrated in different age groups. In chapter 5, neuropharmacological study based on the findings demonstrated in chapter 2 and 4 was performed. Investigation was carried out to determine whether AMPA receptor potentiator drug LY404187 would have beneficial effect to reverse the high frequency micturition seen in aged animals. Finally in chapter 6, all the findings were summarized and a thesis on the effects of ageing on spinal neurons controlling micturition and continence was put forward and proposed based on the results of experiments conducted throughout this study.

Chapter 2

Analysing micturition behaviour in mice at 3, 24 and 32 months of age

2.1 Introduction

2.1.1 Micturition behaviour in human and mouse

Micturition is the release of urine from the urinary bladder through the urethra and the urinary meatus outside the body. It is also known as uresis, urination, or voiding. In healthy adults, the micturition process is under voluntary control. Epidemiological studies suggest that over a 24-hour period, women have seven or eight micturition episodes during waking hours and one or less per night (Lukacz et al., 2009). However, this number is highly varied based upon the total hours of sleep, fluid intake, comorbid medical conditions and other factors. Wyndaele (1998) investigated 38 healthy volunteers and found that three distinct sensations were consistently described during artificial bladder filling. They are first sensation of filling; first desire to void and strong desire to void. The need to urinate is experienced as an uncomfortable feeling of fullness. In male and female, the feeling of fullness and the need to urinate is felt at the lower abdomen region when the bladder is full. The urge to urinate is stimulated by expansion of the bladder. As the bladder walls fill with urine and expand, stretch receptors are stimulated and the micturition reflex is triggered. The normal functional bladder capacity in adult human is 300 to 400ml. The desire to urinate is usually experienced when it contains between 150 and 200 ml and the release of urine is experienced as a lessening of the discomfort.

In humans, the main function of micturition is to dispose waste substances in the urine, by the mean of emptying the bladder. Once outside the body, the urine does not play any important role. While the primary purpose of micturition is the same across the animal kingdom, micturition often serves a social purpose beyond the expulsion of waste material. Thus, in mammals, micturition has additional roles and purposes for survival as an individual as well as a species. In rodents, the presence of urine outside the body is important as demarcation of territory. Besides that, for species survival, the female expels urine during oestrous period as a sexual signal. It signifies to the male that mating is possible. Apart from that, in small rodents such as rats and mice, urine is also use for marking the route along familiar paths

2.1.2 Micturition and continence control during developmental and ageing

Micturition is under the control of a highly complex system involving central, autonomic and somatic nervous system. Effective bladder control is achieved by involvement and integration of neural, smooth muscle and striated muscle coordination to maintain continence and to allow intermittent emptying of the bladder (Thor and de Groat, 2010). During development, involuntary bladder reflex precedes the voluntary bladder control. In infant and young toddler, micturition occur involuntarily until the age of 3-5 years old (Longhurst, 2004). The urine control is achieved in stages. It begins with the sensation of bladder fullness, followed by daytime bladder control and finally night time bladder control. At the end of this period, voluntary voiding is regulated. In mammals, the period of maturation for urinary control varies amongst species. In rat for example, maturation of urinary control takes 2 to 3 weeks (de Groat, 2002). The exact mechanism for the shifting of involuntary to voluntary control of micturition is still largely unknown. However, it is postulated that this shift is probably due to increasing contribution of various

neurotransmitters, changes in the sensitivity of urinary bladder contractility to stimulation, functional maturation of synaptic transmission and reorganisation of the micturition reflex in which the supraspinal micturition reflex is established (de Groat, 2002). The prevalence of urinary incontinence increases considerably with age (Wehrberger et al., 2012). Overactive bladder syndrome is common and distressing disorder of bladder and lower urinary tract, which compromises the storage ability of the bladder in the elderly population. Investigations on the micturition pattern in rats have revealed age associated increases in micturition volume and frequency of micturition episodes which may suggest urinary dysfunction in the elderly (Ranson et al., 2003b).

2.1.3 Hypothesis, aims and objectives

Although in human, the impact of ageing on micturition behaviour is well documented, the precise nature of urinary control in ageing rodents, especially mouse remains unclear. To date, there are relatively few studies that have investigated how bladder function and neural activity are affected by ageing. Since mice are used throughout this study as the animal model to study the effects of ageing on continence control, the micturition behaviour analysis of mice is vital. To address this issue and to determine the significance of ageing on urinary and continence function, urinary output of wild-type female mice was analysed. Understanding how the physiological function alters with age is an important step in the understanding of the neurobiology of micturition and continence control.

The aim of this study is to investigate how natural ageing affects voiding output in mice. The study in this chapter sought to determine whether an increase in age alters the frequency, volume and time intervals between voiding in mice. The micturition profiles of mice were evaluated, voiding pattern in vivo characterised and

age-associated changes of young (3 month-old), aged (24 month-old mice) and aged (32 month-old) were observed. The results in this chapter will serve as a basis of correlation with synaptology studies in the subsequent chapters. It will establish the association between physiology of micturition behaviour and biological effects of ageing on the presynaptic terminals onto the spinal neurones controlling micturition and continence.

2.2 Methods

2.2.1 Animal

Eleven female wild type C57BL/6 mice were used in this experiment, divided into the following age groups young adult (YO) 3 month old (m.o) (n=4), aged (AG) 24 m.o (n=4) and extreme aged (EA) 32 m.o (n=3) were used. The experiments were conducted on small number of animals, as it was not feasible to gather a large sample for the aged and extreme aged. Furthermore, previous ageing studies on micturition behaviour in animal had yielded significant findings despite small sample size being used. Animals were housed in a room with 12 hour dark/light cycle with ad libitum access to food and water. All procedures, care and handling of animals were performed in accordance to the Animals (Scientific Procedures) Act 1986 and all efforts were made to minimise animal discomfort and the number of animal used.

2.2.2 Establishment of volume-area correlation

The micturition analysis was performed on the mice using the voided stain on paper (VSOP) method, as described by Sugino et al. (2008). To establish the volume-area correlation, the mice were placed individually in the metabolic cages for 6 hours duration and urine were collected and pooled. The urine collected was pipetted at a

specified volume and dropped on filter papers (Figure 2.1C). The area of the spot of urine was measured and plotted on the graph volume against area. A linear graph was created (Figure 2.1A) and a mathematical equation was derived at V= 10A – 10, where V is volume (µl) and A is area (cm^2).

2.2.3 Experimental analysis

Experiments were performed during the day and night portion of the animals' circadian clock cycle. Daytime experiments were performed between 0900 to 1300 hour and night time experiments were carried out between 1900 to 2300 hour. To evaluate the micturition behaviour of mice across age categories, conscious mice were placed individually into the modified metabolic cage (diameter 16cm) for precise collection of voided urine. The animals were acclimatised for at least 1 hour to the new environment before data were collected. Experimenting time was for 3 hours duration in the daytime and 3 hours in the night time. The animals were allowed to eat and drink ad libitum and to move freely on the wire-netted grid. A filter paper was placed underneath the wire-netted grid 5 cm below it. Whenever the mice urinated, the urine dropped through the wire-netted grid (3.2 x 0.4cm), landed on the filter paper and made a spot on the filter paper. The filter paper was changed every 30 minutes to avoid multiple drops on the same spot. Care was taken to change it smoothly without disturbing the mice. Any urine drop that landed on the wire-netted grid is manually collected by bringing the filter paper up to touch the grid. The urine spot made by this method has been validated to correspond to the volume of the urine drop. After 3 hours the individual mouse was returned to their respective cages and the filter paper collected for analysis.

2.2.4 Analysis of VSOP

The filter papers were placed under UV light to visualise the dried urine which fluoresces under UV light. The edge of the urine spot was outlined. A 1x1cm rectangle grid paper was placed under and superimposed at the back of the filter paper and the total area of each urine spot was measured. The area of the urine spot and the total number of spots were entered to Microsoft Excel 2013 software for further analysis. Four parameters were derived from the collected data. These include a) frequency (number of void/hour), b) total voided urine volume (µl/hour), c) urine volume per void (µl/void) and d) time between voiding intervals (minutes). Data was analysed for comparison between inter-age groups as well as the difference between daytime and night time voiding behaviour within the same age.

2.2.5 Statistical analysis

Data from each animal were obtained to find the mean value. All quantitative data were expressed as mean ± standard error of the mean (S.E.M). Data was analysed using one-way ANOVA to determine whether there is any significant differences between the means of the three groups. If homogeneity of variances (tested by Levene's test) was violated, a Welch's correction test was run and Games Howell post-hoc test was performed. If there was homogeneity of variances then a post-hoc Tukey's analysis was performed. $P < 0.05$ was taken as statistically significant difference between groups.

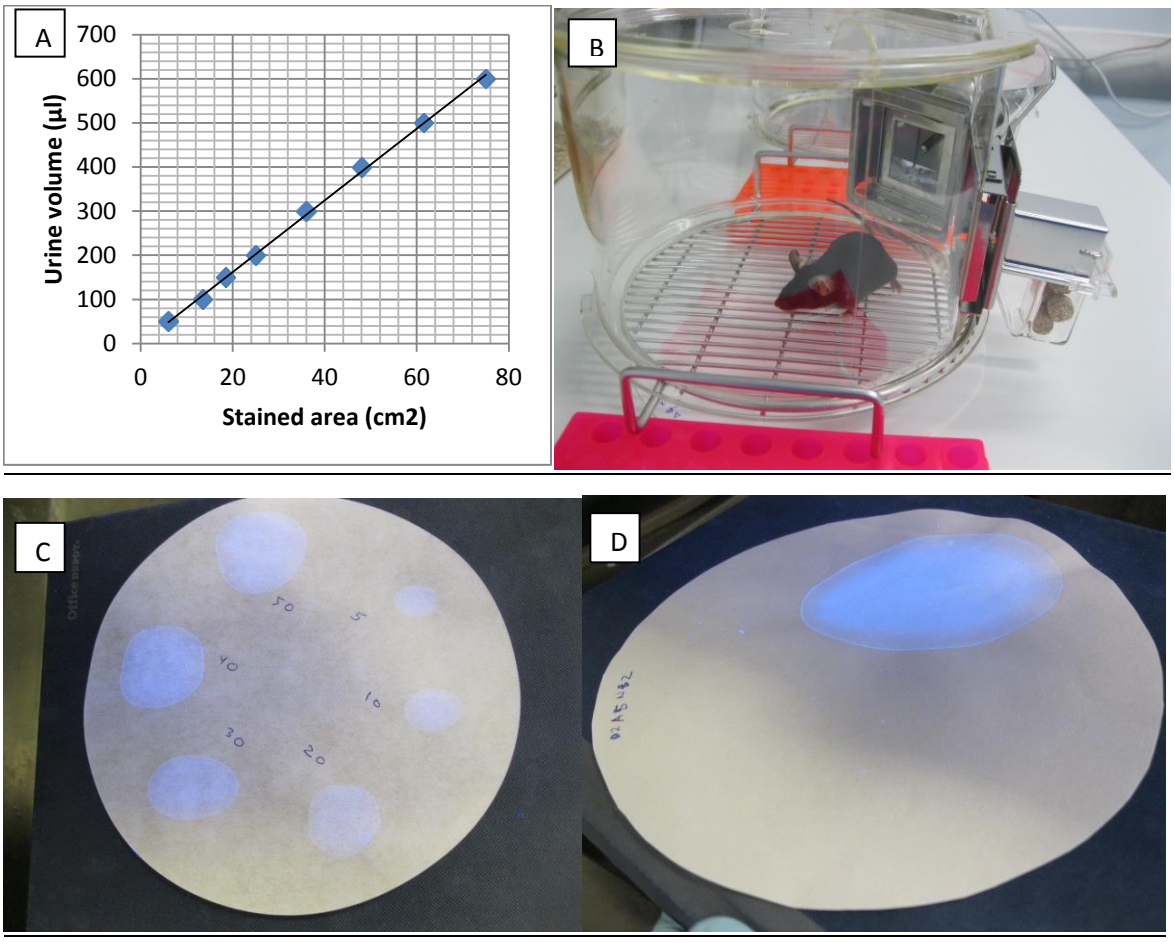

Figure 2.1: Setting up of VSOP method and establishment of volume-area correlation.

A) the graph of urine volume (µl) against voided urine stained area (cm^2). B) The set up for the micturition behavioural analysis. The mouse is allowed to move freely in the modified metabolic cage and a filter paper placed beneath it. C) To establish the corresponding volume to stained area, the filter papers were placed under UV light to visualise the urine which fluoresce under UV light prior to experiment and D) sample of collection of mouse urine under UV light.

2.3 Results

The four parameters of micturition were obtained from young, aged and extreme age mice and the experiments were conducted during daytime and night time. From visual inspection of micturition in mouse, urine was discharged as droplets, and an episode of voiding lasted between 2 to 5 seconds. From the behaviour observed, all the young mice tended to urinate in one corner of the cage. They appear to revisit the same spot in the cage to void. However, the aged and extreme aged mice do not appear to have specific spot to urinate as they were observed to void almost everywhere within the cage.

2.3.1 Frequency (number of voiding/hour) between age-groups

Frequency of micturition during daytime was significantly different between age groups, $F(2,8)= 9.128$, $p=0.009$. Frequency of micturition slightly increased from 3–month old (0.55 ± 0.06 voids/hour) to 24-month old (0.58 ± 0.08 voids/hour) and then doubled at 32-months old of age (1.18 ± 0.19 voids/hour), in that order (Table 2.1). Tukey post hoc analysis revealed that the increase from 3-month old to 32-month old was statistically significant ($p=0.012$) as well as increased from 24-month to 32-month old ($p=0.015$, Figure 2.2A).

For the night experiments the frequency of micturition was significantly different between age groups, Welch's ANOVA $F(2,8)=30.97$, $p=0.012$. Frequency of micturition increased from 3-month old (0.58 ± 0.05 voids/hour) to 24-month old (0.86 ± 0.03 voids/hour) to 32-month old (1.55 ± 0.16 voids/hour). Games-Howell post hoc analysis revealed significant increases from 3-month old to 24-month old ($p=0.016$) and from 3-month old to 32 month old ($p=0.04$, Figure 2.2A)

2.3.2 Total volume of voided urine (µl) / hour between age-groups

There was a positive correlation between increasing age and the total volume of urine produced/hour in both day and night settings. Total volume of voided urine/hour during daytime was significantly different between age groups, Welch's ANOVA $F(2,8)=43.19$, $p=0.007$. Total volume of voided urine increased from 3–month old (89.44 ± 9.41 µl/hour) to 24-month old (193.61 ± 23.81 µl/hour) to 32-months old of age (523.67 ± 62.78 µl/hour), in that order (Table 2.1). Games-Howell post hoc analysis revealed significant increases from 3-month old to 24-month old ($p=0.034$), from 3-month to 32-month ($p=0.034$) and from 24-month to 32-month old ($p=0.044$, Figure 2.2B)

For the night experiments, the total volume of urine/hour was significantly different between age group, Welch's ANOVA $F(2,8)=36.44$, $p=0.005$. Total volume of voided urine increased from 3-month old (119.16 ± 14.10 µl/hour) to 24-month old (300.83 ± 27.47 µl/hour) to 32-month old (634.44 ± 80.56 µl/hour). Games-Howell post hoc analysis revealed significant increases from 3-month old to 24-month old ($p=0.016$), from 3-month to 32-month ($p=0.039$) and from 24-month to 32-month ($p=0.079$, Figure 2.2B)

2.3.3 Volume per void (µl) between age-groups

The volume of urine per void was also positively correlated with increasing age in both day and night experiments. Volume per void during daytime was significantly different between age groups, $F(2,8)=22.83$, $p<0.001$. Mean volume per void increased from 3–month old (166.45 ± 25.89 µl/void) to 24-month old (335.01 ± 17.06 µl/void) to 32-months old of age (464.22 ± 51.36 µl/void, Table 2.1). Tukey post hoc analysis revealed significant increase from 3-month to 24-month

(p=0.009), from 3-month to 32-month (p<0.001) and from 24-month to 32-month (p=0.047, Figure 2.2C).

For the night cycle, the mean volume per void was significantly different between age groups, F(2,8)=12.36, p=0.004. The mean volume per void increased from 3-month old (232.60 ± 12.36 µl/void) to 24-month old (354.57 ± 29.55 µl/void) to 32-month old (452.13 ± 50.10 µl/void). Tukey post hoc analysis revealed significant increase from 3-month to 24-month (p=0.044) as well as 3-month to 32-month (p=0.003, Figure 2.2C).

2.3.4 Time intervals between voiding (minutes) between age-groups

Time interval between voiding during daytime was significantly different between age groups, F(2,8)=5.83, p=0.027. The time interval between voids in 3 month (78.41 ± 13.28 minutes) and 24 month old (89.79 ± 5.50 minutes) mice was not significantly different however, 32 month old mice had significantly fewer minutes between voids (41.89 ± 7.73 minutes) than 24 month old mice (p=0.03) (Figure 2.2D).

For the night experiments, the mean time interval between voiding was significantly different between age groups, F(2,8)=5.68, p=0.029. The mean time interval between voiding decreased from 3-month old (73.45 ± 9.93 minutes) to 24-month old (56.37 ± 1.39 minutes) to 32-month old (38.16 ± 7.09 minutes). Tukey post hoc analysis revealed that the 3 month old mice had significantly longer between voids compared to 32 month old mice (p=0.024, Figure 2.2D)

2.3.5 Frequency (number of voiding/hour) – day vs night in 3,24 and 32 month old mice

In 3-month old mice, the frequency of micturition during daytime (0.55 ± 0.06 voids/hour) was not significantly different from night time (0.58 ± 0.05 voids/hour), $t(6)=-0.333$, $p= 0.75$. However, in 24-month old mice, the frequency of micturition during daytime (0.58 ± 0.83 voids/hour) was significantly different from night time (0.86 ± 0.03 voids/hour), $t(3.6)=-3.162$, $p=0.04$. In 32-month old mice, the frequency of micturition during daytime (1.18 ± 0.19 voids/hour) was not significantly different from night time (1.55 ± 0.16 voids/hour), $t(4)=-1.429$, $p=0.23$.

Table 2.1: Results of micturition analysis in mice using voided stain on paper (VSOP) method

Micturition Analysis		3 months (n=4)	24 months (n=4)	32 months (n=3)
Frequency (no. of micturition/ hour)	AM	0.55 ± 0.06	0.58 ± 0.08	1.18 ± 0.19
	PM	0.58 ± 0.05	0.86 ± 0.03	1.55 ± 0.16
Total volume (µl)/ hour	AM	89.44 ± 9.41	193.61 ± 23.81	523.67 ± 62.78
	PM	119.16 ± 14.10	300.83 ± 27.47	634.44 ± 80.56
Volume (µl) per void	AM	166.45 ± 25.8	335.01 ± 17.0	464.22 ± 51.36
	PM	232.60 ± 12.36	354.57 ± 29.55	452.13 ± 50.10
Time intervals between voiding (min)	AM	78.41 ± 13.28	89.79 ± 5.50	41.89 ± 7.73
	PM	73.45 ± 9.93	56.37 ± 1.39	38.16 ± 7.09

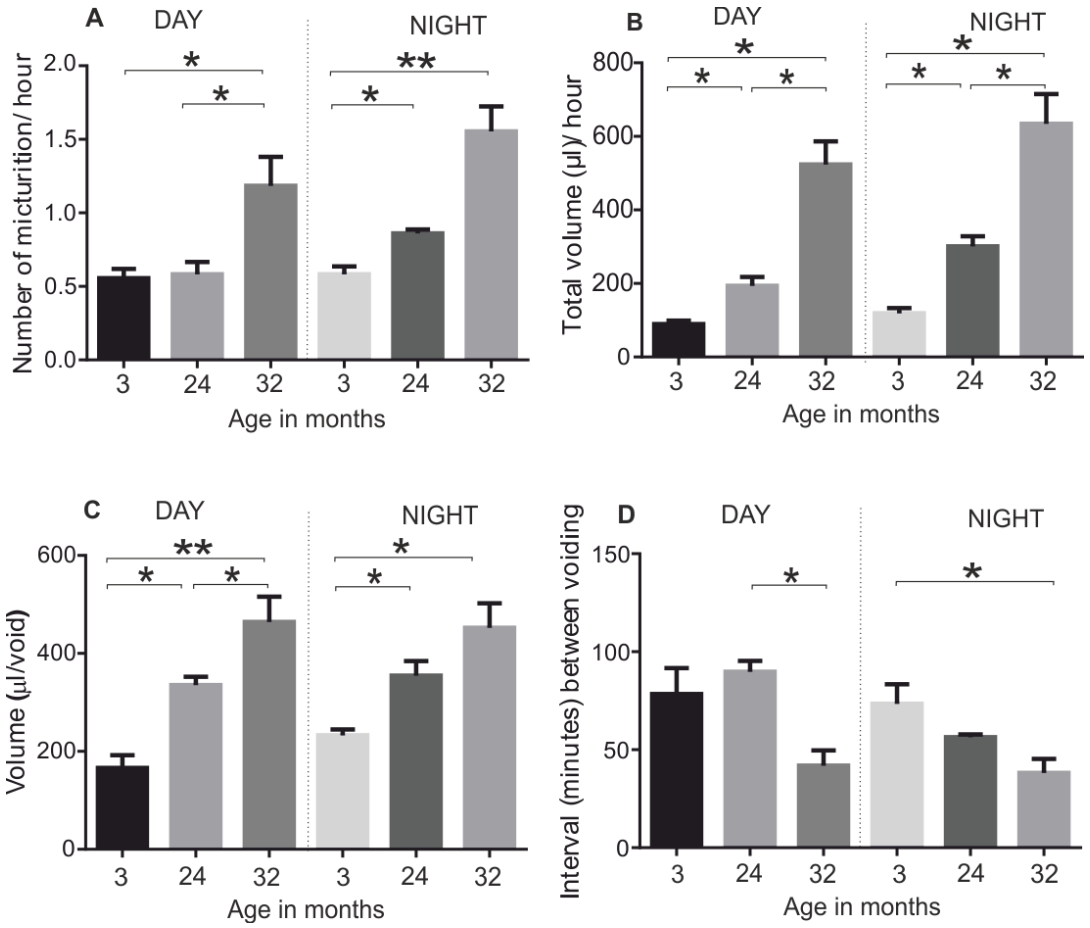

Figure 2.2 Micturition behaviour during day and night time in 3, 24 and 32 month old mice. A) frequency of micturition B) total volume per hour, C) volume per voiding episode and D) time intervals between voiding.

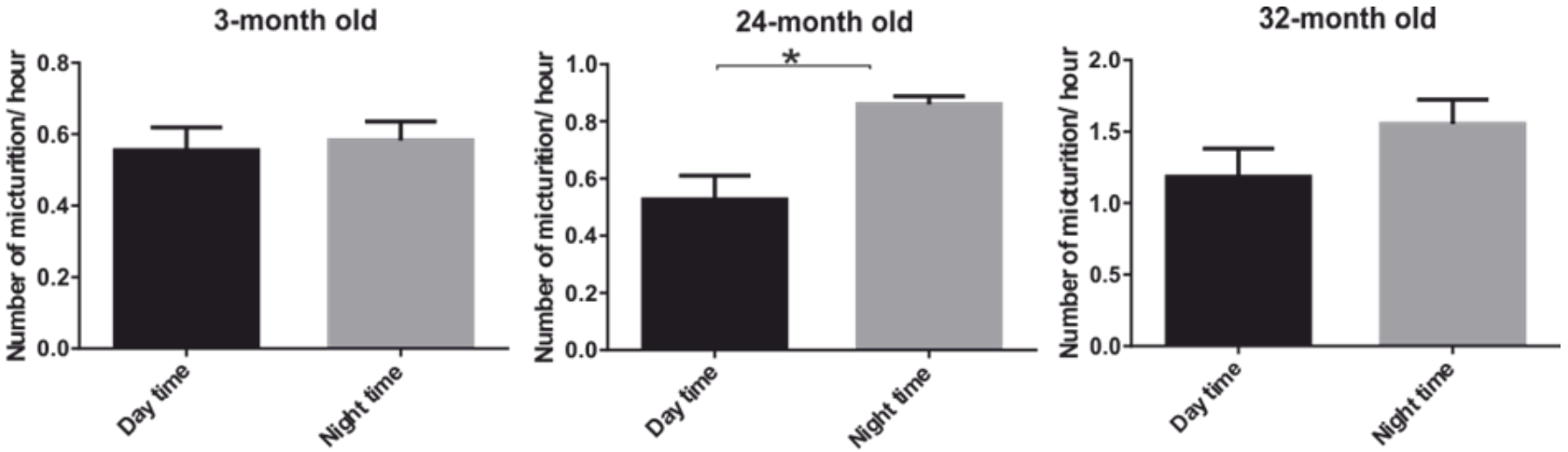

Figure 2.3 Comparison of day and night variation in the frequency of micturition in 3,24 and 32 month old mice

2.4 Discussion

In this study, observations on the behaviour of micturition in conscious, free-moving mice during the daytime and night time was conducted. The functional pattern of micturition is important to determine the physiological regulation on the control of continence and voiding in the mouse subject. This was done prior to analysing the correlative biological changes in the neurological control centre in the spinal cord. Four parameters were obtained namely the frequency, the volume per micturition, total volume and time intervals between voiding episodes. These valuable parameters for continence control were compared between young (3 month-old), aged (24 month-old) and aged (32 month-old) female mice.

