|  |
| --- |
| Chapter 7 |
| General discussion |
| A discussion of the experimental findings in this thesis and future avenues for investigation. |

As each chapter in this thesis includes a detailed discussion of the data presented in the chapter, this discussion is only intended to assemble the major findings of this work, and summarise limitations and possible experiments that could be performed to consolidate and further these findings.

# 7.1 Major findings

This thesis describes experimental work supporting two major experimental findings:-

1. Mediators released from the urothelium directly affect the afferent nerve firing in response to distension in the mouse bladder
2. The K+ sensitivity test currently used in the clinical diagnosis of interstitial cystitis may provide evidence of altered urothelial release of nitric oxide aside from indicating compromised barrier function.

## Mechanosensitivity responses are altered by altered signalling from the urothelium.

Mechanosensitivity, the ability of afferent nerve fibres of the bladder to sense mechanical change, is a crucial component of the micturition reflex. Afferent nerves from components of the lower urinary tract, including both bladder and urethra, constantly transport mechanosensory information to the spinal cord and higher brain regions regarding the degree of distension of the bladder. This signalling is essential in the generation of voiding reflexes, as shown by patients with spinal cord lesions or in spinal cord injured laboratory animals.

Whilst no doubt important, the mechanisms governing the mechanosensory ability of the bladder are unclear. Evidence suggests that the urothelium plays an important role in mechanosensitivity by the release of mediators such as ATP ([Burnstock, 1999](#_ENREF_2); [Ferguson, 1999](#_ENREF_5); [Ferguson *et al.*, 1997](#_ENREF_6)), which directly stimulate purinergic receptors on underlying afferent nerve terminals and initiate an increase in afferent nerve discharge, a mechanism that has shown to be upregulated and proposed as the reason for enhanced pain sensation in patients with interstitial cystitis and painful bladder syndrome ([Kumar *et al.*, 2007](#_ENREF_10)). Similarly, afferent nerve fibres have been shown release mediators in order to communicate with the urothelium and bladder detrusor in order to modulate bladder function. Thus, the model of the ‘sensory web’ was proposed, whereby it was hypothesised that the urothelium and underlying nerve terminals (both afferent and efferent) were involved in cross-talk between these structures, in order to modulate or refine bladder activity ([Apodaca *et al.*, 2007](#_ENREF_1)).

The work in this thesis has investigated the effects of signalling from the urothelium on mechanosensitivity, and has provided evidence to conclude that indeed the urothelium has the ability to release mediators that alter mechanosensitivity responses directly, in this model.

Firstly, data in this thesis showed that inhibition of mediator release from the urothelium by using Ca2+ free Krebs solution caused an increase in the afferent nerve response to ramp distension. Although the possibility that these changes occurred due to a change in Ca2+ regulation could not be ruled out, further experimentation showed that the increased activity was unlikely due to changes in muscle activity of the detrusor, or to changes in ATP breakdown by the reduced activity of ecto-ATPases.

This provided the first piece of evidence in this thesis to support the role of the urothelium in moderating afferent nerve sensitivity, that **inhibition of mediator release from the urothelium directly altered the mechanosensitivity response, causing an augmentation of firing, suggesting that the urothelium is important in releasing mediators that inhibit mechanosensitivity.** Taken with later evidence obtained in further experiments in this thesis, these experiments were also vital in determining the identity of the NO enzymatic pathway responsible for the effect of high K+ on afferent nerve activity in response to distension, as the synthesis and activity of epithelial nitric oxide synthase (eNOS) is dependent on Ca2+.

Secondly, data from high K+ experiments showed that stimulation of the urothelium to induce mediator release resulted in attenuation of the control mechanosensitivity response. This response was attenuated in preparations in which the urothelium was damaged by protamine sulphate administration, providing evidence that the mediator responsible was urothelially released. These experiments demonstrated that **stimulation of mediator release from the urothelium directly altered the mechanosensitivity response, causing an attenuation of afferent nerve firing, further supporting the hypothesis that the urothelium releases mediators that downregulate mechanosensitivity.**

