Ketamine-induced bladder dysfunction

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Abstract

Introduction: Ketamine is a non-competitive antagonist that acts on N-methyl-D-aspartate (NMDA) receptors in the brain and has been used as an anaesthetic agent since the 1960s. It has more recently become popular as a party drug due to it's hallucinogenic effects, however ketamine abusers can develop significant lower urinary tract symptoms with associated damage to the lining of the bladder that can be difficult to treat and can be referred to as ketamine-induced cystitis (KIC).

Aims: i) To explore the neurogenic effects of ketamine, ii) To analyse histological features of ketamine cystitis specimens, iii) To contribute to existing preliminary evidence urothelial cells that express cKit are absent from ketamine-cystitis specimens.

Methods: Immunolabelling of KIC specimens was performed to study specific biomarkers that included nerve growth factor receptor (NGFR), nerve filament protein (NFP), smooth muscle actin (SMA), vimentin, and cKit. Neuronal cell lines (B104 and LAN-5) were used to establish in vitro bioassays to study the effects of ketamine and NGF on neurite outgrowth. Normal human urothelial (NHU) cells were cultured in vitro and treated with ketamine to assess cKit protein expression using both RT-PCR and western blotting.

Results: NGFR was seen to be upregulated in both urothelium and stroma of KIC specimens. SMA was expressed in urothelial cells however this was not seen in correlation with vimentin. cKit was absent in some KIC specimens, corroborated with decreased protein expression in vitro, however cKit transcript was upregulated in ketamine treated NHU cells. Neuronal cells treated with ketamine showed an increase in neurite outgrowth over the control ($p<0.05$).

Conclusions: Upregulation of NGFR appears to be present in KIC specimens and could explain painful bladder symptoms. Whether this is caused directly or indirectly by ketamine has yet to be established. There is an absence of cKit expressing cells in the urothelium from some KIC specimens which requires further investigation.

Contents

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Author's declaration

I hereby certify that I am the sole author of this thesis, and that this is my own work. I declare that this is a true copy of my thesis. All references have been referred to where appropriate, and acknowledgements made to individuals who have provided materials, guidance and support.

1. Introduction

1.1 The urothelium

The urinary bladder and associated urinary tract are lined by urothelium, an epithelium that is highly specialized to facilitate changes in bladder volume and provide a permeability barrier to urine [(1)]. The urothelium is a transitional epithelium and displays a regular stratified architecture, increasing in morphological complexity and differentiation from basal cells, through a variable number of intermediate cells, to the highly differentiated superficial or umbrella cells at the luminal surface[(2)].

The superficial cell layer is primarily responsible for providing the permeability barrier[(3)]; these cells are interconnected by tight junctional complexes, which restrict paracellular ion transport and polarize the cell by limiting diffusion of transport proteins between the apical and basolateral membranes[(4)]. Superficial cells show a unique specialization of the apical plasma membrane, with thickened plaques of asymmetric unit membrane (AUM) on the luminal surface[(5)]. These plaques are composed of a number of component proteins, the uroplakins (UPKs)[(6)], which can be used as objective markers of terminal urothelial cytodifferentiation in many species, including man[(7)]. AUM plays a critical role in limiting transcellular permeability as demonstrated by UPKIIIa deficiency in the transgenic mouse that consequently developed an impaired urothelial water permeability barrier in association with incomplete plaque formation[(8,6)].

Although the uroplakins play a key role in establishing the transcellular permeability barrier for water and possibly some other polar substances, they are not implicated in the formation of the tight junctional complexes that form the paracellular barrier. The relative tightness of tight junctional complexes is associated with the claudins, a family of approximately 24 proteins which form the primary seal-forming fibrils of the tight junction. In the urothelium, the claudins show a differentiation stage-related pattern of expression, with claudin 7 expressed in all but the superficial layer of the urothelium. Claudins 4 and 5 are expressed at the basolateral junctions of superficial cells and claudin 3 is restricted to the joining points at the terminal tight junction between adjacent superficial cells[(9)].