The modified metabolic cage study revealed that aged mice (24 and 32 month) exhibit higher micturition frequency, higher urine volume per void, higher total volume and shorter time intervals between micturition, compared to parameter values of young mice (3 month).

2.4.1 Methods and technical consideration

The micturition behaviour analysis is important to determine if there are changes with ageing on the lower urinary tract system and its correlation with the structural findings. Study on the age-related micturition behaviour in mice is challenging as the urine volume voided per episode of micturition by mouse is very minute, which may be just as little as 50µl or less. In this study, the voided stain on paper technique or VSOP was used to study the micturition pattern in the young, aged and extreme aged mice. This method was first clearly outlined by Hodges et al. (2008) and Sugino et al. (2008), although Birder et al. (2002) had already used this concept. Birder et al. (2002) studied short term urination in freely moving mice in

individual cages on top of a filter paper, by counting the number of spots per hour. However, Sugino et al. (2008) was the first to outline and described this current method. They assessed the voided volume of mouse ranging from 2 to 13 weeks, as well as performed an analysis of the voiding behaviour of cyclophosphamide-induced cystitis mice. Voided volume of ddY mice was quantifiable from as early as 2-week old, indicating the usefulness of this method to measure urine output in mouse model with small bladder capacity. Negoro et al. (2012) and Negoro et al. (2013) further improvised the technique using motorised filter paper. In short, this method provides a simple, noninvasive method of evaluating mouse bladder function. The development of this simple noninvasive method to evaluate bladder physiology/dysfunction clearly has an important role in animal models of bladder disease. In the current study, the volume of urine and the size of urine spot on paper provide a linear mathematical correlation. The results validate and demonstrate that VSOP is a feasible technique to observe the functional micturition and continence control. It is reliable, reproducible and provides critical information on the voiding behaviour in ageing rodents.

2.4.2 Changes in frequency of voiding, total volume, volume per void and time intervals between micturition episodes

It was reported in Wistar rats, the number of micturition episodes showed significant differences between day and night time (higher at night) from 3 to 9 month-old, but no diurnal differences are noted from 12 months onwards (Ranson et al., 2003b). However, the current analysis of micturition characteristics in mouse revealed that during the day, 32 month old mice had a higher frequency of micturition compared to both 3 month and 24 month old mice. During the night, the frequency of micturition in 3-month old mice is significantly less than in 24-month old mice and 32 month old mice. This findings demonstrate an earlier age-related increase in the

frequency of voiding during night time period than during the day. In human, elderly patients complain of having to wake up, having the urge to urinate and the need to visit the toilet several times at night. This is clinically known as nocturia or nocturnal voiding. Subsequently, this affects their general well-being and sleep patterns. Nocturia in the elderly is associated with interrupted sleep, distress, anxiety, sleepiness during daytime and generally poor quality of life. However, although the similar effects of ageing are seen in the mice, as shown in this study, rodents have a distinctive different sleep-wake pattern at night. In contrast to humans, rodents are more awake and active in the darkness of night. It is during this alert and active part of their day that the increase in micturition is observed as in this study reveals. Since night time activity in mouse mimics human day, their age-related increase in voiding behaviour also mimic human experience. Although nocturia is bothersome, patient with incontinence void more in daytime and is the main symptom in human.

Previous studies of effects of ageing on micturition in rodent have been carried out on Fisher 344, Sprague-Dawley and Wistar rat species. These studies have yielded inconsistent results in regards to the total volume of voided urine. Longhurst et al. (1992) compared the micturition behaviour and bladder function of young 6 month old and aged 24 month old Fisher 344 rats and showed that in aged female rats, the number of micturitions during the dark cycle was significantly greater than those during the light cycle. In contrast, no increment of micturition episode or increase in total volume urine was seen in aged male rat. Besides that, Ranson et al. (2003b) reported that although total volume of urine rise steadily with age in Wistar rats, in each age group the total volume of urine voided during the day time is similar to the total volume voided during night time. Volume per micturition was only significantly lower at night at 3 month-old, but from 6 months onwards no significant difference during day and night. However, Chun et al. (1988) reported a 93% increase in urine output in aged (22-24 month old) Fisher 344 rats compared to young (5-7 month

old) rat. The increase in the volume of urine voided may be explained by the size of the bladder being larger in aged rats. Several studies have reported an increase in bladder size. This was observed in studies using rats where there is an increase of about 20% in bladder weight of Fisher rats (Chun et al., 1988) and 66% increase in bladder weight in Sprague-Dawleys rats (Italiano et al., 1995). In the current study, there was a positive correlation between increasing age and the total volume of urine/hour and the amount of urine per void in both day and night settings in mouse. Drawing from the description above regarding the larger bladder size in older rats, it is suggestive that bladder is also larger in the aged mice and its capacity to store urine is higher compared to the smaller bladder of the young mice.

2.4.3 Strength and limitation of study

Generally, the two methods to study the micturition parameters in animal are metabolic cage behavioural study and invasive cystometry study. In studies of the lower urinary tract function, experiments using conscious animals in metabolic cages are the most suitable for examining natural micturition behaviour of the species. However, the information derived from the evaluation is somewhat limited. In order to obtain a detailed profile of reflex micturition activity without influence of mood, in vivo cystometry studies in anaesthesised or unanesthesised animal is necessary. Cystometry studies measure and analyse the responsiveness of bladder muscle to pelvic nerve stimulation and the sensitivity of the pelvic nerve afferents to pressure and volume during bladder distension. Cystometry is usually performed with continuous intravesical instillation of saline via a bladder catheter. Micturition cycles are analysed in regards to voiding pattern, nonvoiding contractions and infused volume. Besides that, basal, filling, threshold and micturition pressures are also recorded.

However, urodynamic and cystometry studies were not carried out on these experiments to determine the voiding pattern and its bladder function. If done in anaesthesised and restrained animal, it may not represent the normal physiological state of micturition in mice. Anaesthetic agent may alter the integrity of neural circuits and synaptic transmission, as well as compromise the contractility of bladder wall. Cystometry, even if done on conscious mice through in vivo preparation may still be inappropriate to assess micturition behaviour. The operative procedures put the aged mice in unnecessary risk as they would poorly tolerate the anaesthesia and surgical procedures.

2.4.4 Conclusion

In this chapter, the frequency of micturition, total volume produced and volume per void are shown to increase during ageing, whilst the interval between voids is decreased with age. These results are similar to those observed in ageing rats. Aged rats show an increase in the volume of micturition (Ranson et al 2003, Chun et al 1988) and frequency of micturition (Chun et al 1988), however to my knowledge this is the first study to have shown the effects of ageing on the micturition characteristics of mice.

This study provides an analysis on the effects of ageing on the micturition behaviour of mouse. The data demonstrate the progressive alteration in the behaviour of micturition and continence control between young and aged mice, which become apparent by 24-month old of age. Taken together, these phenotype of higher micturition episodes suggest that in aged mice, either involuntary bladder contraction during urine storage or weakening of the bladder neck and EUS function may lead to an increase frequency of voiding. In the next chapter, The current study aims to determine the role of central excitatory and inhibitory

terminals onto the somatic motor and autonomic spinal nuclei innervating the bladder and EUS. In the next chapter, the investigation will first determine the location and describe the motor neurones innervating the EUS in mouse by injection of cholera toxin B subunit (CTB) into these muscles.

Chapter 3

Neuroanatomical identification of motoneurones innervating the external urethral sphincter in mouse

3.1 Tracing studies of Onuf's nucleus

The control of continence is highly dependent on the function of the external urethral sphincter (Thor and de Groat, 2010). The EUS is an intrinsic urethral muscle and is alternatively known as the rhabdosphincter muscle. The historical discovery and the unique features of the Onuf's nucleus was discussed in length in Chapter 1. Various retrograde tracing studies have located the origin of the pudendal nerve in different species by injection of tracers into the EUS. The summary of the previous tracing studies are given in Table 3.1 below.

Table 3.1: Tracing studies of Onuf's nucleus and its homologue in different species

Paper (Year)	Species	Location
(Ulibarri et al., 1995)	Mongolian gerbil	L5-S2
(Gerrits et al., 1997)	Golden hamster	S1-S2
(Kuipers et al., 2004)	Guinea pigs	S1
(Sato et al., 1978)	Cat	S1-S2
(Kuzuhara et al., 1980)	Dog	S1-S2

(Nakagawa, 1980)	Saimiri monkey	L7-S1
(McKenna and Nadelhaft, 1986)	Rat	L5-L6
(Schrøder, 1980)	Rat	L6
(Marson, 1997)	Rat	L6

3.1.1 Aim and Objectives

In the previous chapter, behavioural studies of micturition in young and aged female mice were investigated. The observations demonstrated significant age-related changes especially in the frequency of micturition. The increases in frequency with age were observed both during the day and night experiments. This finding indicates similarity of increased number of voiding in elderly humans. To understand the pathophysiology of these higher voiding and lower continence in the aged group, I propose looking into the presynaptic inputs to the motor neurones innervating the bladder and the EUS. The location of the autonomic preganglionic neurones to the bladder in mice are well established. However, while the location of sphincter motor neurones has previously been described in human, baboon, monkey, cat, guinea pig and rats, there is a lack of literature which identifies and discusses the EUS motor neurones in mice. The neuroanatomical features of mice cannot be assumed to be similar to rats as mice are not merely small rats. If mice were to be used in study of ageing, the motor neurones responsible in the innervation of EUS should be determined at the onset of the study. Furthermore the usage of laboratory mice as experimental animals is pertinent and widely used in ageing studies as it is more feasible and cost-effective. Usage of mouse also allows comparative studies to be conducted on transgenic animals and disease-models.

Thus, a detailed description of the characteristic and localisation of Onuf's nucleus in mice is necessary.

The aim of this study is to identify and localise the spinal location of somatic EUS motor neurones in the mouse and serve as a basis for further studies on the neuronal control of micturition and continence in the mice. The following are the specific objectives addressed in this chapter:

1. To identify and localise the spinal location of somatic EUS motor neurones in the mouse.
2. To determine whether retrogradely CTB-labelled neurones and ChAT-labelled neurones colocalise
3. To determine whether the neurones that innervate EUS are the same neurones that innervates the striated perineal muscle surrounding the vaginal wall.

3.2 Materials and Methods

3.2.1 Surgery and retrograde tract tracing study

A retrograde tract tracing study was performed to locate the EUS neurones in adult mice. Adult female wild type C57BL/6 mice (3 month-old, N=4) weighing 20-24g were used. The mice were housed in a room with 12 hour dark/light cycle with ad libitum access to food and water. All surgical procedures, pre- and postoperative care and handling of animals were performed in accordance to the Animals (Scientific Procedures) Act 1986. Animals were anesthetised with isofluorane. In all four animals, a midline ventral suprapubic incision was made and the bladder was exposed. In 2 animals, using a Hamilton syringe, 2 µl of the retrograde marker cholera-toxin B (CTB) (2 µl of 1% in saline) were injected in the proximal urethra, about 3 mm distal to the bladder neck. This part of the urethra contains the EUS

and is located just above the pubic symphysis. In order to expose the EUS, the bladder was gently pulled in a cranial direction. In another 2 animals, 5 µl of CTB was injected on the right and left side of the outer vaginal wall, which is situated just posterior to the urethra. Care was taken to avoid any spillage of the tracer.

3.2.2 Tissue processing

Following recovery from surgery and after a survival time of 3-5 days, all the four animals were anesthetised with sodium pentobarbital 80mg/kg i.p and perfused transcardially with 0.1 M phosphate buffered fixative containing 4% paraformaldehyde. Spinal cords were dissected and the lumbar segments L4 to sacral S2 were identified based on the spinal roots and the dorsal root ganglia (DRG). These segments were removed and post-fixed overnight in the same fixative. These segments were chosen because there is a high probability of pelvic motor neurones to be distributed within these segments. These segments were serially sectioned in transverse plane with a vibrating microtome (Leica VT1000S) at 50 µm thickness. All serially sectioned tissues were processed using immunohistochemistry. These sections were incubated in multiwell plate with anti-CTB antibody raised in rabbit (dilution 1:1000, Millipore) in 0.3% phosphate buffered saline (PBS)-Triton overnight at 4^{o}C. The following day, the sections were washed with PBS and stained with secondary antibody Alexa Fluor 555 donkey anti-rabbit (dilution 1:1000, Invitrogen). The sections were then mounted onto slides, coverslipped and analysed for retrograde labelling. The distribution of retrogradely labeled neurones was examined using a Zeiss LSM510 confocal microscope. The spinal cord sections were imaged using 20x objectives. CTB-IR was used to identify retrogradely labelled motor neurones. This method allowed positive identification of the motor neurone innervating a particular muscle fiber,

since only the innervating neuron was labeled. In control experiments, the omission of primary antibody abolished any significant labelling.

3.2.3 Co-labelling for CTB and choline acetyltransferase (ChAT) immunoreactivity

Several sections of the L6 and S1 segments were taken and dual staining of anti-CTB and anti-ChAT antibodies was performed. The aim was to determine whether there is any colocalisation between the two protein markers. These sections were stained with anti-CTB antibody raised in rabbit (1:1000; Millipore) and goat anti-ChAT (1:500; Millipore) in 0.3% PBS-Triton overnight. The following day, the sections were washed with PBS and stained with secondary antibody Alexa Fluor 555 donkey anti-rabbit (1:1000; Invitrogen) and Alexa Fluor 488 donkey anti-goat. The sections were then mounted onto slides, coverslipped and analysed. The neuronal labelling was examined with Zeiss LSM510 confocal microscope using 20x objectives.

3.2.4 ChAT immunohistochemistry, cytoarchitecture and serial reconstruction of DL nucleus

For the immunocytochemistry study, another different set of three adult female wild type C57BL/6 mice (N=3, age 3 month-old) were perfused and serial sections of the spinal cord (L4-S2) were cut as above were used. Immunohistochemistry using avidin-biotin peroxidase (ABC) method was performed on free-floating spinal cord section. Serial sections of the lumbosacral spinal cord from the animals were taken, washed with PBS and incubated with polyclonal anti-ChAT antibody (1:500; Millipore, goat) for 2 days. After washing, sections were treated with biotin anti-goat (1:200; Jackson) for 4 hours and then incubated in ExtrAvidin-Peroxidase (1:1500

in PBS; Sigma) overnight. The sections were finally washed in Tris HCL buffer (Tris buffer, pH 7.4), pre-incubated in diaminobenzidine tetrachloride (DAB; 50mg/100ml; DAKO) in Tris buffer for 10 minutes then 0.01% H_2O_2 was added to the same solution for 10 minutes. This results in a brown punctate reaction product localised mainly in the cytoplasm when viewed using light microscopy. Sections were then mounted in rostrocaudal order on gelatinised slides. They were dehydrated in serial alcohol dilution and coverslipped for morphological analysis, then imaged for reconstruction of DLN motor neurones. To visualise the rostrocaudal extent of the DLN, a reconstruction of images of the serial sections from a single animal was carried out using the Reconstruct™ software (http://www.bu.edu/neural/Reconstruct.html).

3.3 Results

3.3.1 Retrograde tract tracing

EUS muscle injections resulted in retrogradely labeled neurones bilaterally in the ventral horn, mainly in the ventrolateral part (Figure 3.1A). The labeled neurones were located mainly at the level of L6 and rostral part of S1 segments. This group was observed at the edge of the gray matter in lamina IX, a location corresponding to the DLN nucleus in rats (Schrøder, 1980, McKenna and Nadelhaft, 1986, Marson, 1997). These cells had dendrites projecting dorsolaterally and ventromedially along the edge of the grey matter. On the contrary, injection of tracer into the perineal muscles surrounding the vaginal wall, which lies just posterior to the EUS, resulted in retrogradely labeled neurones bilaterally in the ventral horn, mainly in the ventromedial part, in an area corresponding to the SNB demonstrating that the location of motor neurones innervating the EUS are a different population to those innervating the bulbospongious muscle. The locations of these two nuclei are

clearly distinguished (Figure 3.2B). These results demonstrate that the location of the EUS motor neurones is different from the motor neurones innervating the adjacent perineal muscle surrounding the wall of the vagina. Besides that, no retrogradely labelled cells in RDLN or other region was seen.

3.3.2 Co-labelling for CTB and ChAT immunoreactivity

Retrogradely labelled neurones in the DLN following injection of CTB in the EUS were observed to contain ChAT-IR (Figure. 3.2C). ChAT-IR was observed in the ventral horn where it labels 3 distinct groups of motor neurones: the retrodorsolateral nucleus (RDLN, innervating the flexor digitorum brevis muscle in the foot), dorsolateral nucleus (DLN) and the spinal nucleus of the bulbospongiosus (SNB).

3.3.3 ChAT immunohistochemistry and serial reconstruction of DL nucleus

Based on the above findings from the retrogradely CTB-labeled cells of the EUS and its colocalisation with ChAT, the DLN could be now identified conveniently based on two criteria. Firstly, the cells are typically small to medium in size and form an aggregation or clustered on the ventrolateral part of the gray matter in the L6-S1 segments of the spinal cord. This group is located at the very edge of the border between gray and white matter, ventral to the RDLN and lateral to the SNB. Secondly, these neurones were immunoreactive with ChAT antibody, indicating that they are cholinergic motor neurones (Figure 3.2C). Since the area is distinct, ChAT staining can be used for reconstruction study and subsequent identification of DLN motor neurones.

Serial sections revealed that there is a slight shift on its position, from the ventro-lateral to a mid-ventral position along its rostrocaudal axis. To better visualise the rostrocaudal extent of the DL nucleus, a reconstruction of images of the serial sections was carried out using the Reconstruct™ software. Serial reconstruction of L6-S1 ChAT-IR neurones clearly defined the DLN in the lumbosacral spinal cord (Figure 3.2E and 3.2F).

3.3.4 Morphological features of DLN

Individual sections containing DLN generally show that it is small, thin and almost flat in cross-section (Figure 3.2D). The rostral and caudal borders of this nucleus begin and end abruptly, run along its rostro-caudal axis continuously and the cross-sectional diameter of the nucleus remains similar throughout its length. In addition to that, the shape of the nucleus is similar between left and right sides of the spinal cord.

Based on the serial section reconstruction of the nucleus, the length of the DLN could reach up to 0.65 ± 0.2 mm (N=2). The total number of neurons (based on the number of neuronal nuclei) was counted manually on both sides of the spinal cord. The nucleus of a neurone was selected and counted to avoid counting the same neurone twice. The number of DL motor neurones on the right side was 18.0 ± 1.0 and on the left side was 20.5 ± 0.5. The total number of neurons was 38.5 ± 1.5 per spinal cord. By using the Zen 2011 Imaging Software, individual motor neurones were taken and measured for the diameter (largest length of a straight line passes through the nucleus of the neurone and touching two point on its edge), cross-sectional area and perimeter (circumference length) of motor neurones. Pooled data from motor neurones (n=26, N=2) provided an overall mean diameter of 27.86

± 1.11 µm, a mean cross-sectional area of 242.78 ± 11.31 µm and mean perimeter of 68.41 ± 1.86 µm. Neuronal shape was examined in these cross sectional sections. Shape indices were derived from the ratio of its longitudinal diameter over its perpendicular diameter. Motor neurones in DL nucleus ranged between approximate circular to ellipsoid to elongated spindle shape. Circular neurones accounted for 11% (indices 0.85-1.0), ellipsoid 63% (indices 0.6-0.85) and 26% were fusiform (indices <0.6).

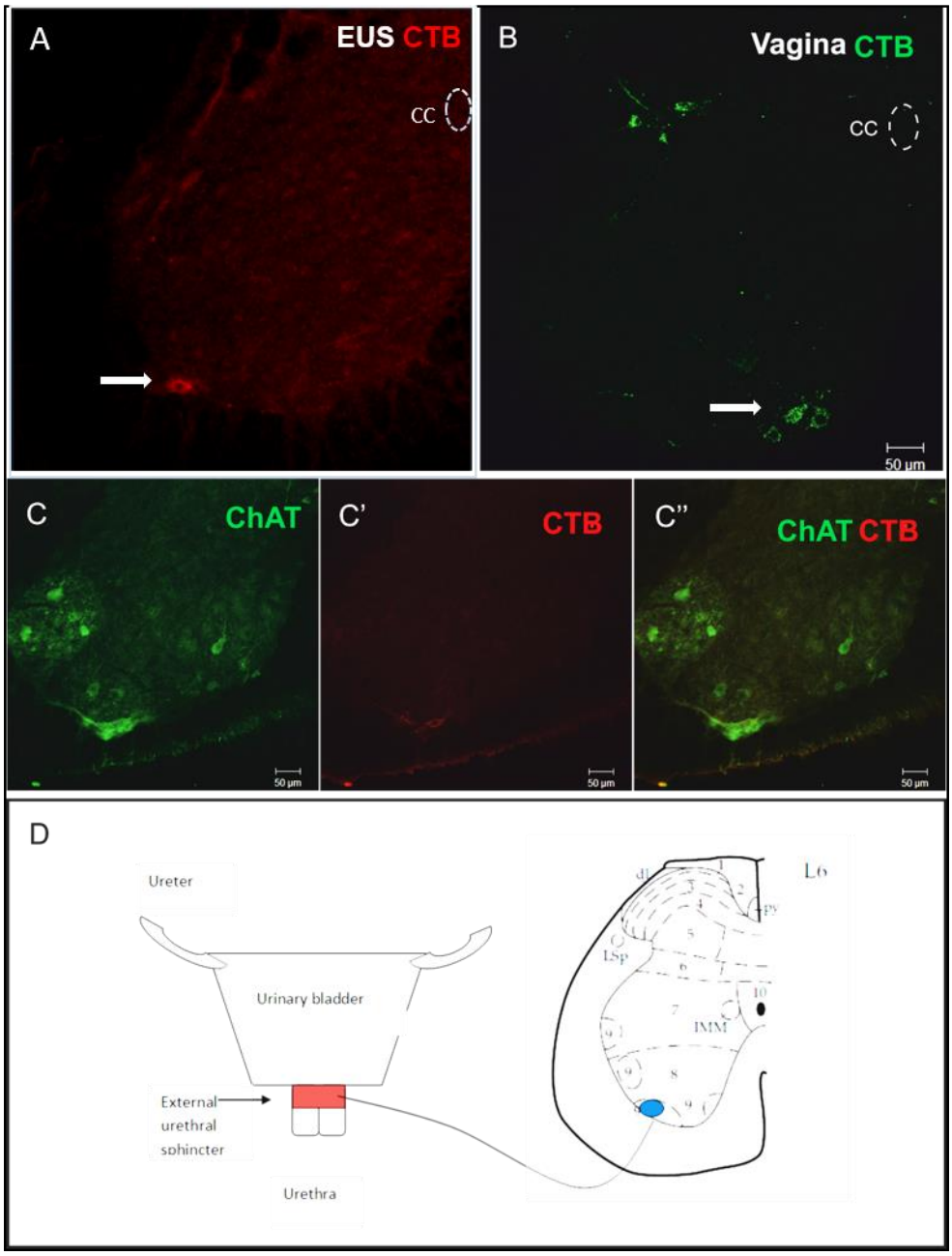

Figure 3.1: Retrograde labelling of DL motor neurones

(A) Motor neurone in the DLN labeled after CTB injection to the external urethral sphincter muscle (arrow). (B) Motor neurones of the vagina wall labeled after CTB injection to the vaginal wall (arrow).(C) The motor neurones of the RDLN, the DLN, and the SNB were ChAT-immunoreactive. (C') The motor neurones in the DLN were labeled after CTB injection to the EUS. (C'') Double labeled neurones. (D) Schematic representation demonstrating the location of the sphincteric motor neurones (blue) in the DLN of sixth lumbar segment of the spinal cord.