Finally, application of a NO inhibitor blocked the inhibition of mechanosensitivity stimulated by high K+ solution administration. This provided evidence to suggest an alternative explanation for the mechanism by which there is pain sensation following administration of high K+ solution in clinical examination. In the clinic, instillation of the bladder with K+ solution has been used as a diagnostic tool in the diagnosis of interstitial cystitis. Patients with an intact, undamaged urothelium perceive no painful sensation during the examination, whereas patients with interstitial cystitis report symptoms of pain and urgency. This was previously proposed to be as a result of diminished urothelial barrier function, thereby allowing the passage of K+ ions across the damaged urothelium and causing depolarisation of afferent nerve endings, and consequently increased sensory nerve activity, and painful sensation. However, the data in this thesis suggests that these clinical observations could be due to the attenuated release of NO (via eNOS pathway) in the bladders of patients with interstitial cystitis, due to a compromised urothelial function and site for NO synthesis, rather than purely as an indication of decreased barrier function.

This data also suggests that there may be a therapeutic potential of the NO pathway specifically via exploration of the eNOS pathway, in the replacement of NO following urothelial damage in interstitial cystitis.

## Compliance and baseline afferent nerve activity

The purpose of the performed experiments was to primarily investigate the effects of urothelial signalling in modulating afferent nerve activity; however other parameters could also be measured in the preparation used.

Bladder compliance was measured primarily to ensure that the observed effects on mechanosensitivity in various experiments were not secondary to a change in detrusor muscle compliance. Incidentally, if the primary line of investigation was to investigate the effects of various compounds on muscle activity, strain gauge preparations or flat-sheet preparations as used previously ([Zagorodnyuk *et al.*, 2009](#_ENREF_16); [Zagorodnyuk *et al.*, 2007](#_ENREF_17)) would have allowed much greater insight into the effects on muscle activity.

The afferent nerve activity between successive bladder distensions was, as previously described, highly variable between preparations. Furthermore, the origin of this firing remains unclear. However, it was important to discount this activity from the mechanosensitivity response, to separate afferent nerve firing at rest from the distension evoked afferent nerve response.

The afferent nerve activity between distensions requires some attention, as many interesting observations were made throughout the course of these experiments in response to different pharmacological agents (as shown in this thesis), for example prolonged afferent nerve discharge following distension, etc. However the methodology used here was unable to provide firm conclusions regarding afferent nerve activity between distensions, and to identify the origin and modulation of this activity. Future experiments could be performed in this model, in which the bladder is not distended, nor continually perfused, thereby reducing the potential for after distension recovery responses or firing as a consequence of sensation of flow through the bladder by afferent nerve terminals.

## The use of an in vitro mouse model to investigate mechanosensitivity

The in vitro mouse bladder preparation used to generate the mechanosensitivity data in this thesis is extremely reliable and robust. Responses to bladder distension are consistent and reproducible over time, enabling comparisons to be made before and after pharmacological intervention. This preparation also offers the unique feature in that afferent nerve activity can be investigated at the level of the primary afferent, without input from efferent or higher brain controlled mechanisms as in other in vivo studies. Whilst this may equally be viewed as a disadvantage when considering the higher levels of control of the bladder in the whole physiological organism and understanding the many levels of control of bladder function, this preparation enables the activity of the peripheral afferent nerves involved in the micturition reflex to be studied in fine detail, and recorded directly, without interference from other control mechanisms or inference of afferent activity from cystometry.

Ideally an in vivo model which combines both voiding cystometry and afferent nerve recordings would be useful as in recent work([IIjima *et al.*, 2007](#_ENREF_9)), or the decerebrate arterially perfused rat preparation that enables the investigation of the complete filling and voiding cycles, both spontaneous and evoked, and the simultaneous recording of bladder intra-luminal pressure, external urinary sphincter–electromyogram, pelvic afferent nerve activity, pudendal motor activity and permits visualisation of the lower urinary tract during investigation ([Sadananda *et al.*, 2011](#_ENREF_14)).