Other differentiation biomarkers of urothelial cells have been identified such as the cytokeratins(CK) with superficial cells marked by expression of CK20, whereas basal cells are marked by expression of CK 5 and 17. In normal urothelium CK20, as a marker of terminal differentiation, is typically expressed in the superficial urothelial cells. In urothelial dysplasia however, CK20 is not restricted to the superficial but can be seen in all layers of the urothelium. Harnden et al showed that normal localisation of CK20 in biopsies from patients with newly diagnosed superficial urothelial papillomas (pTa) can be informative as an indicator for determining non-recurrent disease[10,11].

Preliminary observations at the Jack Birch Unit at the University of York have identified a rare and distinct cKit expressing urothelial cell population in normal human urothelium. This sub-population of cells shows a urothelial (CK+) phenotype but is distinct from CD45+ leucocytes and mast cells that would typically express cKit. The cKit-expressing cells can be located in any one of either the superficial, intermediate, or basal layer of the urothelium, and appear to show a pedicle that connects the cell to the basement membrane. Furthermore, studies of poorly differentiated urothelial carcinoma cell lines suggested an absence of cKit expression, the reasons for which remain unclear. The implications of these findings need to be expanded upon, with the need for further evidence to explore the possible role of cKit positive cells in the urothelium, not only in normal tissue, but also in disease.

Bladder pathology

The urothelium has been proposed to have a sensory role with the transmission of information such as bladder fullness between the mucosa and its underlying nerve and muscle tissues of the bladder being proposed[12,13]. Sensory afferent and efferent nerve processes that innervate the urothelium, and underlying tissues that include a suburothelial layer of myofibroblasts and the detrusor muscle are thought to be key to this sensory role and play a vital role in co-ordinated bladder function[14]. Myofibroblasts arise from fibroblasts that undergo differentiation into smooth muscle-like cells – care should be taken when interpreting histological findings as although these cells can be a found in normal suburothelial tissue, upregulation may indicate some level of fibrosis or scarring. They are extensively linked by gap junctions, have close contact with nerves, and may serve as signaling intermediaries between the urothelium and nerve fibres[15]. Communication between the tissues that comprise the

bladder ensures the proper function of the organ and may provide a mechanism for the urothelium to mediate the spontaneous activity of the smooth muscle or muscle contraction[16,17,18]. As a biomarker therefore, smooth muscle actin may be useful in the characterisation of bladder specimens and to explore the implications of any observations made.

Dysregulation of the sensory function may be important in the pathogenesis of dysfunctional bladder syndromes, such as interstitial cystitis(IC)[19]. IC is a chronic inflammatory disorder of the urinary bladder that manifests as urinary frequency and urgency with bladder pain, in the apparent absence of any infectious agent. The aetiology remains poorly understood, although one hypothesis is that a compromised urothelial barrier is a feature of the disease; whether this is due to an inherent dysfunction of the urothelium itself, or an indirect influence of local factors such as cytokines affecting urothelial differentiation and function remains uncertain[20]. A definitive treatment remains elusive[21,22].

More recognised causative factors that can induce inflammatory changes in the bladder include certain drugs. For example, severe cystitis has been reported in laboratory animals after cyclophosphamide administration[23,24] and in patients receiving the drug as part of their treatment [25]. Cyclophosphamide (CP) is a drug with a wide spectrum of clinical uses, and it has been proved to be effective in the treatment of cancer and nonmalignant disease. CP creates cross-links in DNA, resulting in strand breaks. However, despite preventative measures such as aggressive hydration and the use of the sulfhydryl-containing compound mesna [26], this drug may induce acute inflammation of the urinary bladder [27]. Systemic injection with CP is a widely used method of inducing experimental chemical cystitis in mice and rats. Haemorrhagic cystitis is a common complication of the use of this drug in patients with cancer, and it was shown that acrolein is responsible for the cystitis associated with CP [28,29]. Acrolein is a highly reactive aldehyde derived from precursors formed by the metabolism of CP in the liver and possibly the kidney and bladder, but the mechanism by which acrolein reaches the bladder is unclear[30]. The remainder of the urinary tract is relatively unaffected by CP, and its effect on the bladder has been attributed to the duration of contact with toxic concentrations of acrolein resulting from the urinary storage function of the bladder[28,29].