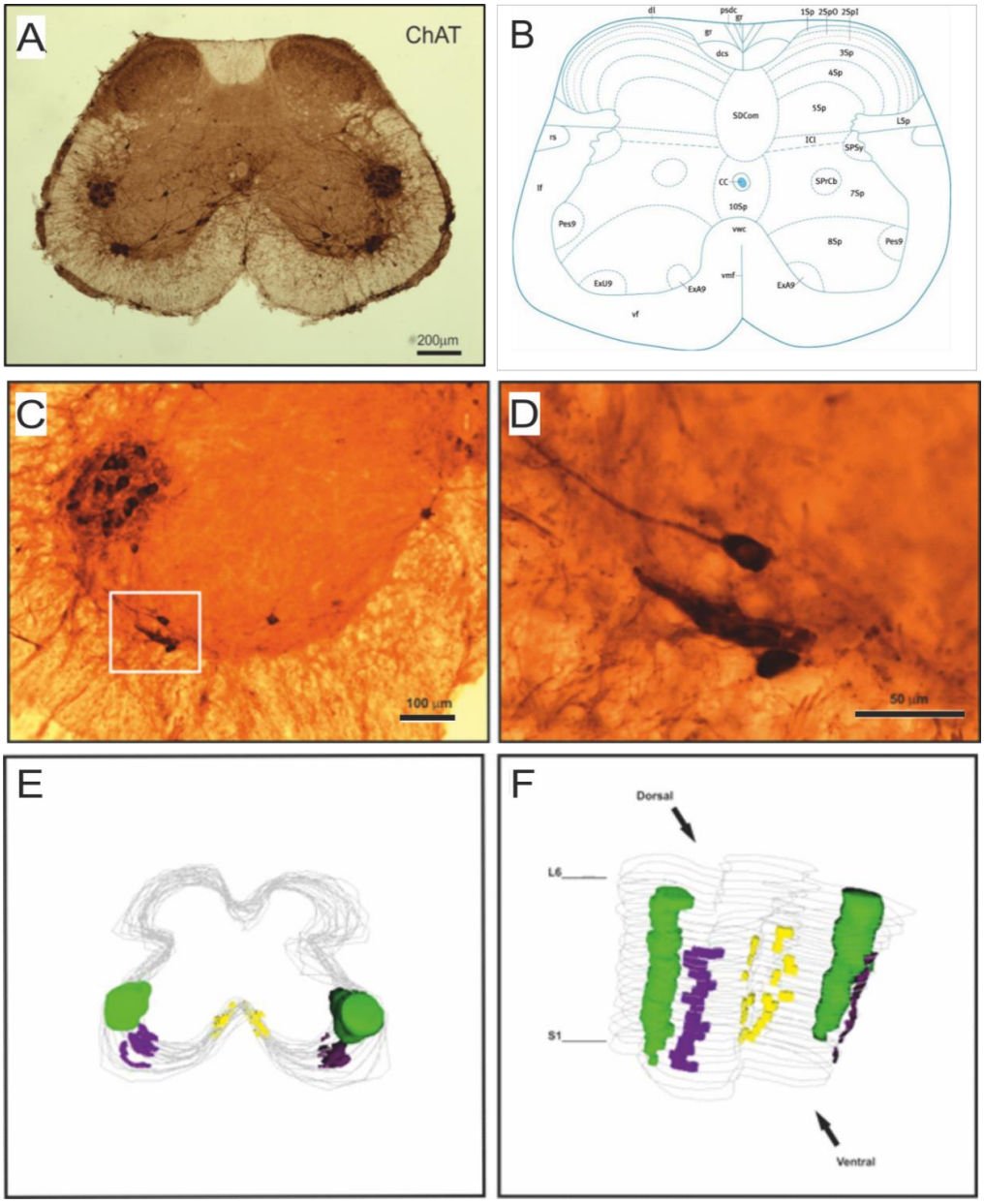

Figure 3.2: ChAT immunoreactivity of lumbosacral spinal cord and reconstruction of DLN

(A, B) A photomicrograph showing the tranverse section of S1 segment of the spinal cord stained with anti-ChAT immunoperoxidase labelling. Onuf's nucleus homologue (DLN) of the lumbosacral spinal cord was seen and corresponded to the external urethral (ExU9) from Allen Brain Atlas. (C) Images of ChAT-stained immunoperoxidase section of mouse L6spinal cord. The ventrolateral position of the DLN motor neurones is shown in the white box. (D) Higher magnification image of DLN shows a small pool of elongated and ellipsoid shape motor neurones. (E) Reconstruction of DLN in the region of lumbosacral L6-S1 shows three separate motor neuronal pools in the gray matter of mouse spinal cord. Cross sectional view of ChAT-stained sections shows the large RDLN (green) on the lateral ventral horn, the DLN (purple) on the ventrolateral position and SNB (yellow) on the medial ventral horn. (F) A 3-D reconstruction from serial sections of a single spinal cord, viewed from the ventrolateral position shows the extent of DLN (purple) initiating from the upper L6 segment and ending at the upper S1 segments.

3.4 Discussion

The present study employed retrograde transport tracing techniques and immunohistochemistry to identify and localise the spinal neurones that innervate the EUS in mouse. The technique is a commonly used method to trace connections in the brain and the spinal cord. In this study, the EUS was surgically localised and injected with CTB. The EUS was injected with the CTB, a cholera toxin conjugate made up of a recombinant of the B subunit only. The CTB is selectively taken up by the axon terminals at the injection sites and is retrogradely transported back to the soma. The non-toxic B subunit is important as it allow the cholera toxin protein complex to attach to the cell surface by binding to ganglioside GM1. An immunohistochemical reaction was then initiated to visualise the location of CTB in slices of spinal tissue, and thus we are able to trace the connections of the spinal cord and the external urethral sphincter muscle in mice. The CTB tracing method has proven to be a powerful tool for retrograde labelling of neurones. It has been used in several neuronal tracing studies and the specificity of this technique has been verified by tracing studies of bladder neurones (Wang et al., 1998) and projections of parabrachial region (Hayakawa et al., 1999). In the current study, the labelling did not appear to be due to spillage of the tracer as motor neurones localised by injection of the EUS and the underlying striated muscle of the vagina showed a markedly different localisation between them.

The organisation of Onuf's nucleus varies among species (Sato et al., 1978, Schrøder, 1980, Kuzuhara et al., 1980, Nakagawa, 1980, Holstege and Kuypers, 1987, McKenna and Nadelhaft, 1986, Gerrits et al., 1997, Kuipers et al., 2004). The present study in mouse showed that the motor neurones innervating the external urethral sphincter muscles are located in the DL nucleus at the level of L6-S1 of the spinal cord. The DL nucleus in mouse was organised into a well-defined cell group

which consist of medium-sized motor neurones. These results demonstrate that the organisation of the DL nucleus in the mouse is in good agreement with that reported in the rat (McKenna and Nadelhaft, 1986, Marson, 1997). In rat, the EUS and the external anal sphincter motor neurones are located in two completely separate nuclei in the same segments of the lumbosacral spinal cord. However, the organisation of DL nucleus in mouse is different from other rodents, such as the hamster. The EUS motor neurones in hamster are clustered within the Onuf's nucleus. This type of arrangement is similar in organisation to that observed in cat, dog (Kuzuhara et al., 1980), monkey (Nakagawa, 1980) and man (Schrøder, 1980).

Once the location of neurones projecting to the external urethral sphincter was elucidated using retrograde tracing with cholera-toxin B chain, this study further continued to establish whether there is co-localisation with ChAT. The co-localisation studies revealed that the nerve cells innervating the EUS (retrogradely labelled) and ChAT are always co-localised. The finding was significant for the identification of DL nucleus in future studies, as usage of anti-cholinergic marker is more feasible to label the EUS motor neurones. The combination of the ChAT-immunoreactivity and the location of ChAT immunoreactive motor neurones was sufficient to distinguish the DL nucleus in the ventral horn, at the level of the sixth lumbar to the first sacral segments of the spinal cord.

The actual total number and diameter of the DL motor neurones are of particular interest in the study of ageing and disorder of micturition. Previously, any loss of systemic function is commonly attributed to the loss of neurons. However, recent studies have showed that this assumption is not necessarily correct. For example, cognitive decline in aged primate does not correspond to significant loss of neocortical neurons with age (Peters and Sethares, 2002). To examine the effects of ageing on spinal motor neurones, data from young adult mouse were obtained as

a basis of comparison for future study. From the serial section reconstruction method, we found that the total number of DL neurones in mouse was 38.5 ± 1.5 neurones per spinal cord, contained within L6-S1 segment measuring 0.65 ± 0.2 mm in length. Since the serial section reconstruction is one of the method to provide an unbiased estimate of the total number of neurons (Coggeshall and Lekan, 1996), this method was employed in the present study.

In this study, female mice were used as experimental models. They were chosen for two main reasons. Firstly, in human, the prevalence of urinary incontinence is documented higher in elderly women (Wehrberger et al., 2012). In order to study this phenomenon in ageing, female animals are deemed as a suitable model, as the motor neurones are not affected by the level of circulating testosterones. DLN is a sexually dimorphic nucleus and is influenced by the level of testosterone hormone (Jordan et al., 1982). In their study, they tested a single dose of testosterone on day two of life to the female rat and noted a masculinized DLN. The number of motor neurones in masculinized DLN was more than female control. In our study, female mice were used and it should be expected that the number of neurons is fewer and the size of the motor neurones smaller as compared to male mouse. In male, the DLN also innervates the ischiocavernosus muscle. Females lack this muscle and this may contribute to the sexually dimorphic features. The second advantage of using female mice, is that it allows the study of the innervation on other female genital structure. While DL innervation to EUS is well established, its innervation to other female sexual structures has not been documented and warrants further investigation.

According to McKenna and Nadelhaft (1986), the homologue of bulbospongiosus muscle in female rat is unclear. However, in their study they have identified a very fine muscle sheet that appears to have its origin on the ventral and lateral surface

of the vagina. Though difficult to distinguish from the connective tissues in gross dissection, this sheet of thin muscle contracted following stimulation of the pudendal nerve. The fibres formed a loop around the vagina and inserted behind the rectum. This feature is similar to the arrangement of the bulbospongiosus muscle observed in male. The description provided by them appears identical to the perineal muscle on the anterior surface of vagina injected with CTB in the current study. This study found that the retrogradely labelled neurones are noted to be located on the ventro-medial group of motor neurones in L6-S1 segments of spinal cord. This group of motor neurones has been reported as the SNB nucleus, and it innervates the bulbospongiosus as well as the external anal sphincter (McKenna and Nadelhaft, 1986, Schrøder, 1980). This means that although the EUS and the bulbospongiosus muscle in female mouse are located adjacent to each other, they are innervated from two different pools of motor neurones in the lumbosacral spinal cord – the DLN and SNB nucleus respectively.

As a conclusion, this study describes the anatomical location and characteristics of the mouse homologue to Onuf's nucleus, the DLN of the spinal cord. Retrograde tracing from the EUS revealed that the motor neurones were located in an anatomically distinct portion of L6-S1 ventral horn, colocalised with ChAT immunoreactivity and were clearly identifiable from neurones labelled retrogradely from the vagina. Reconstruction of ChAT immunoreactivity confirmed that separate motoneuron groups could be identified in the spinal cord. Taken together, this study in this chapter has successfully met the aim to identify, localise and document the spinal location of somatic EUS motor neurones in mouse. These results are important as a basis for synaptological study in the subsequent chapter. Based on these findings, the next investigation will focus on the presynaptic terminals contacting the soma of EUS motor neurones, and how the distribution of the excitatory and inhibitory terminals changes with age.

Chapter 4

Effects of ageing on the presynaptic excitatory and inhibitory inputs to motor neurones in the spinal dorsolateral nucleus and bladder autonomic neurones

4.1 Introduction

4.1.1 Excitatory glutamate and inhibitory GABA/glycine inputs to the Onuf's nucleus/DLN nucleus

Glutamate is a major excitatory neurotransmitter within the spinal cord. Glutamate acts on the DLN motor neurones to excite them and promote EUS closure (Thor, 2004) and penile erection (Aïoun and Rampin, 2005). Close association of VGLUT2-IR terminals with the somata of lumbosacral motor neurones has been widely reported (Alvarez et al., 2004, Todd et al., 2003, Wu et al., 2004). VGLUT2-immunoreactive terminals have also been demonstrated to form close appositions with DLN, RDLN and DM motor neurones (Ranson et al., 2007b). The source of VGLUT2 inputs is likely to include spinal interneurones. *In situ* hybridization studies reveal VGLUT2 mRNA in somata within all spinal regions especially in laminae V, VI, VIII and IX (Landry et al., 2004).

Inhibitory inputs to Onuf's nucleus include GABAergic and glycinergic inputs (Ramirez-Leon et al., 1994). Ramirez-Leon et al. (1994) identified GABAergic and peptidergic innervations to Onuf's nucleus using immunofluorescence in the S1-S2 segments of the cat spinal cord. This study documented the presence of not only GABA but also serotonin, thyrotropin releasing hormone, substance-P, enkephalin

and CGRP-immunoreactive inputs to Onuf's motor neurones. Blok et al. (1997) described an indirect GABAergic pathway inhibition from the PMC to Onuf's nucleus. Injection of the the PMC with WGA-HRP demonstrated anterograde projections from the PMC in the brainstem to the dorsal gray commissure of the sacral cord in cat. More than half of the terminal inputs made contact with GABA-IR interneurones. Thus they postulated that during micturition, the PMC directly stimulates the parasympathetic preganglionic neurones to contract the bladder and indirectly inhibit the motor neurones in the Onuf's nucleus via GABA interneurones to relax the sphincter. When the PMC was injected with the anterograde tracer WGA-HRP, it was found that about 30% of labeled PMC terminals made contact with glycine-IR dendrites in the dorsal gray commissure. The GABA and glycine-IR interneurones are thought to be the major inhibitory transmitters regulating the EUS motor neurones activity during micturition. Electrical or chemical stimulation in the PMC in cats excites the bladder and inhibits sphincter EMG activity (Holstege and Tan, 1987) and hyperpolarizes sphincter motor neurons (Paroschy and Shefchyk, 2000).

4.1.2 Sympathetic and parasympathetic bladder neurones

The sympathetic and parasympathetic preganglionic neurones form the final site for integration of sympathetic and parasympathetic information, respectively. The outputs of sympathetic and parasympathetic neurones are determined by their intrinsic membrane properties and by synaptic inputs from supraspinal regions and local interneurones. Fine control of these properties is vital for maintenance of homeostasis.

Glutamate is the major excitatory neurotransmitter affecting thoracolumbar sympathetic and sacral parasympathetic preganglionic neurones (Chalmers and Pilowsky, 1991, Dampney, 1994). Preganglionic neurones express high level of various AMPA and NMDA receptors subunits while acetylcholine, noradrenaline and serotonin do not have direct excitatory effects. Activation of excitatory inputs to sympathetic preganglionic neurones produces fast excitatory post-synaptic potentials that are blocked by glutamate receptor antagonists.

High proportion of inhibitory transmission onto sympathetic neurones in the spinal cord slice are found to be glycinergic (Dun and Mo, 1989, Spanswick et al., 1994). An important component of control to the sympathetic preganglionic neurones is due to activation from local GABAergic interrneurones (Deuchars, 2007) and descending pathways (Deuchars et al., 1997). GABAergic terminals onto sympathetic neurones were also observed in spinalised animals (Llewellyn-Smith, 2002)

4.1.3 Research Question

In the previous chapter (chapter 2) the micturition behaviour of young and aged mice was investigated. Overall, it has been demonstrated that in ageing, there is a significant increase in the frequency of voiding during day and night time in aged and extreme aged mice. These findings mimic human experience. In the elderly, involuntary passing of urine or incontinence is a common phenomenon. Urinary incontinence increases with ageing and can have a major detrimental effect on the quality of life of the elderly population. The storage and periodic elimination of urine are regulated by a complex neuronal network that co-ordinates the activities of

urinary bladder and the urethral outlet including the bladder neck, urethra and EUS. As the lumbosacral spinal cord contains both autonomic and somatic motor neurons that regulate bladder function, studies of age-associated changes to these neurones, including the inputs onto these neurones, may be key to understanding neurogenic forms of bladder dysfunction in the elderly.

The unique sphincter muscle persistently contracts at rest and only relaxes during the short period of micturition. During micturition, the motor neurones innervating the EUS are inhibited to allow relaxation of urethra and passing of urine. Could the poor continence control be explained by changes in the pattern of innervation to Onuf's motor neurones and bladder autonomic neurones? Does alteration in synaptic inputs to the motor neurones cause alteration in balance between excitatory and inhibitory influence to either somatic or autonomic neurones? These are the questions explored, especially when there is already evidence that morphometric analysis shows no difference in urethral architecture in ageing female rats (Russell et al., 1996). They compared smooth and striated muscle content in the female rat urethra in young (12 month) and old (32 month) animals, using immunohistochemical techniques to detect the presence of smooth muscle myosin and skeletal muscle myosin in the rat urethra. They reported that urethral diameter and external sphincter width do not change with ageing. They also noted that neither the smooth muscle nor the external striated sphincter muscle volume in the urethra is changed with age and conclude that morphometric analysis shows no difference in urethral architecture in aged female rats. This leads to the question - is the neuronal control of the EUS and bladder significantly altered in ageing process? The answer may be particularly important in the etiology of altered micturition and continence control in old age. As such, in this chapter, the excitatory and inhibitory presynaptic terminals contacting DLN motor neurones and autonomic bladder neurones were investigated.

The aim of this study is to identify the potential neurogenic mechanisms resulting in urinary incontinence with emphasis on the pool of spinal neurones that provide the motor innervation to the bladder and external urethral sphincter to regulate bladder function. Excitatory glutamatergic and inhibitory GABAergic and glycinergic innervations of DLN motor neurones, sympathetic and parasympathetic preganglionic neurones in young adult (3 months old), aged (24 months old) and extreme aged (32 months old) female C57BL6 mice were examined using quantitative confocal microscopy.

4.2 Material and Methods

4.2.1 Animals

Female wild type C57BL/6 mice (young adult: 3 months old, or aged: 24 months old, or extreme aged: 32 months old) were used. Animals were housed in a room with 12 hour dark/light cycle with *ad libitum* access to food and water. All surgical procedures, pre- and postoperative care and handling of animals were performed in accordance to the Animals (Scientific Procedures) Act 1986 and all efforts were made to minimise animal discomfort and the number of animal used.

4.2.2 Fluorogold labelling of motor neurones and immunohistochemistry for glutamate/GABA and glycine.

13 female mice (N=5, 3 months old) and (N=5, 24 months old) and (N=3, 32 months old) received an intraperitoneal injection of Fluorogold (FG, 0.1 ml of 1% in distilled water per animal) to label motor and autonomic neurones (Atkinson et al., 2000). Since, the previous micturition studies revealed changes in frequency pattern in aged animals during night experiments, the animals were also planned to

be sacrificed during the dark cycle. A day following FG injection, at 8pm, the animals were sacrificed, perfused with 4% PFA and 50 µm serial sections of the L1 segment for sympathetic preganglionic neurones and L6-S1 segments for parasympathetic and DLN motor neurones were cut. Sections were then incubated in a combination of antibodies against VGLUT2 (1:1000, rabbit, Synaptic Systems 135402), GAD67 (1:1000, mouse, Millipore MAB5406) and GlyT2 (1:15,000, guinea pig, Millipore AB1773) overnight in PBS + 0.3% Triton (Table 4.1). These antibodies dilutions were chosen based on the optimisation protocol that was performed in the lab prior to conducting the current experiments.

To detect the GlyT2, sections were incubated overnight in biotinylated anti-guinea pig (1:500, Jackson Immunoresearch), followed by incubation in Streptavidin Pacific Blue (1:1000, Invitrogen) for 2 hours. GAD67-IR was detected by incubation in Streptavidin Alexa^{555} raised against mouse (1:1000, Invitrogen) for 2 hours and VGLUT2-IR was detected by incubation in Streptavidin Alexa^{488} raised against rabbit (1:1000, Invitrogen) for 2 hours. Sections were then air dried onto slides, mounted under a coverslip using VectaShield mounting medium (Vector labs) and examined using a Zeiss LSM510 confocal microscope

4.2.3 Data analysis

Analysis of the immunofluorescence staining was performed according to the method by Chang and Martin (2009). To quantify the age-associated changes in distribution of VGLUT2, GlyT2 and GAD67-IR contacts onto FG labelled motor neurones, sections were observed under a Zeiss LSM 510 confocal microscope with a 100x oil immersion objective. All sections were imaged using identical settings and parameters. The single optical section image for each neurone was

measured at the level of the cell nucleus. Presynaptic boutons making close appositions (no visible space between the boutons and the motor neurone cell bodies) with FG labelled motor neurones were examined and the number of boutons contacting a motor neurone was divided by the length of the cell body perimeter, which was measured using the Zen 2011 imaging software. The criteria of inclusion for neurones and terminal boutons are those neurones with at least visible nucleus and boutons were determined as punctate making close apposition to the neuronal somata.

The percentage of neurotransmitter-specific terminals coverage was calculated. The calculation was based on the total summation of the three groups of neurotransmitters (VGLUT2, GlyT2 and GAD67-IR inputs) which was considered 100%, for the purpose of representing the three major neurotransmitter inputs, although it is acknowledged that there are other types of terminals besides these. The percentage of individual neurotransmitter-specific terminals contacting the respective spinal nuclei was calculated as (the mean number of each group X 100) / (Total mean of all three groups). The excitatory: inhibitory (E:I) ratio was determined by dividing the percentage of excitatory VGLUT2-IR inputs over the combined percentage of inhibitory GAD67 and GlyT2-IR inputs.

4.2.4 Statistical analysis and figure preparation

All quantitative data were expressed as mean ± standard error of mean (s.e.m), analysed using IBM SPSS Statistics 20 and comparisons made using one-way ANOVA. If homogeneity of variances (tested by Levene's test) was violated, a Welch's correction test was run and Games Howell post-hoc test was performed. If there was homogeneity of variances then a post-hoc Tukey's analysis was performed. Figures were compiled using CorelDraw X7 software and the brightness

and contrast adjusted to optimise the visibility of the immunolabelling. For optimal visualisation, GlyT2-IR detected using Streptavidin Pacific Blue was turned to magenta using the Zen 2011 imaging software.

Table 4.1: Details of the antibodies used in this study are described as below

Primary antibody	Source	Dilution	Secondary antibody	Purpose	Special treatment
VGLUT2	Rabbit, (Synaptic Systems, Göttingen, Germany)	1:1000	Alexa Fluor ^{555} Invitrogen donkey anti rabbit (1:1000)	Marker for glutamate terminals	0.3% Triton
GAD67	Mouse, (Millipore, Harlow, UK)	1:1000	Alexa Fluor ^{488} Invitrogen donkey anti mouse (1:1000)	Marker for GABA terminals	0.3% Triton
GlyT2	Guinea pig, (Millipore, Harlow, UK)	1: 15,000	Biotinylated anti-guinea pig (Jackson Immunoresearch, 1:500) Streptavidin Pacific Blue conjugate, Invitrogen (1:1000)	Marker for glycine terminals	0.3% Triton

4.3 Results

4.3.1 Excitatory and inhibitory inputs onto DLN motor neurones

Intraperitoneal injection of FG resulted in labelled motor neurones in the DLN, DM and RDLN in the ventral horn of the spinal cord (Figure 4.1 A, FG labelled DLN neurones in boxed area). These sections were then processed for triple immunohistochemistry to determine the effects of age on the excitatory and inhibitory inputs onto the DLN motor neurones (Figure 4.1 B-E). There was no overlap in the staining patterns for each antibody tested suggesting there was no colocalisation of these neurotransmitters.

4.3.1.1 Perimeter of DLN motor neurones

The relationship between FG labelled DLN motor neurones and VGLUT2, GAD67 and GlyT2-IR terminals (n=30 motor neurones from N=5 young mice, n=31 motor neurones from N=5 aged mice and n=20 motor neurones from N=3 extreme aged mice) were analysed in single optical sections using laser confocal microscopy. A one-way ANOVA was conducted to determine if the perimeter of DLN motor neurones (µm) was different for groups of different ages. There was no significant difference in the perimeter of the DLN motor neurones between young (71.34 ± 2.22 µm), aged (76.13 ± 2.60 µm) and extreme aged (74.72 ± 2.91 µm) mice, $F(2, 75) = 1.051$, $p=0.355$ (Figure 4.2 A).

4.3.1.2 VGLUT2-IR presynaptic terminals onto DLN motor neurons

VGLUT2-IR in the young adult mouse spinal cord was present as punctate staining throughout the grey matter which was particularly dense in laminae I-II (Figure 4.1B), similar to that previously observed in the rat spinal cord (Todd et al., 2003). In the DLN, VGLUT2-IR terminals were observed in close apposition to FG labelled DLN motor neurones in the young, aged and extreme aged mice (Figure 4.3). The mean number of VGLUT2-IR boutons/100µm was significantly different between age groups, $F(2, 78) = 35.094$, $p < 0.001$. VGLUT2-IR boutons/100µm decreased from the young 3-month old (14.84 ± 0.71, N=5), to aged 24-month old (11.87 ± 0.61, N=5), to extreme aged 32-month old (6.70 ± 0.48, N=3) age groups, in that order. Tukey post hoc analysis revealed significant decrease from young 3-month to aged 24-month (2.98, 95% CI (0.92 to 5.04, p =0.003), decrease from young 3-month to aged 32-month (8.15, 95% CI (5.82 to 10.48, p =0.001) as well as the decrease from 24-months to 32-month (5.17, 95% CI (2.85 to 7.48), p =0.001) (Figure 4.2B). These results and subsequent findings in this chapter are all summarised in Table 4.2

4.3.1.3 GAD67-IR presynaptic terminals onto DLN motor neurons

In the young adult mouse, punctate GAD67-IR was observed throughout the grey matter and was particularly dense in the superficial part of the dorsal horn (Figure 4.1C), similar to that previously described in the rat spinal cord (Mackie et al., 2003). Punctate GAD67-IR surrounded FG labelled DLN motor neurones in the young adult, aged and extremely aged mice (Figure 4.4). The mean number of GAD67-IR boutons/100µm was significantly different between age groups, Welch ANOVA $F(2, 42.423) = 20.907$, $p = 0.001$. GAD67-IR boutons/100µm increased

from the young 3-month old (3.61 ± 0.35, N=5), to aged 24-month old (7.68 ± 0.70, N=5), then remained about the same at aged 32-month old (7.49 ±3.15, N=3). Games-Howell post hoc analysis revealed significant increase from young 3-month to aged 24-month (4.06, 95% CI (2.15 to 5.98, p<0 .001), and significant increase from young 3-month to aged 32-month (3.87, 95% CI (1.93 to 5.82, p <0 .001) but no difference between the 24-month old and the 32-month old age group (Figure 4.2C).