The preparation used in this thesis also enabled a similar physiological stimulus to the stimulus in vivo to be applied to the bladder to elicit afferent nerve firing, rather than comparatively unphysiological stretching via a strain gauge or probing of the tissue as in other flat sheet preparations previously used ([Zagorodnyuk *et al.*, 2009](#_ENREF_16); [Zagorodnyuk *et al.*, 2007](#_ENREF_17)). Despite this, the parameters used in the distension protocol were rather non-physiological, and this is an important consideration as the conclusions regarding mechanosensitivity in this thesis were based on these distension experiments. Previous cystometry in male mice has suggested that the normal bladder filling rat in a wildtype mouse is approximately 36µl/min, which is considerably lower than the 100µl/min rate used to distend the bladder in these experiments ([Igawa *et al.*, 2004](#_ENREF_8)). Furthermore, the high filling rate used rapidly distended the bladder, causing intraluminal pressure to rise quickly, and it is unlikely that this fast increase in intraluminal pressure is comparable with the situation in vivo. In addition, the maximum intraluminal pressure for distension was in the noxious range (50mmHg), in order to activate both Aδ and C fibre afferent nerves. Despite this, previous studies using similar distension protocols have displayed reproducible and relevant results at these parameters and higher, ([Daly *et al.*, 2010](#_ENREF_3); [Daly *et al.*, 2007](#_ENREF_4); [Moss *et al.*, 1997](#_ENREF_11); [Rong *et al.*, 2002](#_ENREF_12); [Rong *et al.*, 2007](#_ENREF_13); [Shea *et al.*, 2000](#_ENREF_15)) therefore these parameters were chosen in this study so as to be able to directly compare these findings with previous work in the field. Unfortunately, using the distension method used in this thesis it is difficult to establish whether it is the change in pressure or the change in volume that causes activation of the afferent nerve fibres, as both are altered simultaneously during distension.

# Further study, clinical perspectives and pharmaceutical opportunities

As is the beauty of all scientific research, there are a plethora of interesting avenues to be explored, and for as many answers that have been provided by the data from the research in this thesis, a multitude of experiments for further investigation have been introduced. Specific suggestions for improvements to methods and limitations of the data have been discussed in each chapter, however, some of the particularly important avenues outstanding for experimentation have been highlighted below.

Whilst there are no doubt innumerable mechanisms that could influence afferent nerve firing and influence mechanosensitivity, the data from this thesis suggests that further investigation of mediator release pathways from the urothelium could yield exciting results and provide greater understanding of mechanisms underlying the maintenance of the healthy bladder and the development of bladder pathology and, importantly, offer new avenues for development of therapeutics for bladder disorders.

As previously described, spontaneous micro-motions of the bladder and simultaneous bursts of afferent nerve firing were observed in some preparations, but as this activity was irregular, and spontaneous, quantification of the response was difficult. It would be interesting to develop a technique to investigate this activity, for example a similar recording set up that could measure intraluminal pressure more sensitively alongside afferent nerve activity, with a protocol devoid of ramp distensions to avoid any interference from the after effects of distension.

Further exploration of the urothelial NO pathway in modulating afferent nerve sensitivity would also be interesting if more time had been available. Firstly, it would be interesting to investigate whether exogenous application of NO donors in protamine sulphate treated bladders could attenuate the augmentation in firing in response to high K+ stimulation. Similarly, in Ca2+ free Krebs experiments it is hypothesised that exogenous application of an NO donor could again attenuate the augmentation in the afferent nerve response to bladder distension.

If more time had been available, NO release assays could have been performed to quantify the release of NO stimulated by each of the high K+ solutions. This release could be measured from both urothelial and mucosal surfaces, and thereby contribute to the strength of the evidence for the K+ stimulated release of urothelial NO demonstrated by experiments in this thesis. This could however prove challenging, as the half-life of NO is extremely small, making measurement of release more difficult.

In particular, time permitting, it would have been extremely interesting to have performed another series of experiments, using a similar method adopted previously in experiments investigating the properties of the urothelial derived inhibitory factor ([Hawthorn *et al.*, 2000](#_ENREF_7)). In these experiments, contractions of a urothelial-denuded muscle strip were inhibited by the presence of a second, intact, bladder strip in the same recording chamber. As the experiments in this thesis have shown that urothelial release of NO can affect bladder afferent nerve activity, it would be interesting to investigate whether the augmentation of firing following high K+ stimulation in the protamine sulphate damaged bladder could be attenuated by the presence of an intact bladder strip. To ensure a high concentration of release, porcine bladder strips could be used, or instead, supernatant from porcine urothelium. However, the exact methodology needs refining and considering more carefully.

It is also important at this stage to acknowledge the limitations of investigating only a tiny proportion of the entire bladder jigsaw as understanding the lower urinary tract , spinal and higher centre control of micturition will with no doubt be required for progression of knowledge. Incidentally bladder responses in the in vivo model with an intact efferent supply may be expected to behave differently, therefore it would also have been beneficial, with further time and resources available to have repeated some of these experiments in an in vivo preparation.