Animal studies have shown increased levels of NGF, NT-3 and BDNF mRNA in rat bladders in response to inflammatory stimulus after chemical irritation with turpentine, and have demonstrated that NGF was associated with sensory changes[31]. In human studies, raised urinary NGF and prostaglandin E2 (PGE2) have been found in patients with interstitial cystitis and painful bladder syndrome (PBS)[32,33,34,35], and one study has shown that chronic ketamine abusers have higher levels of serum BDNF when compared to healthy subjects[36], although the specific implications of this have not been studied further. The neurotrophins are peptides that play a crucial role in the development of the nervous system by determining phenotype-specific neuronal differentiation, and targeting innervation by directing the survival, development, and function of neurons [37,38]. Not only are neurotrophins important in neuronal function, but they have also been found to play an important role in tissue inflammation and repair. In particular, NGF has been found to be produced by several non-nervous tissue types including immune inflammatory cells, epithelial cells, and smooth muscle cells[39,40,41]. p75NGFR is a member of the tumour necrosis family and plays a dual role by transmitting both positive and negative signals that are important in cell survival and cell death[42]. Based on the apparent upregulation of NGFR in disease and after chemical induced damage, it remains unclear whether this suggests a regenerative response or is indicative of some other neurogenic response.

The potential local and systemic effects of drugs and their metabolites excreted through the urinary tract thus poses interesting questions, with particular relevance to ketamine, a drug that appears to cause a response in the bladder that equates to cystitis. Recent studies have suggested that there is a correlation between regular ketamine users or abusers and the development of urinary symptoms such as pain, frequency, nocturia, urgency, and haematuria [43]. On investigation patients are found to have poorly functioning, small capacity bladders associated with fibrosis, which can appear grossly inflamed at cystoscopy [44,45]. Preliminary immunohistochemical observations in ketamine cystitis specimens showed upregulation of p75NGFR in the urothelium and stroma, with the presence of densely positive stromal areas that on histological analysis with Clinical Pathologist Dr Jens Stahlschmidt at Leeds Teaching Hospitals NHS Trust, appeared to resemble nerve fibres, an observation that is not seen in normal urinary tract tissue. As described above, chemical irritation of the bladder has been shown to cause increased levels of neurotrophic factors thus leading to the question of whether

ketamine has neurostimulatory properties either directly or indirectly, and could contribute to the symptoms of pain.

1.2 Introduction to ketamine

1.2.1 History

Ketamine is a phencyclidine derivative that was developed at the Parke-Davis Laboratories USA in the 1960s. It is a non-competitive antagonist that acts on N-methyl-D-aspartate (NMDA) receptors in the brain that control synaptic plasticity and memory[46,47]. Ketamine creates a "dissociative anaesthesia" characterized by a separation between the thalamocortical and limbic systems, thus blocking sensory input such as pain, whilst maintaining vital reflexes such as blood pressure, pulse, and respiratory rate[48].

1.2.2 Medical use, pharmacokinetics, and bioavailability

Ketamine is used in both human and veterinary anaesthesia with doses ranging from 1-4.5mg/kg as an intravenous infusion, and also as an analgesic in chronic pain and palliation, with parenteral administration in the range 0.125–0.3 mg/kg/hr, or oral doses ranging from 50mg-1g daily[49,50]. Pain threshold elevation has been shown to occur at plasma ketamine concentrations above 160ng/ml. The pharmacokinetics of ketamine in analgesic doses after intravenous, intramuscular, and oral administration suggest a terminal half-life of 186 minutes and with a relatively short half-life this is deemed to be one of the benefits of ketamine. Absorption after intramuscular injection is rapid and has a high bioavailability at 93%, however only 17% of the oral dose is absorbed because of extensive first-pass metabolism in the liver[47]. The primary metabolic pathway involves hepatic N-demethylation via the cytochrome P450 system to form norketamine, a pharmacologically active metabolite with one-fifth to one third the anaesthetic potency of ketamine[51,52]. Norketamine is hydroxylated to form hydroxyl-norketamine which in turn can be conjugated to more water soluble derivatives and excreted through the urinary tract. Faecal excretion accounts for less than five percent of an injected dose of ketamine[53,54].

Another potential use of ketamine is within mental health subjects. Ketamine is thought to have antidepressant properties, with recent studies suggesting the antidepressant effect through the modulation of glutamatergic signaling[55-60]. In major depressive disorder (MDD), ketamine has shown reproducible antidepressant effects within a short

period of time in a condition that inherently carries a long and risky period of latency when treated with more conventional mono-aminergic antidepressants such venlafaxine[61]. The limitations to translation of this to clinical practice in humans are evident by the nature of ketamine's anaesthetic properties and its side effects thus making clinical trials in humans at this stage impossible, however the mechanism of action will potentially open new avenues to explore alternative therapies which are fast-acting and effective.