4.3.1.4 GlyT2-IR presynaptic terminals onto DLN motor neurons

GlyT2-IR in the young adult mouse was similar to that previously reported in the rat spinal cord (Zafra et al., 1995b, Mackie et al., 2003) with low levels in laminae I and II and higher levels of punctate staining throughout the remainder of the grey matter (Figure 4.1D). GlyT2-IR boutons were present around FG labelled DLN motor neurones in young adult and aged mice (Figure 4.5). The mean number of GlyT2-IR boutons/100µm was significantly different between age groups, $F(2, 80) = 16.216$, $p < 0.001$. GlyT2-IR boutons/100µm increased from the young 3-month old (6.46 ± 0.44, N=5), to aged 24-month old (8.42 ± 0.63, N=5), to extreme aged 32-month old (11.76 ± 0.81, N=3) age groups, in that order. Tukey post hoc analysis revealed significant increase from young 3-month to aged 24-month (1.95, 95% CI (0.01 to 3.91, p =0 .049), increase from young 3-month to aged 32-month (5.30, 95% CI (3.07 to 7.52, p < 0 .001) as well as the increase from 24-months to 32-month (3.34, 95% CI (1.13 to 5.56), p =0 .002) (Figure 4.2D).

Table 4.2 VGLUT2, GAD67 and GlyT2-IR inputs in close apposition to DLN, sympathetic and parasympathetic preganglionic neurones in young, aged and extreme aged mice.

	VGLUT2-IR terminals/100μm of soma			GAD67-IR terminals/100μm of soma			GlyT2-IR terminals/100μm of soma		
	Young	**Aged**	**Extreme aged**	**Young**	**Aged**	**Extreme aged**	**Young**	**Aged**	**Extreme aged**
DLN (mean)	14.84 ± 0.71	11.87 ± 0.61	6.70 ± 0.48	3.61 ± 0.35	7.68 ± 0.70	7.49 ±3.15	6.46 ± 0.44	8.42 ± 0.63	11.76 ± 0.81
% of terminal inputs	59.6	42.4	25.8	14.5	27.5	28.9	25.9	30.1	45.3
Sympathetic (mean)	12.32 ± 0.64	9.75 ± 0.71	4.45 ± 0.48	5.84 ± 0.53	5.61 ± 0.37	5.09 ± 0.51	10.54 ± 0.53	9.41 ± 0.47	9.29 ± 0.57
% of terminal inputs	42.9	39.4	23.6	20.3	22.6	27.0	36.7	38.0	49.3
Parasympathetic (mean)	8.30 ± 0.32	9.99 ± 0.89	7.47 ± 0.52	4.67 ± 0.55	7.45 ± 0.51	7.77 ± 0.77	7.24 ± 0.61	8.02 ± 0.45	9.80 ± 0.50
% of terminal inputs	41.1	39.2	29.8	23.1	29.3	31.0	35.8	31.5	39.1

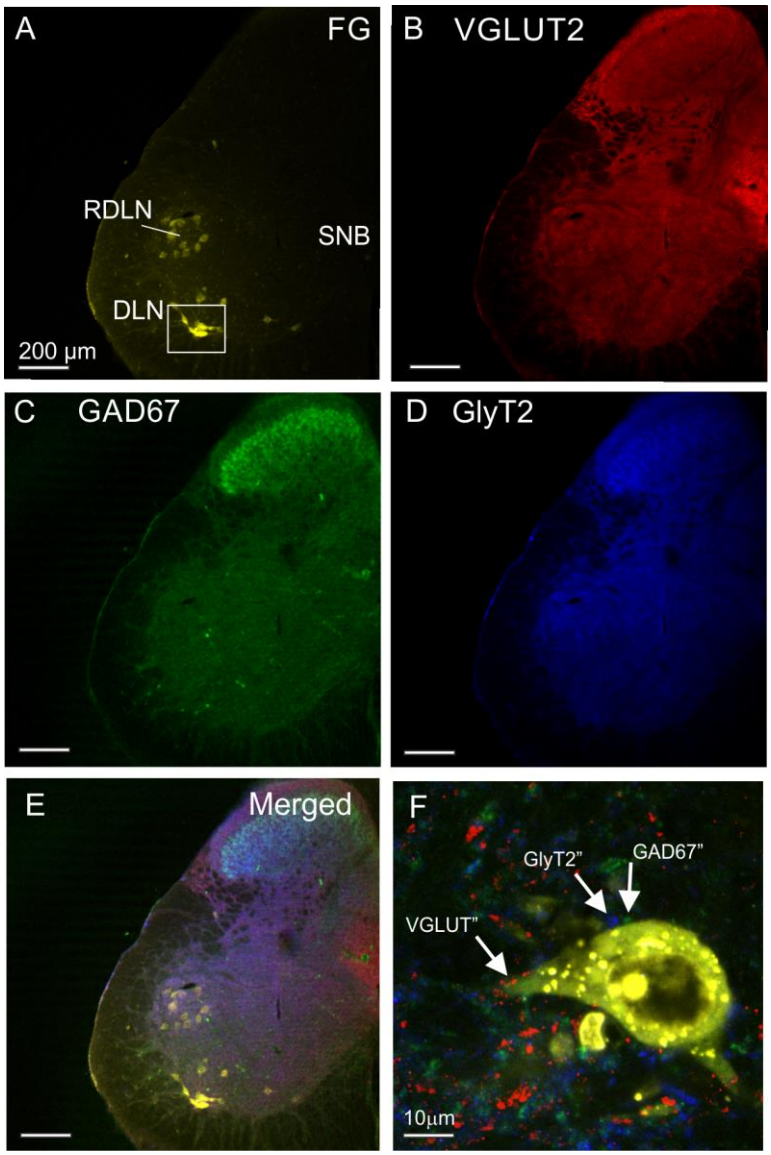

Figure 4.1 Representative confocal images of L6 segment of the spinal cord of young mice, triple labelled for VGLUT2 (red), GAD67 (green) and GlyT2 (blue).

(A) FG labelled motor neurones were observed in the ventral spinal cord in the RDLN, DLN and SNB (boxed area shows the DLN). (B) VGLUT2-IR appeared as punctate labelling throughout the grey matter, particularly dense in laminae I and II. (C) GAD67-IR was observed throughout the grey matter of the spinal cord and was particularly dense in the superficial part of the dorsal horn. (D) GlyT2-IR was present in low levels in laminae I and II and higher levels of punctate staining was present throughout the remainder of the grey matter. (E) Merged image to visualise the density and distribution of immunoreactive labelling (F) A close up image provides better depiction of a FG-labelled motor neurone with all 3 neurotransmitter-specific labelings.

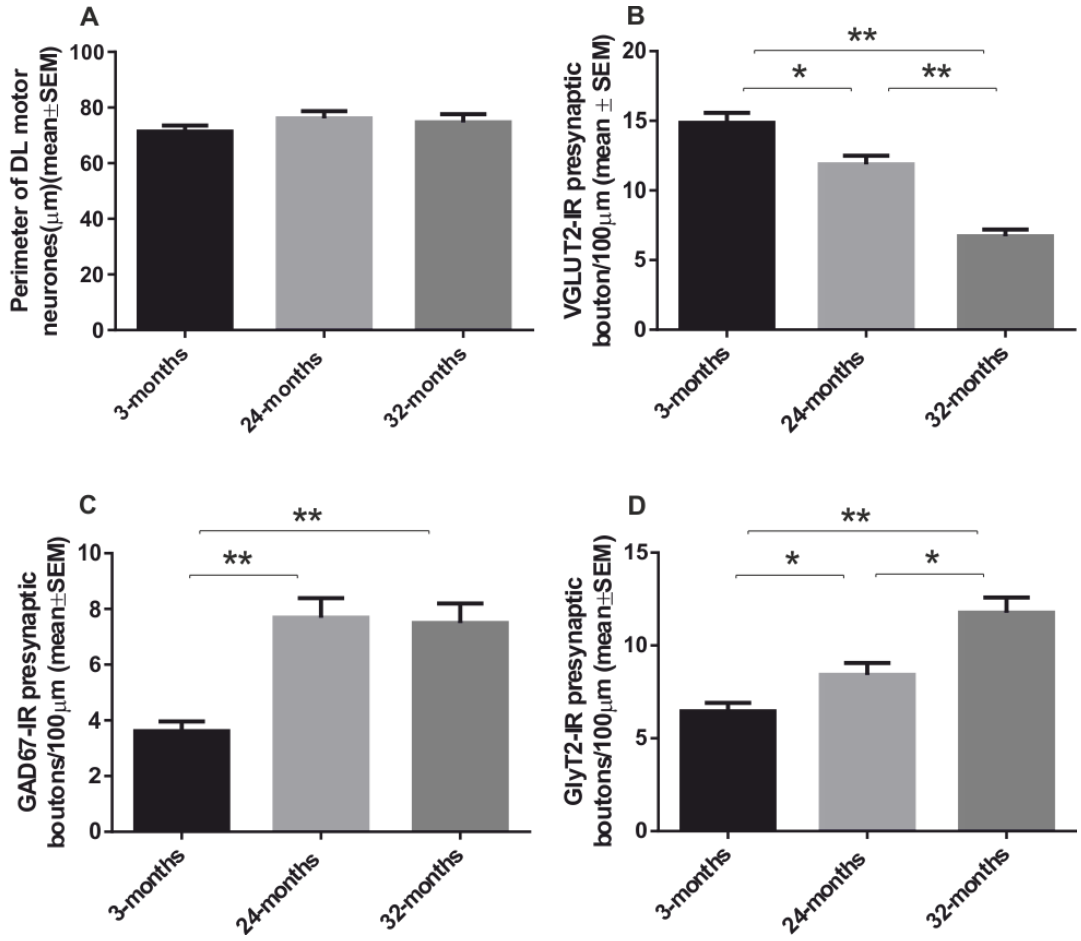

Figure 4.2 The number of excitatory VGLUT2-IR terminals contacting DLN motor neurones decreases with age while the number of inhibitory GAD67-IR and GlyT2-IR terminals contacting DLN motor neurones increases with age.

(A)There was no significant difference in the perimeter of the DLN motor neurones between young, aged and extreme aged mice. (B) The frequency of VGLUT2-IR terminals contacting the FG labelled DLN neurones was significantly lower in aged and extreme aged animals compared to young adult animals. (C) The number of GAD67-IR terminals contacting DLN motor neurones was significantly increased in the aged and extreme aged mice compared to the young adult. (D) The number of GlyT2-IR terminals contacting DLN motor neurones was significantly increased in the aged and extreme aged mice compared to the young adult

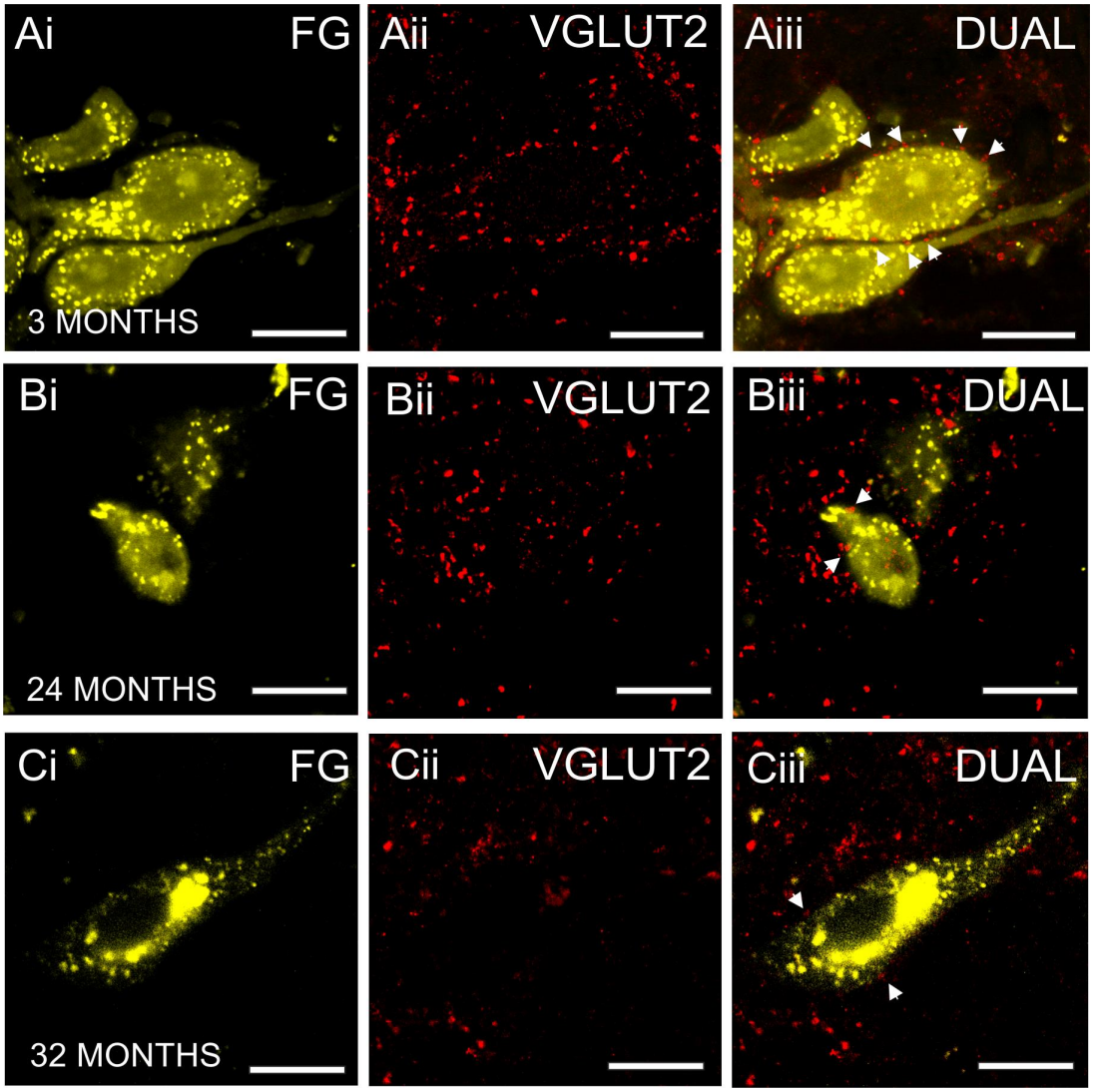

Figure 4.3 The number of excitatory VGLUT2-IR terminals contacting DLN motor neurones decreases with age.

In young adult, aged and extreme aged mice, FG labelled DLN motor neurones (Ai, Bi and Ci respectively) were surrounded by VGLUT2-IR terminals (Aii, Aiii, Bii, Biii, Cii and Ciii). Arrows point to examples of terminals in close apposition with DLN motor neurones. Scale bar 50µm.

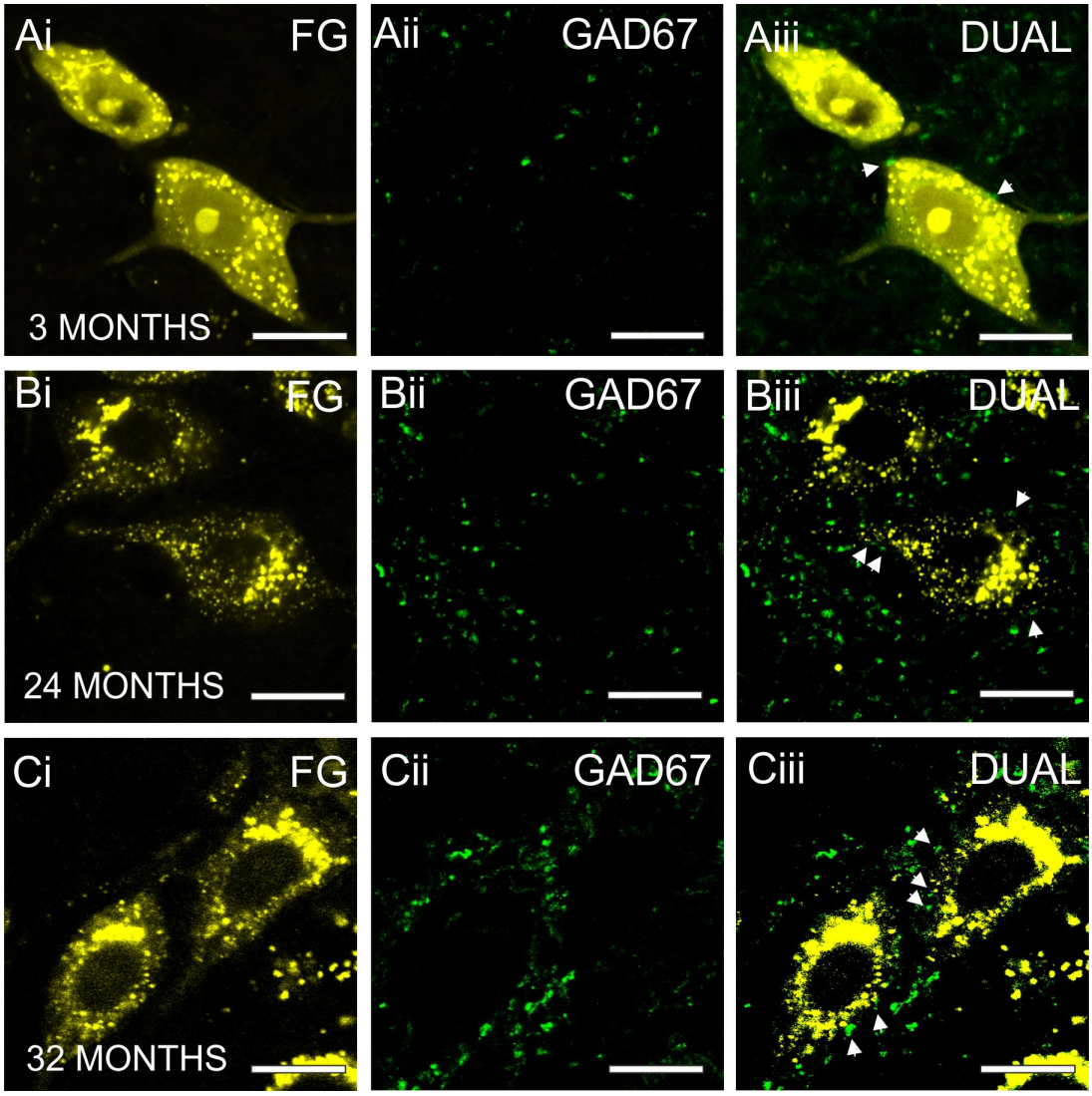

Figure 4.4 The number of inhibitory GAD67-IR terminals contacting DLN motor neurones increases with age.

GAD67-IR terminals were observed to contact FG labelled DLN motor neurones in the young adult (Ai-Aiii), aged (Bi-Biii) and extreme aged mice (Ci-Ciii). Arrows point to examples of terminals in close apposition with DLN motor neurones.

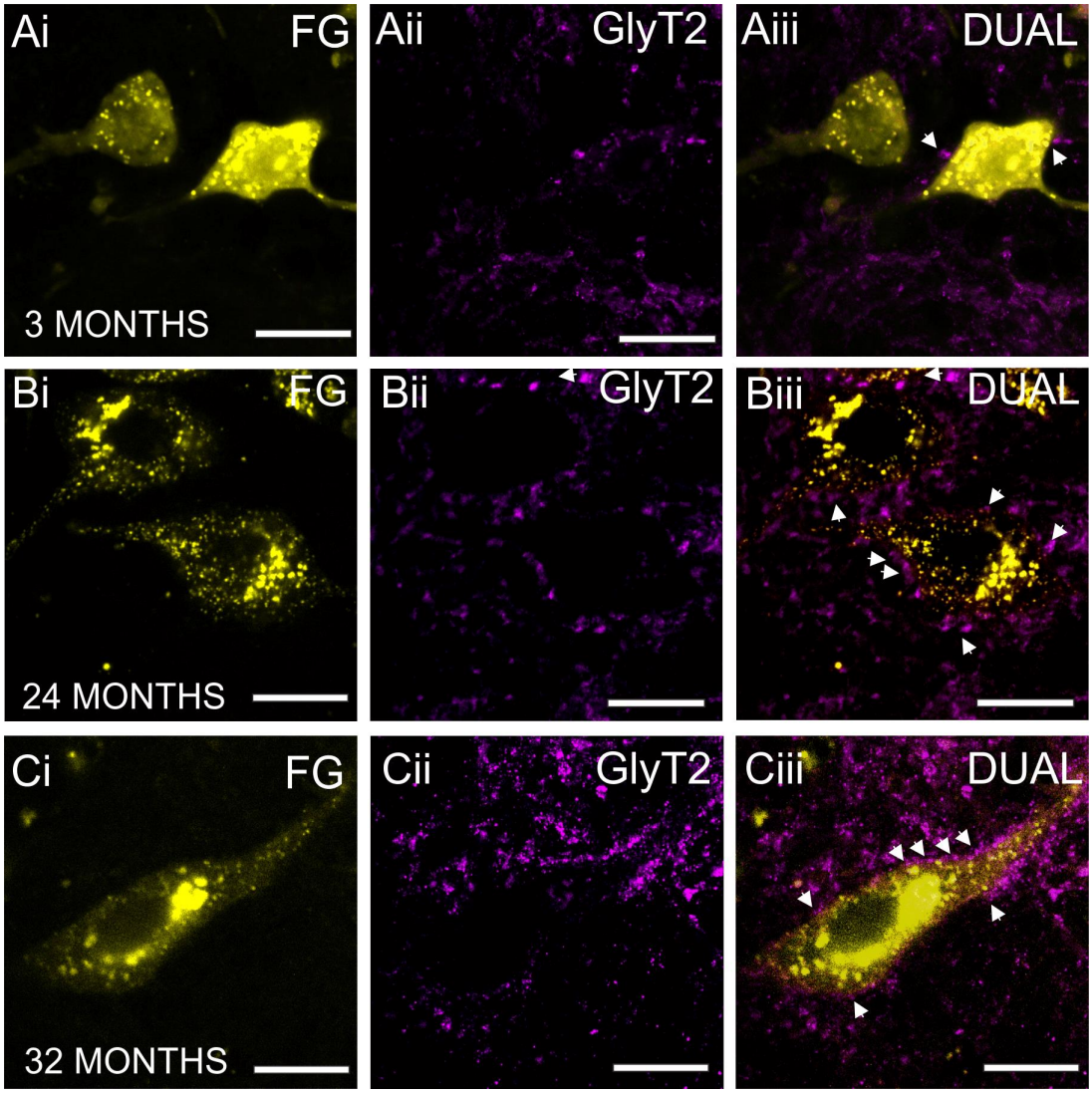

Figure 4.5 The number of inhibitory GlyT2-IR terminals contacting DLN motor neurones increases with age.

GlyT2-IR terminals were also observed to contact FG labelled DLN motor neurones in the young adult (Ai-Aiii) aged (Bi-Biii) and extreme aged mice (Ci-Ciii). Arrows point to examples of terminals in close apposition with DLN motor neurones.

4.3.2 Excitatory and inhibitory inputs onto sympathetic preganglionic neurones in the IML

Intraperitoneal injection of FG resulted in labelled sympathetic motor neurones in the IML in the L1 segment of spinal cord. The relationship between FG labelled sympathetic preganglionic neurones (n=41 neurones from N=4 young mice, n=37 neurones from N=4 aged mice and n=20 neurones from N=3 extreme aged mice) and VGLUT2, GAD67 and GlyT2-IR terminals were analysed using confocal microscopy as above.

4.3.2.1 Perimeter of sympathetic preganglionic neurones

Mean perimeter of sympathetic preganglionic neurones was significantly different between age groups, Welch's ANOVA $F(2, 44.772) = 9.47$, $p < 0.001$. The mean perimeter of sympathetic preganglionic neurones remained unchanged between the young 3-month (54.36 ± 1.29 µm, N=4) and aged 24-month old (55.61 ± 1.43 µm, N=4), however, it increased in the 32-month old (68.91 ± 3.07 µm, N=3). Games-Howell post hoc analysis revealed no significant difference between the 3-month old and the 24-month old age group, but a significant increase from young 3-month to aged 32-month (14.54, 95% CI (8.18 to 20.90, $p<0$ .001), and from aged 24-month to extreme aged 32-month (13.30, 95% CI (6.83 to 19.77, $p <0$ .001) (Figure 4.6A).