In the wider context of the bladder research field, the impression is given that research should focus in particular on the diagnostic procedures of clinical intervention of bladder disorders, as there is a limit to the usefulness of bladder diaries and urodynamics. It would be interesting to investigate the composition of the urine from patients with different types of bladder dysfunction to ascertain whether the urine contains any mediators that could be measured as biomarkers for disease.

Further understanding of the modulation of sensory pathways by urothelial signalling could offer new insight into the mechanisms of control and fine-tuning of afferent sensitivity in the bladder, potentially offering an exciting new direction for therapy of bladder dysfunction.

Apodaca, G., Balestreire, E., & Birder, L. A. (2007). The Uroepithelial-associated sensory web. *Kidney International, 72*, 1057-1064.

Burnstock, G. (1999). Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. *J Anat, 194 ( Pt 3)*, 335-342.

Daly, D., Chess-Williams, R., Chapple, C., & Grundy, D. (2010). The inhibitory role of acetylcholine and muscarinic receptors in bladder afferent activity. *European Urology, 58*, 22-28.

Daly, D., Rong, W., Chess-Williams, R., Chapple, C. R., & Grundy, D. (2007). Bladder afferent sensitivity in wildtype and TRPV1 knockout mice. *The Journal of Physiology, 583*, 663-674.

Ferguson, D. R. (1999). Urothelial function. *BJU Int, 84*(3), 235-242.

Ferguson, D. R., Kennedy, I., & Burton, T. J. (1997). ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes - a possible sensory mechanism? *Journal of Physiology, 505*(2), 503-511.

Hawthorn, M. H., Chapple, C. R., Cock, M., & Chess-Williams, R. (2000). Urothelium-derived inhibitory factor(s) influences on detrusor muscle contractility in vitro. *British Journal of Pharmacology, 129*(3), 416-419.

Igawa, Y., Zhang, X., Nishizawa, O., Umeda, M., Iwata, A., Taketo, M. M., Manabe, T., Matsui, M., & Andersson, K. E. (2004). Cystometric findings in mice lacking muscarinic M2 or M3 receptors. *Journal of Urology, 172*, 2460-2464.

IIjima, K., de Wachter, S., & Wyndaele, J. (2007). Effects of the M3 receptor selective muscarinic antagonist Darifenacin on bladder afferent activity of the rat pelvic nerve. *European Urology, 52*, 842-849.

Kumar, V., Chapple, C. R., Surprenant, A., & Chess-Williams, R. (2007). Enhanced ATP release from the urothelium of patients with painful bladder syndrome: A possible pathphysiological explanantion. *The Journal of Urology, 178*(4), 1533-1536.

Moss, N. G., Wallace Harrington, W., & Tucker, S. (1997). Pressure, volume, and chemosensitivity in afferent innervation of urinary bladder in rats. *American journal of physiology, 272*, 695-703.

Rong, W., Spyer, K., & Burnstock, G. (2002). Activation and sensitisation of low and high threshold afferent fibres mediated by P2X receptors in the mouse urinary bladder. *The Journal of Physiology, 541*, 591-600.

Rong, W., Winchester, W. J., & Grundy, D. (2007). Spontaneous hypersensitivity in mesenteric afferent nerves of mice deficient in the sst2 subtype of somatostatin receptor. *Journal of Physiology, 581*(2), 779-786.

Sadananda, P., Drake, M. J., Paton, J. F. R., & Pickering, A. E. (2011). An exploration of the control of micturition using a novel in situ arterially perfused rat preparation. *Frontiers in Neuroscience, 5*(62).

Shea, V. K., Cai, R., Crepps, B., Mason, J. L., & Perl, E. R. (2000). Sensory fibers of the pelvic nerve innervating the rat's urinary bladder. *The Journal of Neurophysiology, 84*, 1924-1933.

Zagorodnyuk, V. P., Brookes, S. J. H., Spencer, N. J., & Gregory, S. (2009). Mechanotransduction and chemosensitivity of two major classes of bladder afferents with endings in the vicinity to the urothelium. *The Journal of Physiology, 587*, 3523-3538.

Zagorodnyuk, V. P., Gibbins, I. L., Costa, M., Brookes, S. J. H., & Gregory, S. (2007). Properties of the major classes of mechanoreceptors in the guinea pig bladder. *Journal of Physiology, 585*, 147-163.