4.3.2.2 VGLUT2-IR presynaptic terminals onto sympathetic preganglionic neurons

VGLUT2, GlyT2 and GAD7-IR terminals were observed to surround FG labelled sympathetic preganglionic neurones in the IML in young and aged animals (Figure 4.7-4.9). The mean number of VGLUT2-IR boutons/100µm was significantly different between age groups, $F(2, 90) = 29.386$, $p < 0.001$. VGLUT-IR boutons/100µm decreased from the young 3-month old (12.32 ± 0.64, N=4), to aged 24-month old (9.75 ± 0.71, N=4), to aged 32-month old (4.45 ± 0.48, N=3) age groups, in that order. Tukey post hoc analysis revealed significant decrease from young 3-month to aged 24-month (2.57, 95% CI (0.45 to 4.69, p =0 .013), decrease from young 3-month to extreme aged 32-month (7.87, 95% CI (5.42 to 10.31, $p < 0$ .001) as well as the decrease from aged 24-months to extreme aged 32-month (5.29, 95% CI (2.73 to 7.85), $p < 0$ .001) (Figure 4.6B and Figure 4.7).

4.3.2.3 GAD67-IR presynaptic terminals onto sympathetic preganglionic neurons

The mean GAD67-IR boutons/100µm in the young 3 month old was 5.84 ± 0.53 (N=4), in aged 24-month old was 5.61 ± 0.37 (N=4), and in extreme aged 32-month old was 5.09 ± 0.51 (N=3) but the differences between these age groups were not significant, Welch's ANOVA F(2, 53.522) = 0.561, p = 0.574 (Figure 4.6C and Figure 4.8).

4.3.2.4 GlyT2-IR presynaptic terminals onto sympathetic preganglionic neurons

The mean GlyT2-IR boutons/100µm in the young 3 month old was 10.54 ± 0.53 (N=4), in aged 24-month old was 9.41 ± 0.47 (N=4), and in extreme aged 32-month old was 9.29 ± 0.57 (N=3) but the differences between these age groups were not significant, $F(2, 95) = 1.753$, $p = 0.179$ (Figure 4.6D and Figure 4.9).

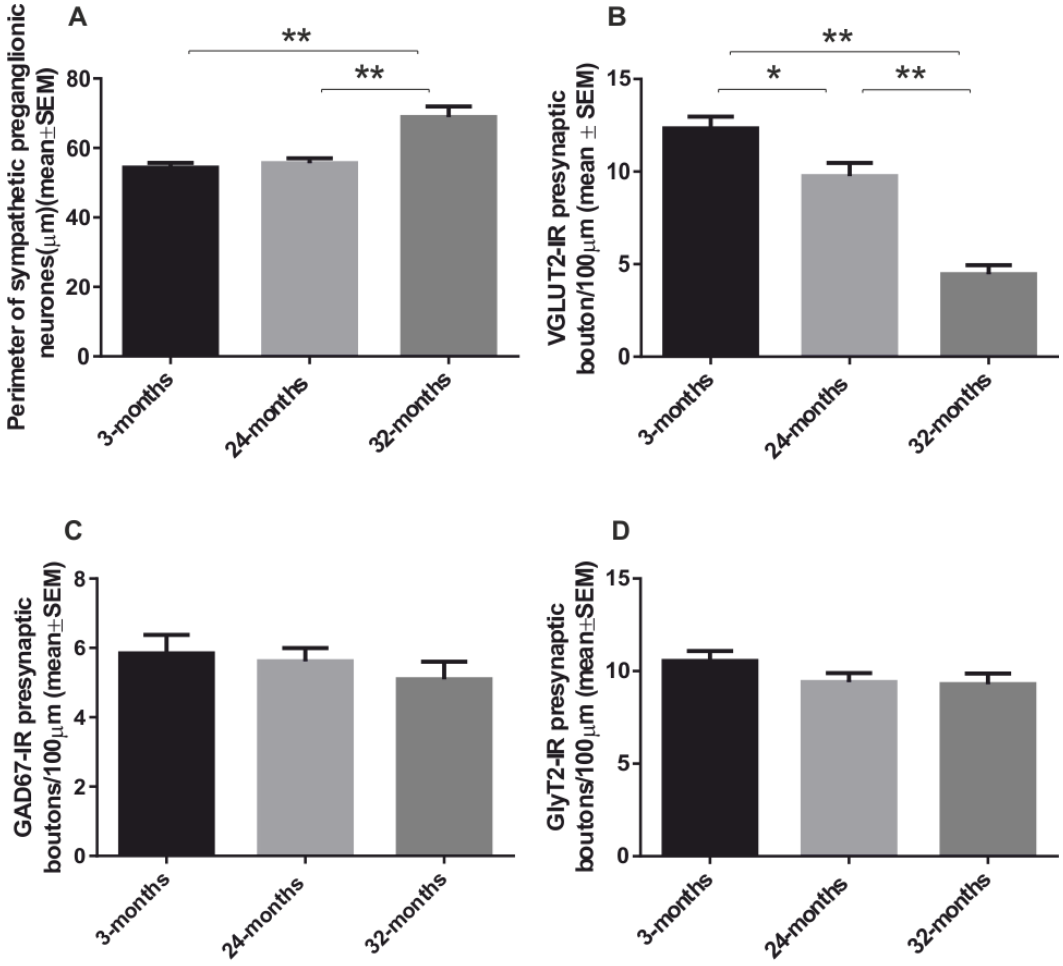

Figure 4.6 The number of excitatory VGLUT2-IR terminals contacting sympathetic preganglionic neurones in the IML decreases with age yet there is no change in inhibitory (GAD67 or GlyT2-IR) inputs.

(A) There was a significant increase in the perimeter of the sympathetic neurones in extreme aged mice. (B) There was significantly fewer VGLUT2-IR terminals contacting sympathetic neurones in the aged and extreme aged mice compared to the young adults. However, (C) and (D) show that the numbers of inhibitory terminals onto sympathetic preganglionic neurones did not change with age.

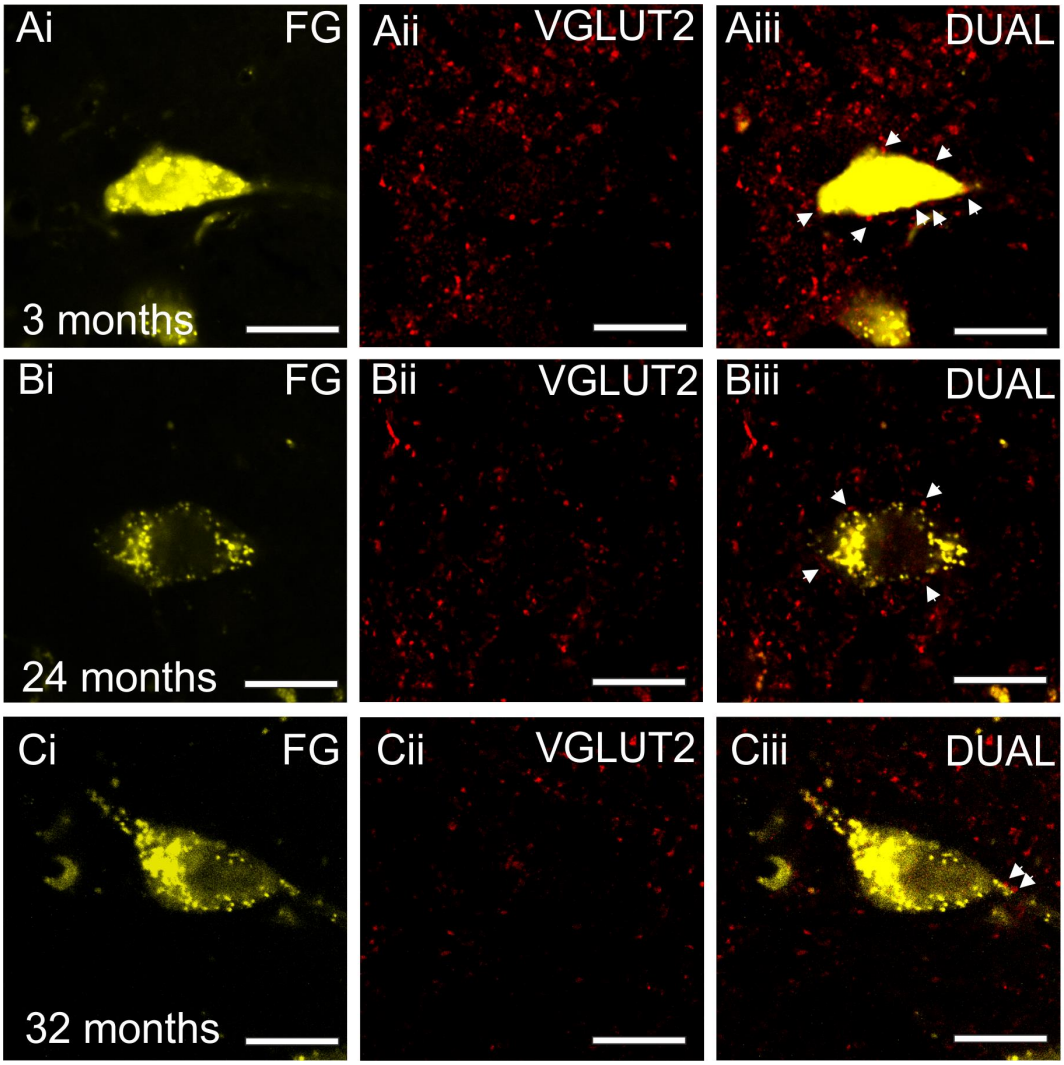

Figure 4.7 The number of excitatory VGLUT2-IR terminals contacting sympathetic preganglionic neurones decreases with age.

VGLUT2-IR terminals were observed to contact FG labelled sympathetic motor neurones in the IML in the young adult (Ai-Aiii), aged (Bi-Biii) and extreme aged (Ci-Ciii) mice. Arrows point to examples of terminals in close apposition with sympathetic preganglionic neurones.

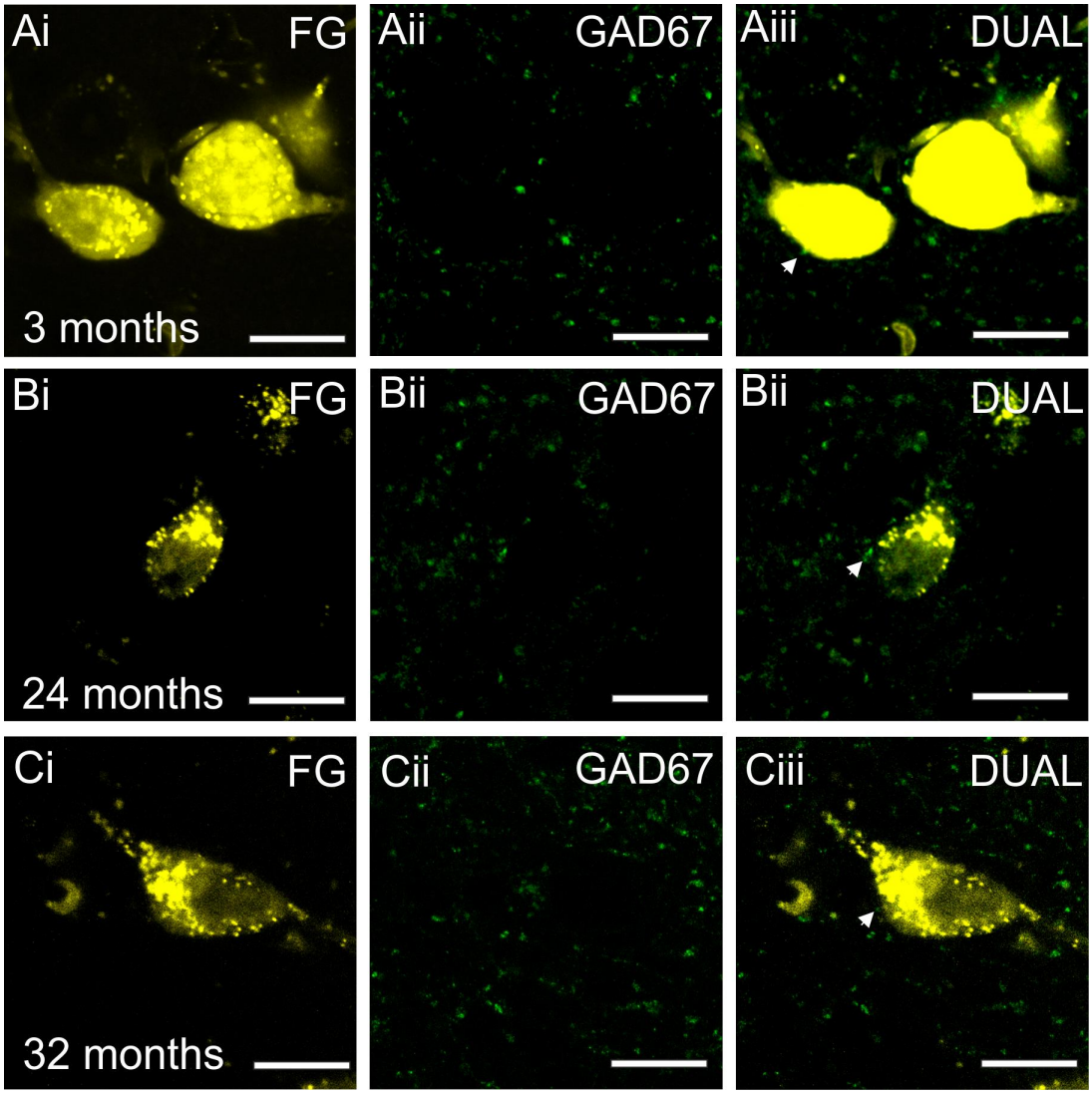

Figure 4.8 The number of inhibitory GAD67-IR terminals contacting sympathetic preganglionic neurones did not change with age.

GAD67-IR terminals were also observed to contact FG labelled sympathetic preganglionic neurones in both the young adult (Ai-Aiii), aged (Ci-Ciii) and extreme aged (Di-Diii) mice. Arrows point to examples of terminals in close apposition with sympathetic preganglionic neurones.

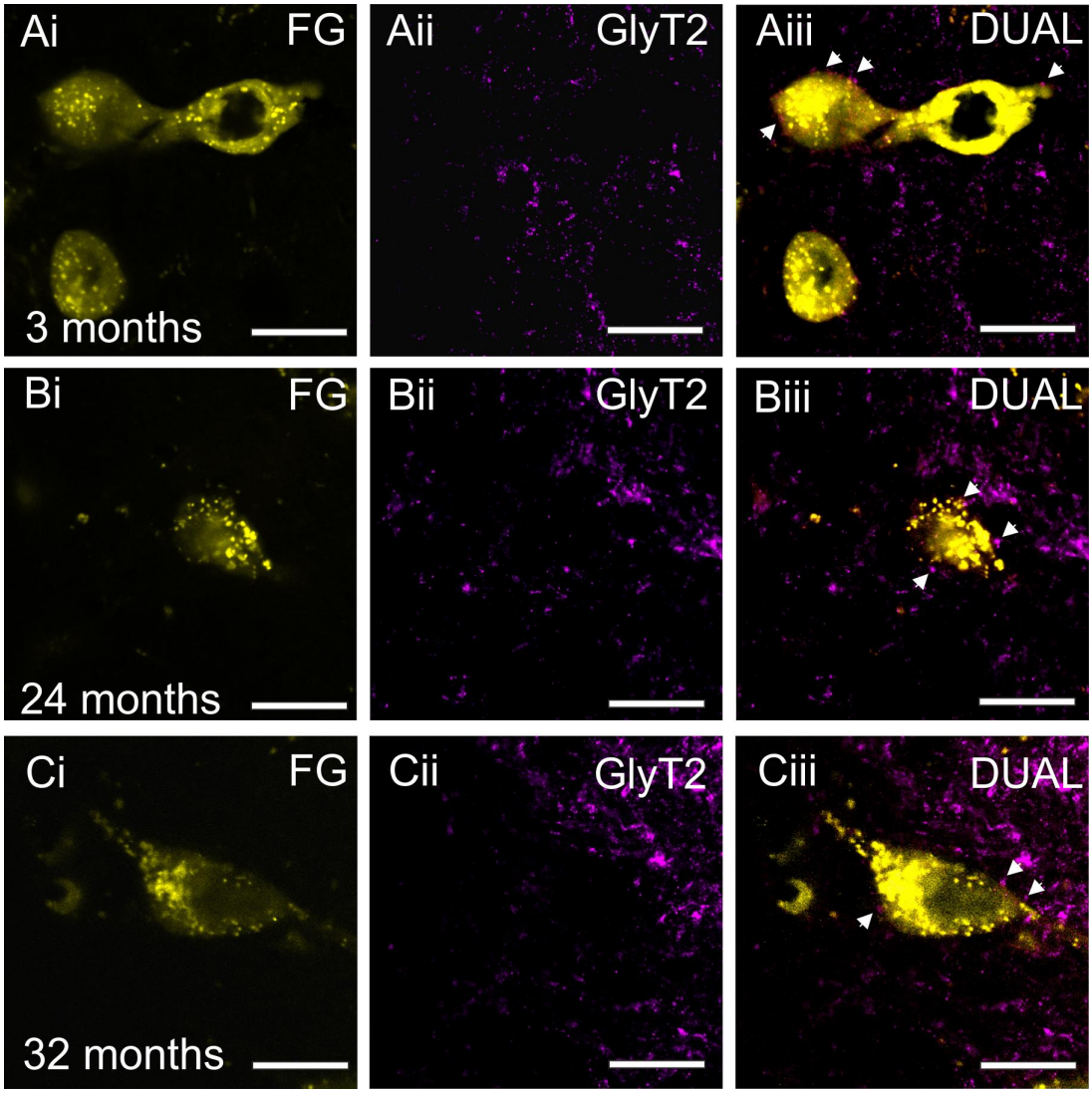

Figure 4.9 The number of inhibitory GlyT2-IR terminals contacting sympathetic preganglionic neurones did not change with age.

GlyT2-IR terminals surrounded FG labelled sympathetic preganglionic neurones in both the young adult (Ai-Aiii), aged (Bi-Biii) and extreme aged (Ci-Ciii) mice. Arrows point to examples of terminals in close apposition with sympathetic preganglionic neurones.

4.3.3 Excitatory and inhibitory inputs onto parasympathetic preganglionic neurones

Intraperitoneal injection of FG resulted in labelled parasympathetic motor neurones in the IML in the L6-S1 segments of the spinal cord. VGLUT2, GlyT2 and GAD7-IR terminals were observed to surround FG labelled parasympathetic motor neurones in the IML in both young, aged and extreme age animals (Figure 4.11-4.13). The relationship between FG labelled parasympathetic preganglionic neurones (n=22 neurones from N=4 young mice, n=20 neurones from N=4 aged mice and n=18 neurones from N=3 extreme aged mice) and VGLUT2, GAD67 and GlyT2-IR terminals were analysed using confocal microscopy as above.

4.3.3.1 Perimeter of parasympathetic preganglionic neurones

Mean perimeter of parasympathetic preganglionic neurones was significantly different between age groups, $F(2, 56) = 8.038$, $p = 0.001$. The mean perimeter of parasympathetic preganglionic neurones remained unchanged between the young 3-month (64.83 ± 2.07 µm, N=4) and aged 24-month old (61.62 ± 2.04 µm, N=4), however, it increased in the extreme aged 32-month old (74.06 ± 11.18 µm, N=3). Tukey post hoc analysis revealed no significant difference between the 3-month old and the 24-month old age group, but a significant increase from young 3-month to extreme aged 32-month (9.22, 95% CI (1.46 to 16.99, p=0.016), and from aged 24-month to extreme aged 32-month (12.44, 95% CI (4.76 to 20.11, p = 0.001) (Figure 4.10A).

4.3.3.2 VGLUT2-IR presynaptic terminals onto parasympathetic preganglionic neurons

The mean number of VGLUT2-IR boutons/100µm in the young 3 month old was 8.30 ± 0.32 (N=4), in aged 24-month old was 9.99 ± 0.89 (N=4), and in extreme aged 32-month old was 7.47 ± 0.52 (N=3) but the differences between these age groups were not significant, Welch's ANOVA F(2, 32.574) = 2.971, p = 0.65 (Figure 4.10B and Figure 4.11).

4.3.3.3 GAD67-IR presynaptic terminals onto parasympathetic preganglionic neurons

The mean number of GAD67-IR boutons/100µm was significantly different between age groups, $F(2, 58) = 8.107$, $p = 0.001$. GAD67-IR boutons/100µm increased from the young 3-month old (4.67 ± 0.55, N=4), to aged 24-month old (7.45 ± 0.51, N=4), then remained the same at extreme aged 32-month old (7.77 ± 0.77, N=3). Tukey post hoc analysis revealed significant increase from young 3-month to aged 24-month (2.78, 95% CI (0.77 to 4.79, $p = 0$ .004), and significant increase from young 3-month to aged 32-month (3.10, 95% CI (1.00 to 5.19, $p = 0$ .002) but no difference between the 24-month old and the 32-month old age group (Figure 4.10C and Figure 4.12).

4.3.3.4 GlyT2-IR presynaptic terminals onto parasympathetic preganglionic neurones

The mean number of GlyT2-IR boutons/100µm was significantly different between age groups, $F(2, 57) = 5.864$, $p = 0.005$. GlyT2-IR boutons/100µm increased from

the young 3-month old (7.24 ± 0.61, N=4), to aged 24-month old (8.02 ± 0.45, N=4), then remained the same at extreme aged 32-month old (9.80 ± 0.50, N=3). Tukey post hoc analysis revealed significant increase from young 3-month to extreme aged 32-month (2.56, 95% CI (0.73 to 4.39, p = 0 .004), but no difference between the young 3-month old and 24-month old, and between 24-month old and the extreme aged 32-month old age group (Figure 4.10D and Figure 4.13).

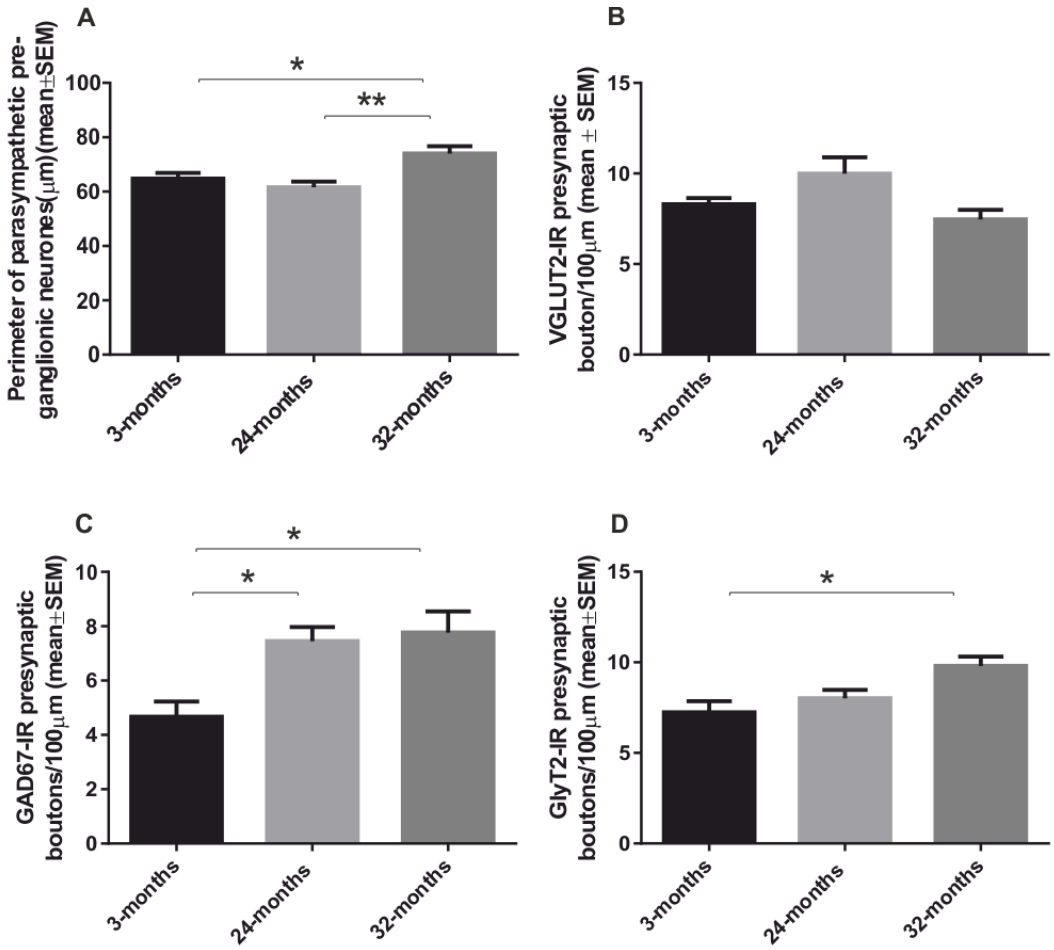

Figure 4.10 The number of excitatory VGLUT2-IR terminals contacting parasympathetic preganglionic neurones in the IML did not change with age yet there is an increase in inhibitory (GAD67 or GlyT2-IR) inputs.

(A) There was a significant increase in the perimeter of the parasympathetic neurones in extreme aged mice. (B) There was no change in the number of VGLUT2-IR terminals contacting parasympathetic neurones in different age groups (C) The number of GAD67-IR terminals contacting parasympathetic neurones was significantly increased in the aged and extreme aged mice compared to the young adult. (D) The number of GlyT2-IR terminals contacting DLN motor neurones was significantly increased in the extreme aged mice compared to the young adult.

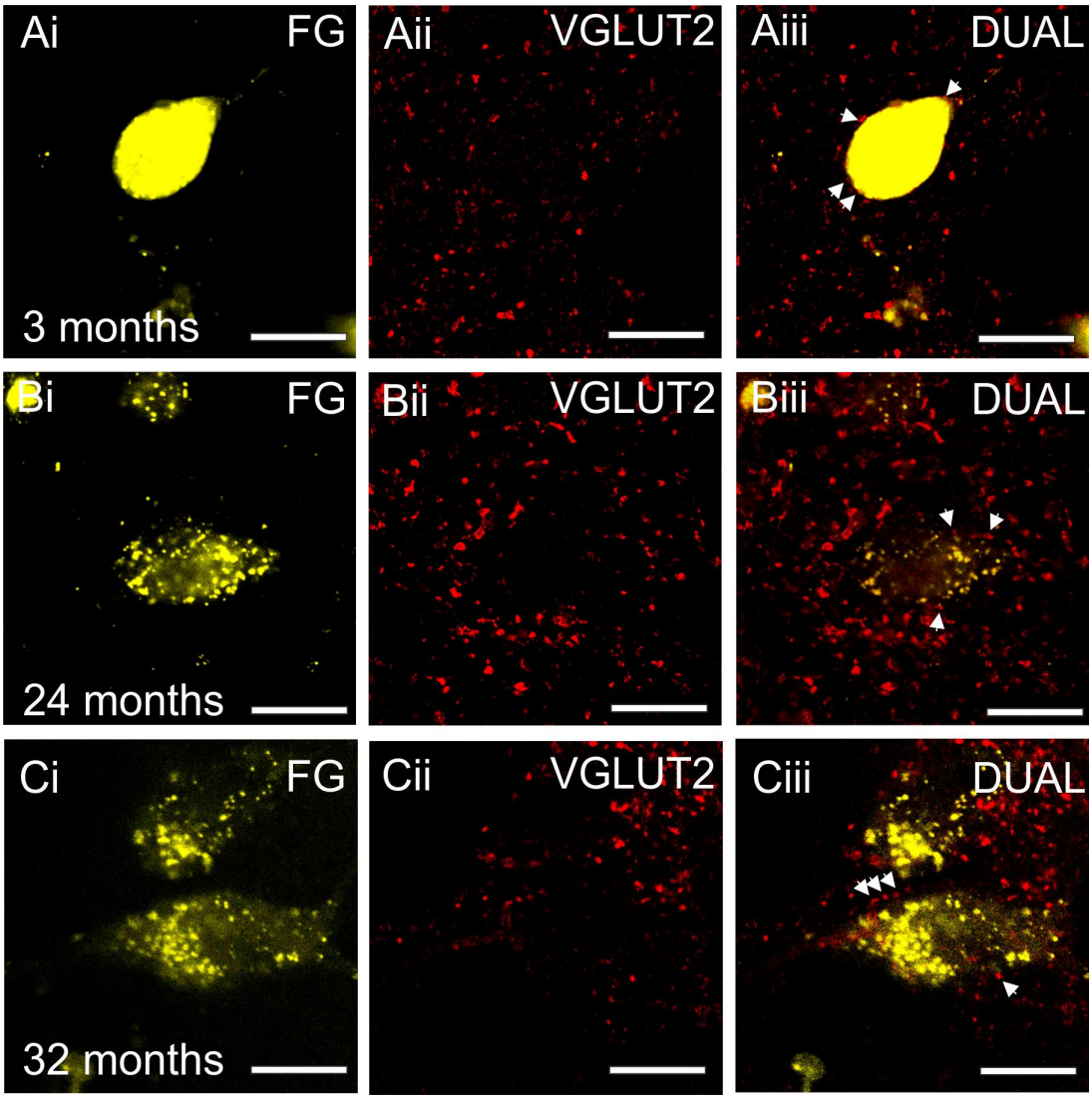

Figure 4.11 The number of excitatory VGLUT2-IR terminals contacting parasympathetic preganglionic neurones did not change with age.

VGLUT2-IR terminals were observed to contact FG labelled parasympathetic preganglionic in the IML in the young adult (Ai-Aiii), aged (Bi-Biii) and extreme aged (Ci-Ciii) mice. Arrows point to examples of terminals in close apposition with parasympathetic preganglionic neurones.

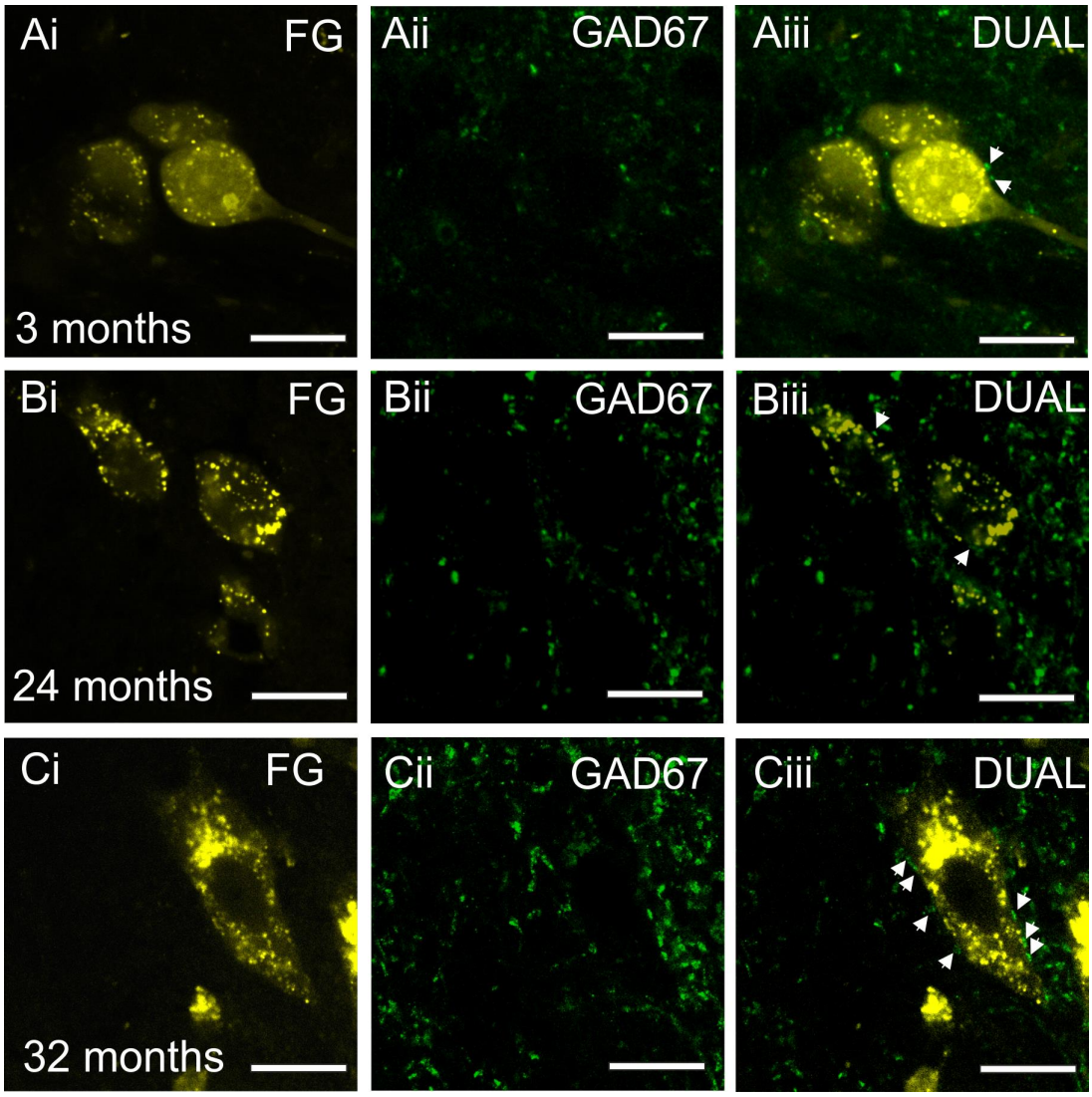

Figure 4.12 The number of inhibitory GAD67-IR terminals contacting parasympathetic preganglionic neurones increased with age.

GAD67-IR terminals were observed to contact FG labelled parasympathetic preganglionic neurones in the young adult (Ai-Aiii), aged (Bi-Biii) and extreme aged (Ci-Ciii) mice. Arrows point to examples of terminals in close apposition with parasympathetic preganglionic neurones.

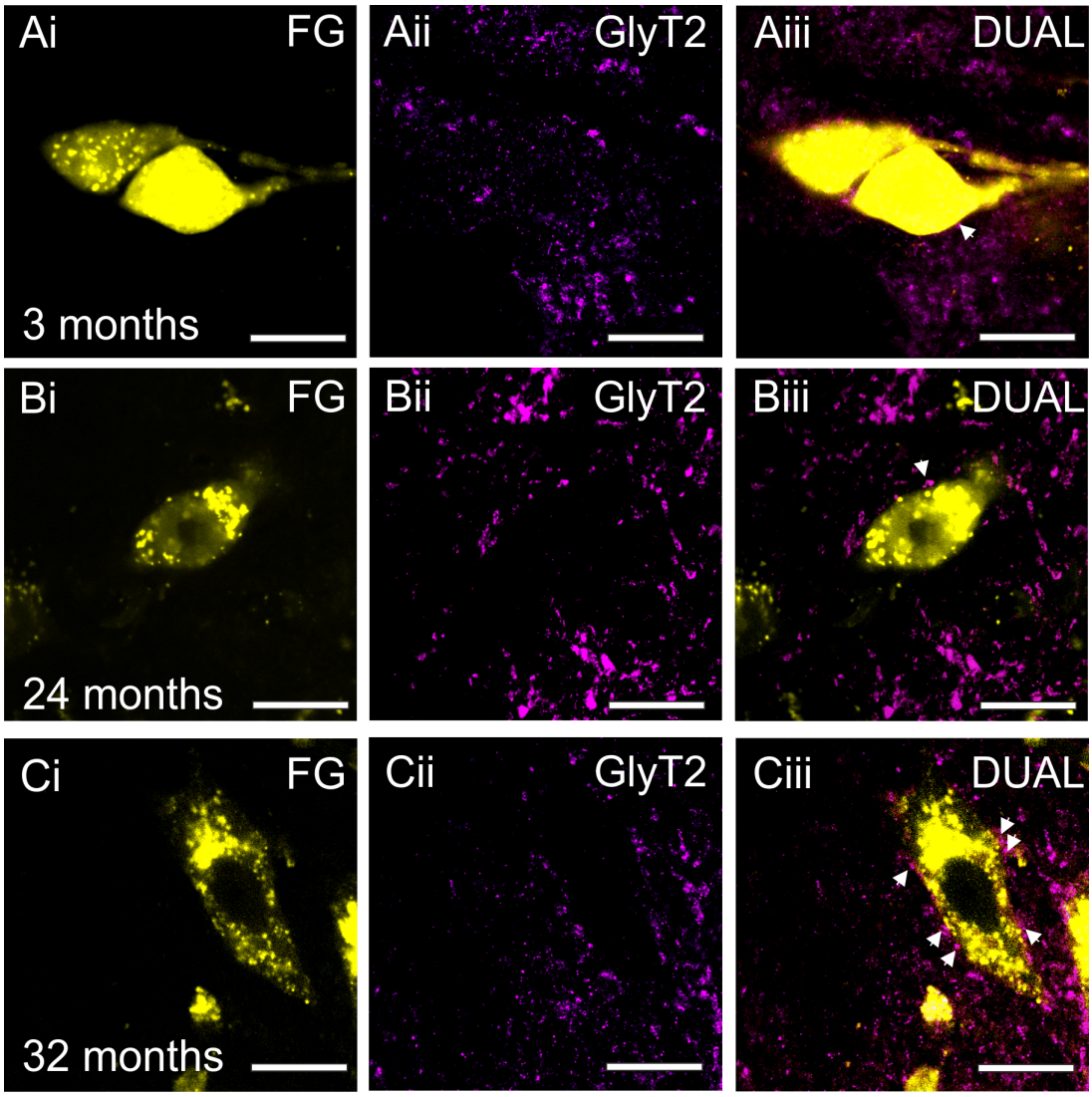

Figure 4.13 The number of inhibitory GlyT2-IR terminals contacting parasympathetic preganglionic neurones increased with age.

GlyT2-IR terminals surrounded FG labelled parasympathetic preganglionic neurones in both the young adult (Ai-Aiii), aged (Bi-Biii) and extreme aged (Ci-Ciii) mice. Arrows point to examples of terminals in close apposition with parasympathetic preganglionic neurones.

4.3.3.5 The E:I ratio of inputs onto the DLN, sympathetic and parasympathetic preganglionic neurones

When the number of terminals onto DLN neurones that were excitatory (VGLUT2) or inhibitory (GAD67 and GlyT2) were calculated and expressed as a percentage of the total number of the three types of inputs onto the cell, the percentage of excitatory inputs to the DLN decreased with age from 60% in 3 month old mice to 26% in 32 month old mice whereas the percentage of inhibitory inputs increased from 40% in 3 month old mice to 74% in 32 month old mice. The E:I ratio of inputs onto the DLN lowered substantially from 1.5 at 3 months to 0.3 at 32 months as a result of this net change (Figure 4.14).

Apart from that, there was a gradual decrease in the percentage of excitatory terminals onto the sympathetic neurones. This decreased from 43% in 3 month old mice to 39% in 24 month old mice and 24% in 32 month old mice. Whilst there was no change in the number of inhibitory inputs on the sympathetic motor neurones, when taken as a percentage of total inputs on the cell bodies the percentage of inhibitory inputs increased from 57% in 3 month old mice to 61% in 24 month old and 76% in 32 month old mice. The E:I ratio therefore changed from 0.7 in 3 month old mice, to 0.6 in 24 month old mice and decreased further to 0.4 in 32 month old mice (Figure 4.14).

The percentage of excitatory inputs to parasympathetic neurones decreased slightly from 41% in 3 month old animals to 39% in 24 month old animals and 30% in 32 month old animals. There was a net increase in inhibitory input to these cells from 59% in 3 month old animals to 61% in 24 month old animals and 70% in 32 month old animals. The E:I ratio was 0.8 in 3 month old, 0.7 in 24 month old and 0.3 in 32

month old mice (Figure 4.14). All results indicating pattern of increment or decrement of neurotransmitter-specific terminals contacting DLN, sympathetic and parasympathetic preganglionic nuclei are summarised in a diagram (Figure 4.15).

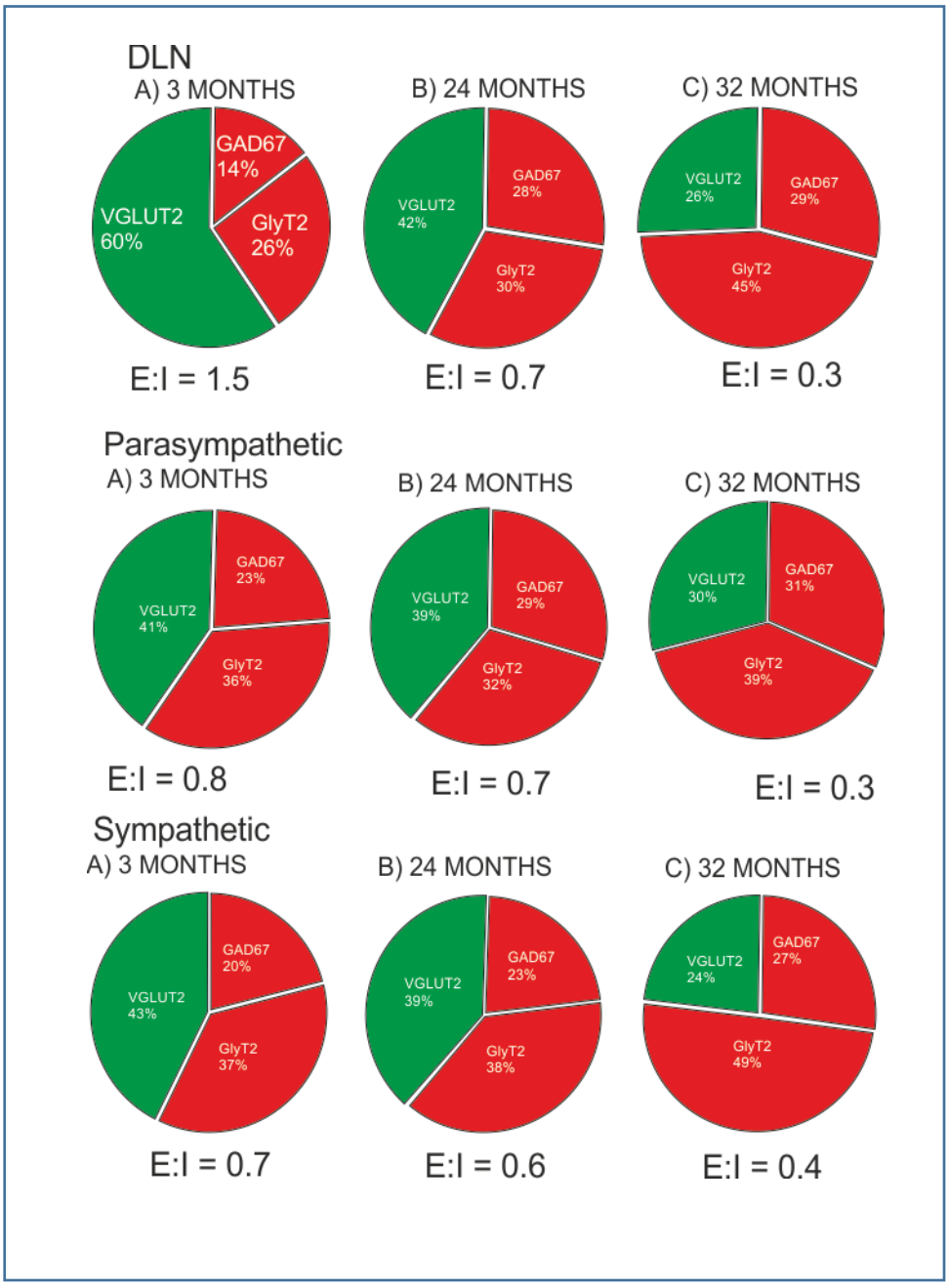

Figure 4.14 Pie charts illustrating the percentage of total inputs to DLN, sympathetic and parasympathetic neurones that were excitatory (green; VGLUT2) and inhibitory (red; GAD67 and GlyT2) in the different ages studied. The ratio of excitatory to inhibitory inputs (E:I) is shown under each pie chart

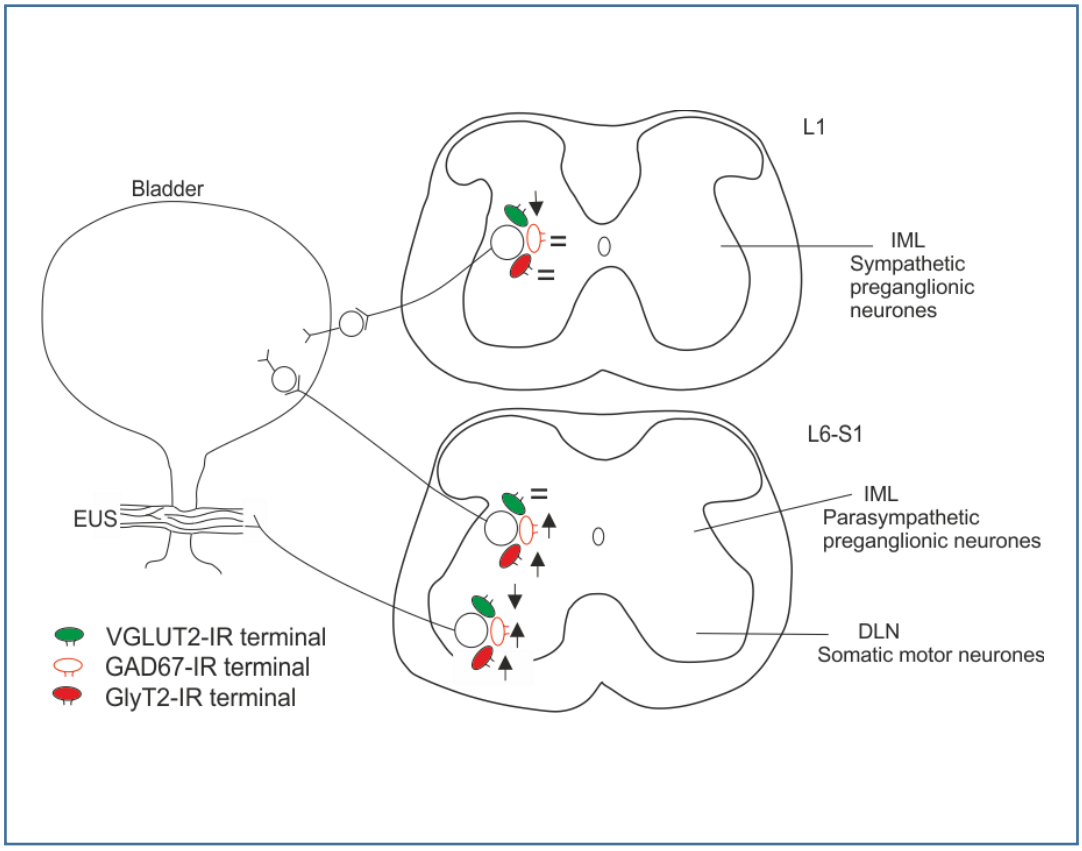

Figure 4.15 Schematic diagram of the changes in the inputs onto DLN and autonomic motor neurones during ageing.

As a summary, it was found that in the DLN, the axosomatic glutamatergic inputs were reduced but inhibitory GABAergic and glycinergic inputs were increased. In the sympathetic preganglionic neurones, the number of excitatory glutamatergic terminals contacting sympathetic preganglionic neurones in the IML also decreases with age, yet there is no change in inhibitory GABAergic and glycinergic inputs. In the parasympathetic preganglionic neurones, the number of excitatory glutamatergic terminals contacting parasympathetic preganglionic neurones in the IML did not change with age, yet there is an increase in inhibitory GABAergic and glycinergic inputs.

4.4 Discussion

4.4.1 Detection of glutamate, GABA and glycine in nerve terminals

In the previous chapter, using CTB retrograde tracing method, the motor neurones innervating the external urethral sphincter were traced and verified to be located in the ventral horn of the sixth lumbar to first sacral segments of the spinal cord. The study confirms that sphincter motor neurones in mouse reside in spinal DLN and form a solid anatomical basis for further synaptology studies. In this current chapter, that knowledge of the location of the EUS motor neurones was applied. The DLN is easily identified based on the location of FG labelling. FG is a reliable marker for motor and autonomic neurones, giving a bright yellowish labelling of neuronal soma and relatively easy to use by intraperitoneal injection (Schmued and Fallon, 1986).

Glutamatergic, GABAergic and glycinergic axon terminals on motor and autonomic neurones were detected with antibodies to VGLUT2, GAD67 and GlyT2, respectively. The two forms of glutamic acid decarboxylase enzymes, namely the GAD65 and GAD67, are responsible for synthesis of the inhibitory transmitter GABA (Kaufman et al., 1991). GAD67 was chosen in this study because this isoform is the major one found in boutons on spinal motor neurones (Mackie et al., 2003). Detection of GAD67 immunohistochemistry yields excellent axon terminal labelling, but labelling is not prominent in the GABAergic neuronal somata, which makes it an ideal marker of GABAergic boutons. The two forms of glycine transporter are glycine transporter 1 (GlyT1) and GlyT2. GlyT1 is an astroglial glycine transporter while GlyT2 is expressed in neuronal glycinergic boutons in mammals, where it is presumed to function in the presynaptic reuptake of transmitter at glycinergic synapses (Roux and Supplisson, 2000). Although

immunoreactivity against the neuronal GlyT2 isoform is a reliable marker for glycinergic neurones, GlyT2 antibody labels primarily axons terminals and makes it an excellent marker for glycinergic boutons (Zafra et al., 1995a).

The vesicular glutamate transporters (VGLUTs) regulate storage and release of glutamate in the brain and spinal cord. The VGLUT1 and VGLUT2 isoforms are widely expressed and differentially distributed in the CNS. Cerebral cortex, hippocampus and cerebellar cortex express predominantly mRNA for VGLUT1. By contrast, the brainstem and deep cerebellar nuclei express almost exclusively VGLUT2 (Fremeau Jr et al., 2004). The regional differences in expression of these transporters represent functionally distinct circuits

4.4.2 Effects of ageing on the presynaptic excitatory and inhibitory inputs to DLN motor neurones

In chapter 2, micturition behavioural study by using voided stain on paper technique (VSOP) revealed that aged (24 month old) and extreme aged (32 month old) mice exhibit higher micturition frequency, higher urine volume per void, higher total volume voided and shorter time intervals between micturition, compared to young mice (3 month old). The phenotypes of higher frequency of voiding with shorter intervals between voiding suggest either increased activation of bladder contraction during urine storage or weakening of bladder neck function and continence control in ageing.

In the DLN, the number of excitatory VGLUT2-IR boutons progressively decreased from the young 3-month old to aged 24-month old and continued to decrease in extreme aged 32-month old. However, the inhibitory inputs to DLN showed the opposite effects. Both showed an increase in the number of terminals contacting

DLN neurones. Significant decline in the ratio of excitatory to inhibitory presynaptic densities was observed from the young (E:I = 1.43) to the aged (E:I = 0.73) and to the extreme aged mice (E:I = 0.34). Other studies have reported similar age-related changes in synaptic inputs onto the DLN. Ranson et al. (2007a) documented age-related increases in vesicular acetylcholine transporter (VAChT) terminals, but observed a decrease in serotoninergic inputs in aged rats (Ranson et al., 2003a). Ranson et. al., (2007a) performed immunohistochemistry study on VGLUT1 and VGLUT2 on the pelvic motor neurones of rats. They described the numerous close appositions of VGLUT2 boutons on DLN, compared to the scarce apposition of VGLUT1 terminals surrounding the soma of DLN motor neurones in young rats. However, no comparison on the effect of ageing on the distribution of VGLUT2 between the young and aged on DL nucleus were reported. That gap of information is addressed in this current study.

Likewise, the distribution of immunoreactive GABAergic and glycinergic terminals within DLN matched previous descriptions (Ramirez-Leon et al, 1994, Blok et. al 1997). However, the current study as outlined in this chapter is the first to report the effects of ageing on the inhibitory inputs to the sphincter motor neurones. It was found that a significant age-related increase occurred in both inhibitory neurotransmitter-containing terminals in DLN which eventually altered the presynaptic excitatory-inhibitory inputs ratio to the DLN.

The significant decrease in glutamatergic inputs to the somata of DLN motor neurones in aged mice can be interpreted as a loss of powerful excitatory control onto sphincter motor neurones. Subsequent possible outcomes are reduced firing of action potentials of the DLN and reduced signaling to the urethral sphincter. Together with the gradual increase in inhibitory GABA/glycine innervation to DLN motor neurones; this in turn could lead to an increase in relaxation of the sphincter

and weakening of the bladder neck control in the aged animals, manifested as higher voiding frequency at shorter intervals. These results correlate with the study on the micturition behaviour of young and aged animals in chapter 2.

4.4.3 Effects of ageing on the presynaptic excitatory and inhibitory inputs to bladder autonomic neurones

In the sympathetic nucleus, there is a decline in presynaptic excitatory-inhibitory (E:I) ratio from young (0.75) to aged (0.65) to extreme aged (0.31). In the sacral parasympathetic nucleus, there was a decline in presynaptic E:I ratio from young (0.70) to aged (0.64) to extreme aged (0.42).

In the bladder sympathetic preganglionic neurones, a decrease in glutamate-immunoreactive and serotonin-immunoreactive boutons has been reported in ageing (Santer et al., 2002, Ranson et al., 2003a). The present findings showed that GABA/glycine inputs to sympathetic preganglionic neurones were unchanged in IML, while in an EM study, Santer et al. (2002) described it as an unchanged synaptic apposition length from boutons immunoreactive for glycine or gamma-aminobutyric acid (GABA) to IML in rats.

The sympathetic preganglionic nucleus and somatic DLN can be grouped together as they work in a synergistic way to promote continence. DLN motor neurones control EUS sphincter and sympathetic preganglionic neurones control the detrusor muscle of bladder and its internal sphincter to promote bladder relaxation for filling of urine and storage. In ageing, the current study showed that the input of excitatory neurotransmitter VGLUT2 was reduced to both the DLN and sympathetic neurones

in the aged group compared to the young mice. This may reflect reduced excitation of both groups of neurones and in turn, reflects weaker excitation of the EUS and internal bladder sphincter. This may also suggest weaker pelvic floor contraction to withhold urine, and poor bladder relaxation during the phase of storage. These effects are not pro-continence, and may be translated as responsible for increased frequency of voiding and leakage of urine in extreme cases. In addition to that, specific to the DLN, the inhibitory inputs of GABAergic and glycinergic axon terminals to the DLN motor neurones are significantly increased in ageing. This may promote overall net increase of inhibitory effects on DLN motor neurones and weaker stimulation of EUS which will also contribute to the incontinence.

The parasympathetic preganglionic nucleus works in the opposite way. Parasympathetic preganglionic neurones innervate the urinary bladder and stimulate contraction of detrusor muscle of the bladder and promote micturition. In ageing, the excitatory glutamatergic inputs to the parasympathetic preganglionic neurones neither significantly increased nor decreased. On the other hand, GABA and glycine innervation to these neurones increased. The net inhibitory influence appeared to increase. However, the difference in the E:I ratio between the young and extreme aged mice in the parasympathetic nucleus was relatively smaller (difference of 0.28) compared to the effects on E:I ratio of DLN (difference of 1.13) and sympathetic (difference of 0.44). As such, these differential changes may reflect a predominant age-related change in the presynaptic E:I ratio onto DLN and sympathetic efferents more than parasympathetic efferent, which may suggest a weaker continence control.

4.4.4 Excitatory/ inhibitory synaptic imbalance in ageing

The findings in this chapter indicate an altered excitatory/inhibitory balance that occurs in aged animals versus young animals. These alterations of the excitatory/ inhibitory balance has been noted and documented elsewhere in the spinal cord (Santer et al., 2002, Ranson et al., 2003a, Ranson et al., 2005, Ranson et al., 2007a, Ranson et al., 2007b). All these findings indicate selective vulnerability of changes in different neurotransmitters.

Elsewhere, in the cerebral cortex, (Majdi et al., 2007) reported a gradual decline in the density of excitatory and inhibitory presynaptic boutons, however the ratio of excitatory-inhibitory density was not significantly altered. (Casu et al., 2002) showed significant age-related decrease in population of cholinergic boutons in opposition to proximal and distal dendrites of layer V pyramidal neurones. Besides that, Palomba et.al (2008) revealed that the number and areas of GABAergic presynaptic boutons on the hypothalamic suprachiasmatic were significantly decreased in old versus young mice. These findings show a marked reduction in the GABAergic terminal synaptic network of the ageing biological clock.

Various brain regions display different vulnerability with respect to structural and neurochemical changes during ageing. It appears that there is a selective age-related vulnerability in CNS regions during ageing. However, whether all neurotransmitter system are equally affected and what triggers and drives these important changes is still largely unknown and requires further investigation.

Studies on the interaction between different neurotransmitter systems such as glutamate and GABA/glycine in ageing are providing new clues to understand the age-related changes in micturition circuits of the spinal cord. Specifically the interaction between glutamate, GABA and glycine could be of interest to understand the functional neurochemical substrates of ageing as it has been useful to understand the neuropathology of overactive bladder and urinary incontinence. In fact, this type of interaction correlating with behavioral parameters could provide significant advances in our understanding of ageing. This approach might also be the basis for the development of therapeutic tools aimed not only to compensate deficits of just one single neurotransmitter, but to rebalance the possible deficits in the interaction of multiple neurotransmitters in a specific circuit of the spinal cord. In the next chapter, an attempt to reverse the effect excitatory glutamate inputs decline on the ageing neuro-urinary system by enhancing AMPA receptor-mediated glutamatergic neurotransmission was performed.

Chapter 5

The effects of AMPA receptor potentiator LY404187 on micturition behaviour in aged 24 months old female mice.

5.1 Introduction

Urinary incontinence and lower urinary tract symptoms belongs to the most frequent urological disorders in late life and their occurrence increase with age (Irwin et al., 2011). In the previous chapter (chapter 2), the effects of ageing on the micturition behaviour of mice were studied. By 24 month of age, female wild-type mice voided more frequently with higher volumes of micturition, than younger mice

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The decline in continence control can partly be explained by changes in the synaptic inputs to spinal neurones controlling micturition that occur during normal ageing. In chapter 4, a significant alteration of excitatory-inhibitory balance on all neurones controlling lower urinary function was observed. The largest changes were observed by inputs onto the soma of the sphincter motor neurones, a major reduction of glutamatergic inputs, together with significant increase on the inhibitory GABAergic and glycinergic inputs. These data suggest the pathophysiology of incontinence in ageing that profound loss of glutamatergic presynaptic inputs and the shift in the pattern of innervation of aged neurones may contribute to behavioural impairment and poor sphincter control in the elderly.

However, to date there are no adequate options of pharmacotherapies targeting the issue of incontinence in the elderly. While national and international guidelines for

continence care do exist, pharmacological and non-pharmacological management of incontinence is both still far from satisfactory. An exception to this is the use of duloxetine, a selective serotonin and norepinephrine reuptake inhibitor (Thor, 2004). Duloxetine works in managing stress urinary incontinence by inhibition of the presynaptic neuronal reuptake in the sacral spinal cord. This results in elevated level of serotonin and norepinephrine in the synaptic cleft and lead to an increase in the nerve stimulation to the EUS, improve sphincter tone and stronger urthral contraction. But as a whole, effective and well-tolerated clinical drugs have not been developed in targeting urge urinary incontinence in ageing. One wonder at the possibility of reversing the effects of ageing on the urinary function, by supplementing additional boost on the glutamatergic excitatory influence to these motor neurones. Subsequently, the next strategy involves introducing an AMPA receptor potentiator and to observe its effects on the micturition behaviour of aged 24 months old mice.

5.1.1 AMPA receptors in the Onuf's nucleus/DLN

Ionotropic glutamate receptors consist of two subtypes: α-amino-3-hydroxy-5-metyl-4-isoxazole-propanoic acid (AMPA) and N-metyl-D-aspartate (NMDA) (Sprengel and Seeburg, 1993). AMPA receptors are found in many parts of the brain and distributed throughout the central nervous system. Ionotropic AMPA receptors have four different subunits: GluR1, GluR2, GluR3, and GluR4. The four subunits assemble to form functional receptors with a central channel pore. The N terminus is located extracellularly and the C terminus intracellularly. The receptor allow passage of sodium and potassium ions and are non-selective cation channels.

Glutamate acting on AMPA-type ionotropic glutamate receptor mediates the majority of fast excitatory synaptic transmission in the mammalian central nervous system.(Dingledine et al., 1999). Their activity-dependent insertion at the

postsynaptic membrane activates AMPA receptors and contributes to the induction of long term potentiation (LTP). Thus, AMPA receptors is suggested to be a major neural substrate for the formation and development of learning and memory (Lynch and Baudry, 1984). Deficits in glutamatergic neurotransmission and AMPA receptor antagonism is often implicated in cognitive deficits and degenerative neurological disease (Segovia et al., 2001). In a study to find the relationship between reduced AMPA receptors in ageing and spatial memory performance in mouse, Magnusson (1998) showed decrease of NMDA and AMPA receptors in the hippocampus was significantly correlated with age-related declines in spatial learning in Morris water maze. In contrast, enhancing glutamatergic neurotransmission or AMPA receptor-mediated transmission appear to enhance cognition and may therefore be therapeutically beneficial where cognitive function is impaired (Lynch et al., 1997). Many early cognitive enhancers drugs such as piracetam and aniracetam were reported to positively modulate AMPA receptors (Copani et al., 1992). In electrophysiological studies, aniracetam increases the peak amplitude and reduces the rate of decay of the ion current generated by AMPA.

Study has also indicated that the glutamatergic pathway in the central nervous system plays an important role in the control of micturition in rats (Yoshiyama and De Groat, 2005). Glutamate is a major excitatory neurotransmitter and the glutamatergic pathway activated via AMPA receptors play a crucial role in the control of activity of Onuf’s nucleus. Williams et al. (1996) have used subunit specific immunocytochemistry to study the distribution of AMPA receptors in the human motor system of the spinal cord, brainstem, and motor cortex. They reported that motor neurones of Onuf's nucleus was shown stained with GluR1, GluR 2/3 and GluR4 antibodies. In addition to that, Kawamorita et al. (2015) demonstrated that the blockade of spinal glutamatergic AMPA receptors significantly attenuates the urethral closure reflex during sneezing in the rats. The result indicates that the

glutamatergic mechanism via AMPA receptors plays an important role in the active urethral closure reflex during sneezing at the spinal level in rat.

5.1.2 AMPA receptor potentiators

AMPA receptors potentiators are positive allosteric modulators that increase current flux through the ion channel in the presence of an agonist, by reducing desensitisation and/or deactivation (Partin et al., 1994, Yamada and Tang, 1993). They are also known as ampakines. A number of different classes of AMPA receptor potentiators have been developed including pyrrolidinones such as piracetam and aniracetam (Gouliaev and Senning, 1994), structurally related benzamide compounds, such as CX-516 and 1 –BCP (Staubli et al., 1994) and thiazide derivatives, such as cyclothiazide, diazoxide and IDRA-21 (Yamada and Rothman, 1992, Zivkovic et al., 1995).

Many of these compounds have demonstrated cognitive enhancing effects in rodent models of cognition (Stäubli et al., 1994). Stäubli et al. (1994) studied the effects AMPA receptor potentiator on the induction of LTP in the hippocampus of rats. Intraperitoneal injections of the drug markedly increased the degree and duration of long-term potentiation and improve retention of memory in a radial maze task and in an odor-matching problem. Besides that, AMPA receptor potentiators have been suggested to be efficacious in animal models of depression (Knapp et al., 2002). They tested the activity of a series of nootropic drugs, including piracetam, aniracetam, fluoxetine, and desimpramine in a behavioural test linked to depression. The nootropic drugs reduced submissive behaviour over time, suggesting that ampakines may have antidepressant property.

Since in the current study, glutamate-IR presynaptic terminals were found to be reduced in DLN and sympathetic preganglionic neurones in aged mice, it is worth to consider AMPA-receptor potentiator as potential treatment for urinary incontinence. Furthermore, in a recent interesting development, ampakines was found to stimulate motor output in Pompe disease (respiratory insufficiency) mouse model. Pompe disease diminishes respiratory motor drive but administration of ampakine CX717 robustly increased phrenic and hypoglossal inspiratory bursting (ElMallah et al., 2015).

5.1.3 AMPAkines LY404187

LY404187 (*N*-[2-(4'-cyanobiphenyl-4-yl)-propyl]propane-2-sulfonamide) is a member of biarylpropylsulfonomide class of AMPA receptors potentiators. LY404187 is a selective AMPA receptor positive allosteric modulator for GluA1i, GluA2i, GluA3i and GluA4i. It has been developed to enhance glutamatergic synaptic transmission both in vitro and in vivo, and is highly potent and centrally active (Ornstein et al., 2000, Lindén et al., 2001). The augmentation of synaptic activity is due to direct potentiation of AMPA receptor function and enhance AMPA receptor-mediated neurotransmission by reducing desensitization of the ion channel.

Recent study has characterized the effects of LY404187 on pre- and postsynaptic functions at CA3–CA1 synapses in neonatal rats by using whole cell patch-clamp recording (Song et al., 2012). They found that LY404187 did not appear to alter the probability of presynaptic release, as evidenced by the lack of significant changes in both the amplitude and the paired-pulse facilitation ratio (an index of release probability) of NMDA excitatory postsynaptic current (EPSCs). However, LY404187

enhanced both the amplitude and 1/CV2 (CV: coefficient of variation) of AMPA EPSCs and the number of the synapses exhibiting AMPA EPSCs.

5.1.4 Aim

AMPA receptor potentiators enhance AMPA receptor-mediated glutamatergic neurotransmission and may have therapeutic potentials as continence promoter. While increased in AMPA receptor mediated neurotransmission was found to be beneficial in depression, anxiety and neurodegenerative management, preclinical study addressing AMPA receptor in relation to urinary incontinence is lacking. No investigation has been conducted on the potential effects of AMPAkines on the age-associated decline in micturition function. Thus, the aim of the current study was to determine and compare the effects of AMPA receptor potentiator LY404187 (0.5mg/kg subcutenously) upon micturition behaviour modification by using micturition study analysis in aged 24 months old mice.

5.2 Material and method

5.2.1 Animal

Eight aged female C57BL/6 mice (N=8, age 24 months old) were divided into two group, vehicle-only group (N=4) and LY404187 group (N=4).All animal and experimental procedures were approved by the Central Biological Unit of University of Leeds. All efforts were made to minimise animal discomfort and the number of animal used. Experiments were performed during the day and night portion of the animals' circadian clock cycle. Daytime experiments were performed between 0900 to 1300 hour and night time experiments were carried out between 1900 to 2300 hour.

5.2.2 Drugs preparation and administration

LY404187 (CAS number 211311-95-4, TOCRIS Bioscience) is available as a white solid compound. To form the stock solution at 50mM, 10mg of LY404187 powder was dissolved in 30% ethanol, followed by gentle warming at 60^{o}C and rapid shaking of the container. 0.1ml of the working solution (containing 10µg of LY404187) was injected to the mice (weight 20-25g) subcutaneously. The AMPA receptor potentiator LY404187 (0.5mg/kg) or vehicle (30% ethanol) were administered subcutaneously 60 minutes before micturition study were recorded. The dosage was decided based on the work done by Jones et al. (2005) and Fowler et al. (2004). The time interval between injection and data recording was chosen based on a study which indicated that peak plasma levels of the AMPA receptor potentiator LY404187 are observed for 1 to 2 hours after subcutaneous administration.

5.2.3 Micturition analysis

The micturition analysis was performed on the mice according to the method described by Sugino et al. (2008) as outlined in detail in chapter 2 (section 2.2.3). Briefly, conscious aged mice were placed individually into the modified metabolic cage collection of voided urine. After the subcutaneous injection of either vehicle or LY404187, the animals were left in the metabolic cages for at least 1 hour before data were collected. Experimenting time was for 3 hours duration in the daytime and 3 hours in the night time. A filter paper was placed underneath the wire-netted grid 5 cm below it. Whenever the mice urinate, the urine drops through the wire-netted grid (3.2 x 0.4cm), landed on the filter paper and made a spot on the filter paper. The filter paper is changed every 30 minutes to avoid multiple drops on the same spot. Any urine drop that landed on the wire-netted grid is manually collected

by bringing the filter paper up to touch the grid. After 3 hours the individual mouse was returned to their respective cages and the filter paper collected for analysis.

The filter papers were placed under UV light to visualise the dried urine which fluoresces under UV light. The edge of the urine spot is outlined. A 1x1cm rectangle grid paper is placed under and superimposed at the back of the filter paper and the total area of each urine spot was measured. The area of the urine spot and the total number of spots were entered to Microsoft Excel 2013 software for statistical analysis. Four evaluated micturition parameters were derived from the collected data including frequency (number of void/hour), total voided urine volume (µl/ hour), urine volume per void (µl/void) and time between voiding intervals (min).

5.2.4 Statistical analysis

Data from the each experiments were pooled. All quantitative data were expressed as mean ± standard error of mean (s.e.m), analysed using IBM SPSS Statistics 20 and comparisons made using independent sample t-test. $P < 0.05$ was taken as statistically significant difference between samples.

5.3 Results

5.3.1 Effects of LY404187 on frequency of micturition

Before and after drug administration, mice were generally inquisitive, with periods of movement and grooming. LY404187 produced no overt changes in this behavioural pattern. The micturition profiles on number of micturition/hour during day time showed no significant difference between vehicle (0.69 ± 0.07) and LY404187 (0.52 ± 0.05), $t(6)=1.897$, $p=0.107$. The micturition profiles on frequency/hour during night time also did not show any significant difference between vehicle (0.63 ± 0.07) and LY404187 (0.74 ± 0.12), $t(6)=-1.268$, $p=0.253$ (Table 5.1)

5.3.2 Total volume of micturition

The micturition profiles on total volume/hour during day time showed no significant difference between vehicle (225 ± 23.01 µl/hour) and LY404187 (171.50 ± 5.12 µl/hour), $t(6)=2.250$, $p=0.065$. The micturition profiles on total volume/hour during night time showed no significant difference between vehicle (267 ± 36.34 µl/hour) and LY404187 (226 ± 22.19 µl/hour), $t(6)=0.966$, $p=0.371$.

5.3.3 Volume per episode of voiding

The volume per micturition during day time showed no significant difference between vehicle (328.55 ± 30.55 µl) and LY404187 (334.43 ± 32.30 µl), $t(6)=-0.132$, $p=0.899$. The volume per micturition during night time showed no significant difference between vehicle (385.47 ± 30.26 µl) and LY404187 (302.60 ± 26.05 µl), $t(6)=2.075$, $p=0.083$.

5.3.4 Time intervals between voiding

The time intervals between voiding during day time showed no significant difference between vehicle (76.50 ± 8.00 minutes) and LY404187 (104.58 ± 24.69 minutes), t(6)=-1.082, p=0.346. The time intervals between voiding during night time showed no significant difference between vehicle (81.79 ± 13.10 minutes) and LY404187 (78.06 ± 7.18 minutes), t(6)=0.249, p=0.811.

Table 5.1 Effects of AMPA receptors LY404187 on the frequency, total volume, volume per void and time inervals between voiding in aged 24 month old mice.

Micturition Analysis	Vehicle (n=4)		LY404187 (n=4)
Frequency (no. of micturition/ 9hr)	AM	0.69 ± 0.07	0.52 ± 0.05
	PM	0.63 ± 0.07	0.74 ± 0.12
Total volume (µl)/ 9hr	AM	225 ± 23.01	171.50 ± 5.12
	PM	267 ± 36.34	226 ± 22.19
Volume (µl) per void	AM	328.55 ± 30.55	334.43 ± 32.30
	PM	385.47 ± 30.26	302.60 ± 26.05
Time intervals between voiding (min)	AM	76.50 ± 8.00	104.58 ± 24.69
	PM	81.79 ± 13.10	78.06 ± 7.18

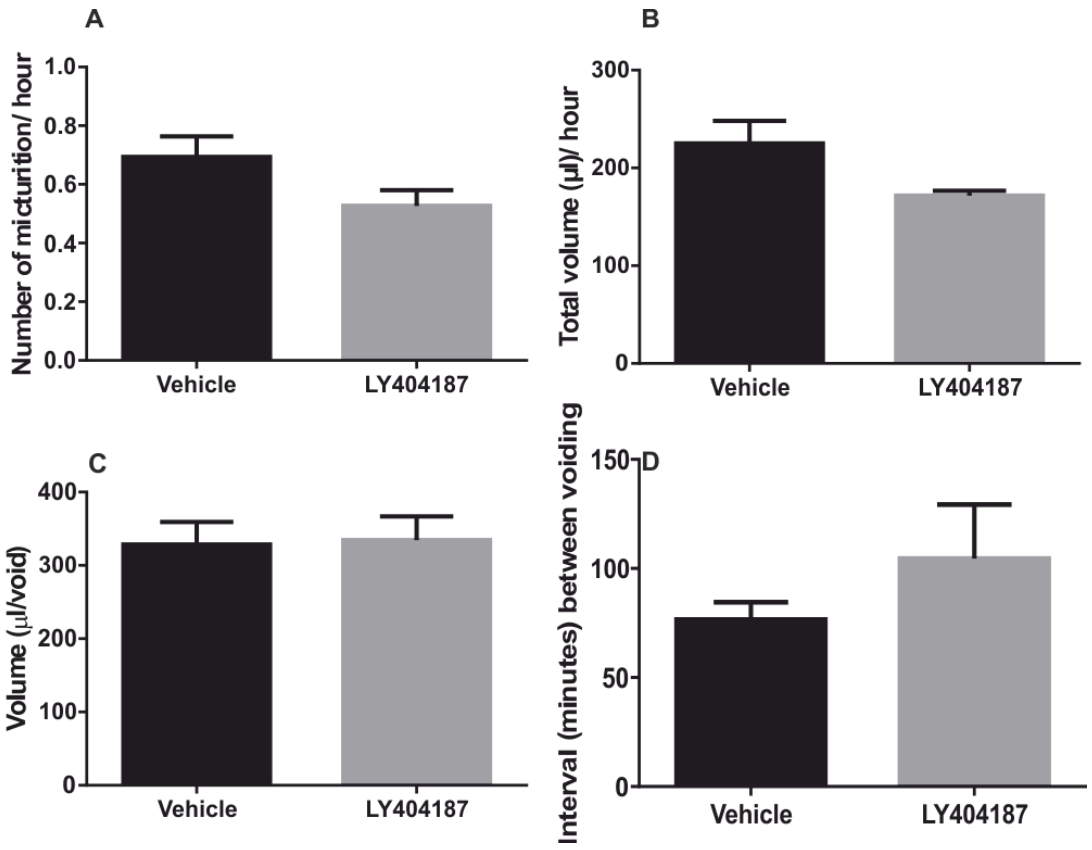

Figure 5.1 Effects of LY404187 on micturition behaviour in aged 24 months old mice during day time.

A) Frequency of micturition B) Total volume per hour C) Volume per voiding episode, and D) Time intervals between voiding. No significant changes were observed in any of these micturition profiles

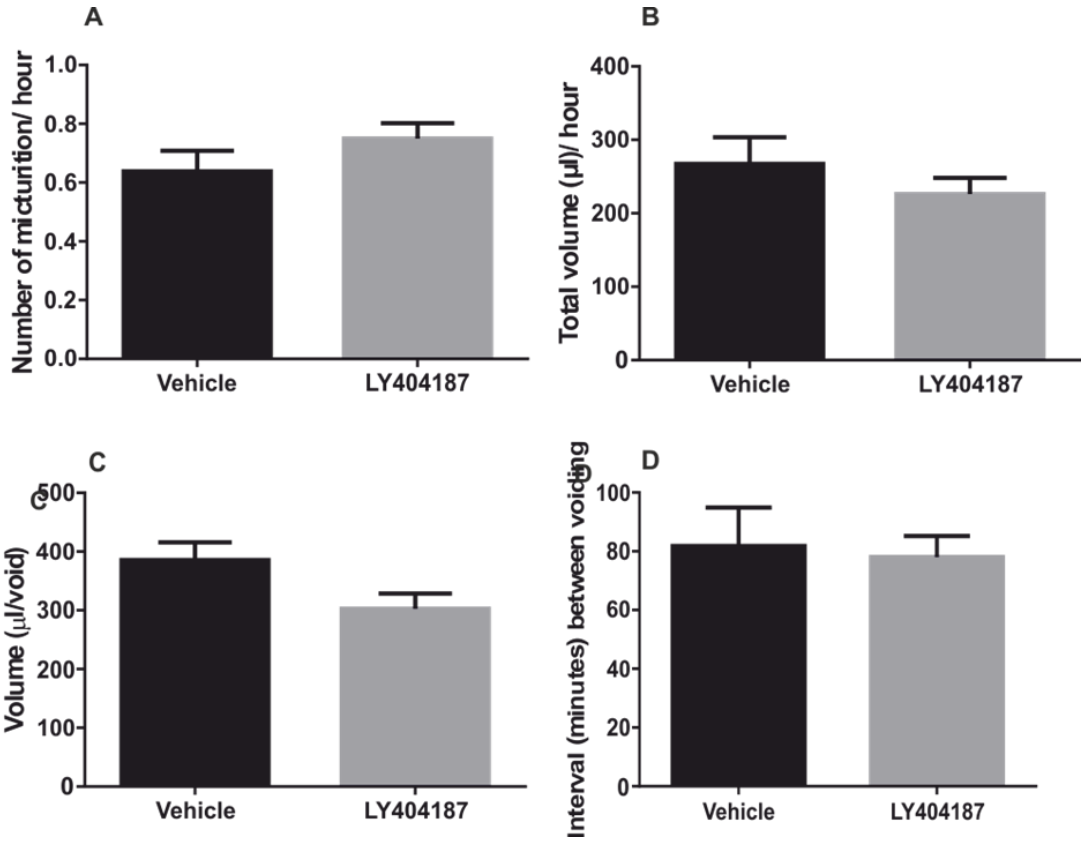

Figure 5.2 Effects of LY404187 on micturition behaviour in aged 24 months old mice during night time.

A) Frequency of micturition, B) Total volume per hour C) Volume per voiding episode, and D) Time intervals between voiding. No significant changes were observed in any of these micturition profiles.

5.4 Discussion

The aim of the current study was to determine and compare the effects of AMPA receptor potentiator LY404187 (0.5mg/kg subcutenously) upon micturition behaviour in aged 24 months old mice. At that given sample size and dosage, this experiment did not provide any evidence of changes in the micturition profile in the aged mice.

In clinical urology, despite the prevalence of overactive bladder and urge incontinence, treatments are still generally poor. Focus on the management of urge incontinence have been primarily put on lifestyle modifications and urinary care in the elderly. Most of the drugs currently approved for urinary incontinence targets on the detrusor muscle of the bladder to relax it. Amongst these are the anti-muscarinics and β-adrenergic agonists. Anti-muscarinic drug that is used for urge incontinence includes oxybutynin, tolterodine, trospium amd hysoscyamine and act non-selectively at both M3 and M2 receptors on the bladder. However, despite being the most widely used class of drugs for these conditions, an analysis of 51 clinical trials by Hay-Smith et.al (2006) has revealed that the efficacy of anti-muscarinics was unclear. Less than 50% claimed having improvement after taking anti-muscarinics and only 15% of patients with urge incontinence are dry. Other promising drugs on treating overactive bladder are 5HT/NE reuptake inhibitors (Thor, 2004). This condition can be reversed with the 5-HT reuptake blocker fluoxetine. In patients with clinical depression and urge incontinence, tricyclic antidepressants and the combined 5-HT/NE reuptake blockers are useful in both depression and urge incontinence. To achieve improved therapeutic efficacy, treatment strategies may have to go beyond anti-muscarinic and 5HT/NE reuptake inhibitors, especially when there are new evidence that demonstrated significant reduction of glutamate terminals onto DLN motor neurones and sympathetic

neurones, and its corresponding behavioural micturition effects in advance age. Thus, new drug therapies are desperately needed. Glutamate must be recognised as a key element in the pathophysiology of urinary incontinence and the role and potential of AMPA receptor potentiators in enhancing glutamatergic neurotransmission should be seen as the novel target in combating this condition.

In the previous chapter, we have shown that deficits in glutamatergic transmission may contribute to continence decline in the elderly. Elsewhere in the brain, studies on AMPA receptor potentiator have provided evidence that AMPAkines may have therapeutic benefits where glutamatergic hypofunction occurs. AMPA receptor potentiator has been shown to have been effective in rodent models of depression, suggesting antidepressant property (Li et.al, 2001). In vivo, they are active in rodent models of memory and cognition and has been shown to enhanced cognitive function (Quirk and Nisenbaum, 2002, O'Neill et al., 2004b) and in Parkinson Disease (O'Neill et al., 2004a), apart from possible application in treatment of schizophrenia and ADHD. These effects appear to be mediated through multiple mechanism of action secondary to AMPA receptor potentiation. One prominent effect of these molecules also appears to increase brain derived neurotropic (BDNF) expression both in vitro and in vivo (Mackowiak et al., 2002).

Thus it has been suggested that AMPA receptor potentiators may be beneficial in the treatment of incontinence by centrally acting on the CNS. In this study, for the first time, investigation was conducted using AMPA receptor potentiator LY404187, to determine whether AMPA receptors potentiation leads to improvement of micturition profiles in aged subjects. A subcutaneous dose of 0.5 mg/kg per animal was administered an hour prior to observation. The present study demonstrate that the AMPA receptor potentiator, LY404187 did not significantly alter or modify the

micturition behaviour of aged female mice during day or night time experiments after administration of LY404187. Although there was a decrease in the number of micturition (approximately 24% fewer episodes) and longer intervals between voiding (approximately 37% longer) during daytime, it however did not reach statistical significance. Perhaps, a larger sample size may be required in future studies to test the effectiveness of AMPA receptor potentiator in treating urinary incontinence.

In this study, animals received a single subcutaneous injection of LY404187 at the dose of 0.5mg/kg and subjected to behavioural analysis an hour after injection. LY404187 dose was selected based on the study by Jones et al. (2005) which found that subcutaneous doses of LY404187 (0.5mg/kg) were needed to produce significant increases in blood oxygenation level dependent (BOLD) contrast as a marker of neuronal activity in brain regions including the hippocampus, lateral and medial habenulae and superior and inferior colliculi in rat. The increase in BOLD signal reflect increases in excitatory neurotransmission. In addition to that, Fowler et al. (2004) have performed systematic study on the effects of LY404187 on cerebral glucose utilisation and c-fos expression in rat on a serial subcutaneous dosing preparations. They reported that at dose of 0.5 mg/kg, LY 404187 produced significant elevations in glucose utilization in 28 of the 52 anatomical regions analysed, which included rostral neocortical areas and the hippocampus, as well the dorsal raphe nucleus, lateral habenula, and locus coeruleus. LY404187 (0.5 mg/kg) also produced increases in c-fos immunoreactivity in the cortex, locus coeruleus, and the dorsal raphe nucleus. Thus, this dosage of LY404187 was chosen in the current experiment. However, a series of testing with different dosage was not done to determine whether the effects of LY404187 on micturition behaviour may be observed in dose-dependent manner. Future studies should

probably consider using higher dosage to observe the therapeutic effects of LY404187.

Another avenue to be investigated is the role of NMDA receptors on micturition behaviour. According to Yoshiyama and De Groat (2005) the lower urinary tract is controlled by the glutamatergic system not only through AMPA receptors, but also via NMDA receptors. In describing the pharmacological activity of LY404187 in acutely isolated rat cerebellar Purkinje neurons and cultured rat hippocampal neurons, (Gates et al., 2001) noted that these compounds appear to have at least a 10–100-fold selectivity for AMPA receptors over other NMDA or kainate receptor channels and voltage-gated calcium ion channels in neurons. Thus LY404187 had minimal activity on NMDA receptor responses. Therefore the contribution of NMDA receptors to urethral continence reflex need to be investigated in any future study.

5.4.1 Conclusion

As a conclusion, although AMPA receptor potentiators may have therapeutic benefits elsewhere in the CNS where glutamatergic hypofunction occurs, this study did not provide evidence that LY404187 at the dose of 0.5mg/kg subcutaneously have therapeutic use in the treatment of incontinence in ageing. The potential of AMPA receptors and LY404187 and the contribution of NMDA as a novel target in promoting continence receptors should be explored further in the future.

Chapter 6

General Discussion

The current understanding of the pathophysiology of urinary incontinence is limited. The main goal of the research presented in this thesis was to examine potential changes in neuronal circuitry underlying urinary incontinence in the mouse model. This study has combined micturition behavioural study, anatomical, surgery and neuronal tracing method, immunofluorescence and quantitative confocal microscopy to determine the relationship between micturition character in ageing animal and its corresponding age-related changes in the spinal neuronal nuclei controlling the lower urinary tract.

In chapter 2, the frequency of micturition, which may also reflect the power of continence, was determined using the voided stain on paper (VSOP) technique. The micturition characteristics of 3, 24 and 32 month old mice revealed that increasing age increases the frequency of micturition, total volume of urine produced and volume/void in night and daytime experiments. In addition, 32 month old mice had a shorter interval between voids than the younger animals. Ageing caused an increase in the frequency of micturition, total volume produced, and volume of urine produced per void, whilst the interval between urine voids decreased with age. Such changes in total volume of urine produced and the volume of urine produced per void is likely to be related to the changes in the size of the mice due to age i.e an increase in body size and bladder volume in older mice compared to younger mice correlates to an increase in urine produced. A similar increase in the volume of micturition has also been shown in aged rats (Chun et al., 1988, Ranson et al., 2003b).

Since little research has previously been carried out to characterise the location of the pudendal motor neurones of the DLN in the mouse, in chapter 3, the location and structure of the DLN innervating the external urethral sphincter was determined using retrograde labelling techniques and immunohistochemistry for choline acetyltransferase (ChAT). Such tracing studies revealed that neurones innervating the mouse external urethral sphincter muscles are present in the DLN, a well-defined group of medium-sized motor neurones located between L6-S1 of the spinal cord. The nature of the inputs onto retrogradely labelled (by injection of Fluorogold) DLN neurones and autonomic neurones in the spinal cord which control micturition were also determined using immunohistochemistry to detect excitatory (glutamatergic) and inhibitory (GABA and glycinergic) terminals in 3, 24 and 32 month old mice. The net excitatory inputs are reduced while the net inhibitory inputs are increased in the spinal nuclei controlling bladder and urethral function in aged mice. The present study documented a decline in excitatory glutamate-IR presynaptic terminal inputs in DLN and sympathetic neurones, and an increase in inhibitory GABA-IR and glycine-IR inputs in DLN and parasympathetic neurones. Prominent changes in the excitatory/inhibitory ratio are observed in DLN and sympathetic nuclei of aged mice. The shift in the dominant inputs and alteration in the ratio of excitatory/inhibitory balance, may suggest a reduction in excitability of DLN motor neurones and sympathetic preganglionic neurones. This phenomenon can be explain through the concept of summation of synaptic potentials.

Neurones in the central nervous system are typically innervated by thousands of synapses and binding of neurotransmitter creates postsynaptic potentials (PSPs). The PSPs produced by each active synapse can sum together to determine the behavior of the postsynaptic neurone. PSPs are deemed excitatory if they increase

the probability that an action potential will occur, and inhibitory if they decrease the chances.

Activation of either one of the excitatory synapses such as glutamatergic synapse produces a short term depolarisation and an excitatory postsynaptic potential (EPSP). While a single depolarisation may not have much of an effect on the postsynaptic neuron, repeated depolarisations caused by high frequency stimulation can causes all EPSPs to sum together. If the summation of the EPSPs is sufficient to reach the threshold potential, a postsynaptic action potential results. In contrast to glutamate, the neurotransmitters GABA and glycine mainly function to trigger hyperpolarization of the postsynaptic membrane and create inhibitory postsynaptic potentials (IPSPs). Summation with other IPSPs can cancel out EPSPs and determines whether the postsynaptic potential will reach threshold and cause an action potential to fire in the postsynaptic neurone.

Thus, the production of an action potential depends on the balance between excitation and inhibition. If the sum of all EPSPs and IPSPs results in a depolarisation of sufficient amplitude to raise the membrane potential above threshold, then the postsynaptic cell will produce an action potential. Conversely, if inhibition prevails, then the postsynaptic neurone will remain silent. The balance between EPSPs and IPSPs changes continually over time, depending on the number of excitatory and inhibitory synapses active at a given moment and the magnitude of the current at each synapse.

As mentioned earlier, the current study showed a shift in the dominant inputs and alteration in the ratio of excitatory/inhibitory balance, which may suggest a reduction in excitability of DLN motor neurones and sympathetic preganglionic neurones.This may lead to decrease the capacity of Onuf's motor neurones to sustain tonic contraction of the EUS, and sympathetic neurones to maintain detrusor muscle relaxation and internal sphincter contraction during bladder filling. This study proposed that the shift in the excitatory-inhibitory synaptic balance may be the cause for the weak sphincter control in the elderly and may explained the underlying mechanism of urinary incontinence in ageing population.

6.1 Implications

This thesis reports an altered excitatory/inhibitory balance that occurs in aged animals versus young animals. Crucially, there is a selective decrease in excitatory synapses onto neurones that control continence – the number of VGLUT2 terminals decreased onto both DLN motor neurones and sympathetic preganglionic neurones. Such alterations of the excitatory/ inhibitory balance has been noted and documented elsewhere in the central nervous system (Majdi et al., 2007, Casu et al., 2002, Palomba et al., 2008). As for the DLN itself, (Ranson et al., 2007a) documented age-related increases in acetylcholine terminal boutons but decreased in serotoninergic inputs have been observed in aged rats (Ranson et al., 2003a). In the bladder sympathetic preganglionic neurones, decrease in glutamate-immunoreactive and serotonin-immunoreactive boutons were noted (Ranson et al., 2003a, Santer et al., 2002). Our findings also concur with study on GABA/glycine inputs to sympathetic where the inhibitory inputs were unchanged (Santer et al., 2002). In the bladder parasympathetic nucleus, study on the serotoninergic inputs reveals that they were unchanged in ageing (Ranson et al., 2003a). The current

findings and all the previous studies which have shown age-related changes to the synaptic inputs to the bladder and EUS neurones are summarised in Figure 6.1.

As our understanding of the spinal control of the LUT increase, the more likely we are to understand the causes of the disorders where voluntary control of the LUT is lost and find ways to treat these dysfunctions from which so many humans suffer. The results in this thesis and previous studies suggest potential treatments. Since there is a net loss of excitatory terminals onto continence promoting neurones enhancing excitation may be one strategy. One such compound, a so-called cognitive enhancer that positively modulates AMPA receptors, was tested in these studies. Unfortunately, no significant outcomes were detected, for reason discussed in chapter 5. Other potential treatments could, for example, include serotonin receptor modulation since Ranson et.al (2003) reported a decrease in serotoninergic inputs onto continence promoting neurones. Indeed, perhaps combination therapies of both may lead to symptom relief of these patients.

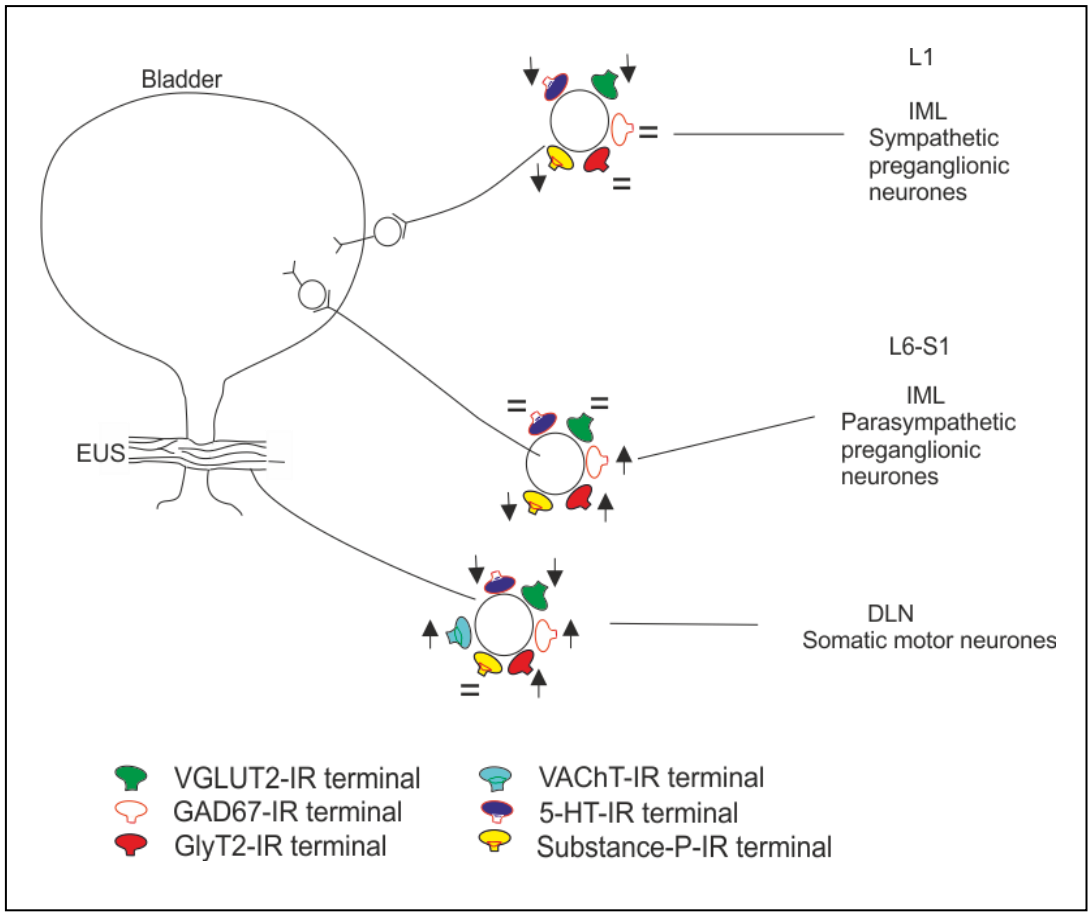

Figure 6.1 Schematic diagram of the changes in the inputs onto DLN and autonomic preganglionic neurones during ageing.

6.2 Potential mechanisms of changes in presynaptic terminals

From the observations on the inputs of synaptic terminals in different spinal nuclei controlling micturition and continence, it is noted that the pattern of terminal axosomatic innervations varies according to neurotransmitter-specific terminals as well as differences in neuronal subtypes. For instance, it was found that glutamatergic terminals were reduced in DLN and onto sympathetic preganglionic neurones, but not those opposing the parasympathetic preganglionic neurones. Two fundamental questions arise. First, what drives the changes in synaptic inputs and what are the mechanisms that underlie the axonal pruning and synaptic

elimination in ageing? Secondly, why are these changes region-specific being prominent in one area but not in the other site of the CNS?

A recent development to potentially explain loss of specific synapses is the discovery of the role of complement mediated immuno-inflammation in ageing CNS. Recent work suggests that complement proteins localized to presynaptic terminals leads to synaptic elimination in ageing. In a study by (Stephan et al., 2013), they investigated the role of C1q in the cognitive decline of normal brain aging. C1q is the initiator protein of the classical complement cascade and plays a critical point of regulation in this pathway. Binding of C1q to cell membranes triggers a proteolytic cascade of complement proteins, resulting in C3 opsonisation or tagging and phagocytosis by macrophages. (Stephan et al., 2013) used immunohistochemistry to show in the aging brain that C1q protein levels dramatically increase up to 300-fold and was localized in close proximity to synapses. Moreover, aged C1q-deficient mice exhibited significantly less cognitive and memory decline in certain hippocampus-dependent behaviour tests compared with their wild-type littermates. Since the C1q-immunoreactivity was detected inside most or all microglia, they suggest accumulation of C1q protein released by microglia onto perisynaptic sites may mediates synapse elimination in the ageing CNS. Interestingly, C1q was also localised to selective populations of inhibitory interneurones in the hippocampus.

A similar observation has been documented in a developmental study of the mouse retinogeniculate system, where C1q and C3 localized to retinogeniculate synapses. The presynaptic terminals of retinal ganglion cells are eliminated by phagocytic microglia expressing complement receptors (Stevens et al., 2007). Thus, the microglia is implicated for mediating clearance of unused or weak synapses during early postnatal synaptic pruning. Besides that, synapse alteration and elimination

are both often observed in the early stages of chronic neurodegenerative disorders, such as Alzheimer's disease (Selkoe, 2002), multiple sclerosis (Michailidou et al., 2015) and ocular neuropathies glaucoma (Rosen and Stevens, 2010). Glaucomas are also characterized by extensive synapse elimination that precedes the subsequent death of retinal ganglion cells and progressively leads to vision loss. C1q expression colocalised with adult retinal synapses at early stages of glaucoma in DBA/2J mice (Stevens et al., 2007). Complement mediated synaptic elimination is probably one of the underlying mechanisms involved in this process.

Another complement, the C3 has also been studied in relation to normal ageing brain. (Shi et al., 2015) examined behaviour as well as electrophysiological and synaptic changes in the brains of C3-deficient mice compared with wild-type mice at postnatal day 30, 4 months, and 16 months of age and found that region-specific and age-dependent synapse loss in aged WT mice that was not observed in C3 knockout mice. They also observed that age-dependent neuron loss in hippocampal CA3 that followed synapse loss in aged WT mice, neither of which were observed in aged C3 knockout mice, suggesting a prominent role for complement protein C3 in mediating aged-related and region-specific changes in synaptic function and plasticity in the aging brain.

Thus, the growing evidence points to phagocytic elimination of synapses mediated by complement C1q and C3 that is detrimental to synapses during aging. However, the extent of synaptic pruning throughout life especially in ageing still needs to be addressed. Furthermore, various brain and spinal cord regions display different vulnerability with respect to structural and neurochemical changes during ageing. What drives these differences in selective age-related vulnerability during ageing is still largely unknown. In this respect, it is interesting to note that C1q was expressed

and potentially released by inhibitory terminals in the hippocampus (Stephan et al., 2013). If this was the case in the spinal circuits investigated here, it could provide a potential explanation for the selective reduction of excitatory terminals. Inhibition of these complement pathways could therefore provide a strategy for future therapies. Future investigations could therefore perform micturition analysis and synaptology of spinal micturition circuitry in mice in which relevant complement proteins have been knocked out.

Summary

In summary, for the first time this study established the pattern of micturition in mice and provides a neuroanatomical report regarding the presynaptic inputs and the excitatory: inhibitory balance onto the DLN, sympathetic and parasympathetic preganglionic neurones innervating the bladder and EUS in different age groups. This study confirms that sphincter motor neurones in mouse reside in spinal DLN and form a solid anatomical basis for further synaptology studies. Behavioural study in mice reveals that aged mice had increase frequency of nocturnal micturition and mimic incontinence in elderly human. The comparison of excitatory and inhibitory presynaptic terminals on the spinal nucleus controlling micturition and continence demonstrate that net excitatory inputs is reduced while net inhibitory is increased, affecting the ratio between neuronal excitation and inhibition. Although administration of a dose-specific AMPA receptor potentiator did not reverse the high frequency pattern in the aged mice, more experiments will be required to determine if this was related to dosage of the drug. Overall findings suggest and propose that an underlying mechanism is the change in the balance of excitatory – inhibitory inputs on these motor neurones. These may contribute to an explanation of the pathophysiology of incontinence that occurred in the ageing population.

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