1.2.3 Side-effects and potential for abuse

The main disadvantage of ketamine as an anaesthetic is the high incidence of hallucinations, nightmares, and other transient psychotic effects, although these can be reduced by concurrent administration of benzodiazepines such as diazepam or midazolam. For reasons that are not fully understood, children suffer less from these side-effects and therefore ketamine is still regularly used in paediatric anaesthesia, but rarely used in adult anaesthesia. Within the last 20 years however, the side effects of ketamine, such as hallucinations and "near death experiences", have provoked its status as a recreational drug, popular amongst party-goers, and evidenced by a study looking at the profile of urine taken from individuals at a rave party in Taiwan that showed presence of ketamine in 47% of samples[62]. Ketamine abuse has also become more prevalent in the UK and goes by various different nicknames such as K, super K, Vitamin K, and special K, and carries a street value of approximately £21 per gram. The 2010/11 British Crime Survey, commissioned by the Home Office, showed an increase in reported use of the drug among those aged 16-24, and estimated that 300 000 people had used ketamine at least once, a figure that has risen from an estimated 140,000 users in 2007, after ketamine was added to the list of drugs surveyed following the drug's classification in 2006. It is currently controlled as a Class C drug under the Misuse of Drugs Act 1971 although there are regularly calls for re-classification of the drug into a higher class.

In addition to the psychogenic side effects of ketamine, evidence suggests a correlation between ketamine abuse and the development of urinary symptoms and histological change within the bladder, the histopathological mechanism of which remains poorly understood. One hypothesis is that the metabolites of ketamine are excreted in the urine and this may produce a directly damaging effect on the urothelium[45]. The contact time

of urine containing the hydroxyl-norketamines is obviously variable between individuals depending on the bladder capacity and voiding frequency, and this also may provide an insight into the route of exposure as has been shown with other chemical induced cystitis such as post-cyclophosphamide treatment. Although there is much in the literature about clinical presentation, clinical findings and the aetiology of ketamine abuse [44,45,63-65], the actual pathophysiological mechanism of urothelial damage remains poorly understood. Current pharmacotherapies such as anticholinergics have little benefit on symptoms, and although cessation of ketamine use can improve symptoms, in many instances the damage to the urinary tract is irreversible and may require major surgery. This means removing the bladder (cystectomy) with creation of a "neobladder" using a section of the small intestine.

1.2.4 Histopathological features of ketamine cystitis

To date, few groups have studied the histological features of human bladder specimens exposed to ketamine. Oxley et al[66] have published work on human bladder specimens exposed to ketamine and revealed mucosal ulceration with urothelial denudation, and urothelial atypia that may resemble carcinoma-in-situ (CIS). High levels of p53 and Ki67 expression were described that supported a malignant transformation event, however, the absence of Cytokeratin 20 (CK20) expression was taken by the authors as indicative of a benign pathology even though in fact loss of expression was originally described as non-informative[10]. One can only speculate about whether CK20 was absent due to loss of superficial urothelium, or that ketamine had induced changes leading to the down regulation of CK20. This illustrates the importance of clear communication between surgeons and pathologists to highlight any history of ketamine use in order to prevent a mis-diagnosis of CIS, the management of which is very different to that of ketamine-induced change.

1.2.5 In vitro studies with ketamine

In vitro studies using cortical neurons from monkeys showed that ketamine induced apoptosis is associated with an increase in translocated transcription factor NF-kB, down-regulation of PSA-NCAM (neuronal specific marker), and upregulation of NMDA receptors. The authors suggest that the upregulation of NMDA receptors is vital in the role of ketamine–induced apoptosis, and this is based on evidence that when the

synthesis of NR1, a key subunit protein of the NMDA receptor, is blocked, neuronal cell death is reduced[67].

Takadera et al[68] have shown similar evidence of NMDA receptor involvement in rat cortical neurons, whereby ketamine-induced cell death could be blocked using an agonist of the NMDA receptor. The urinary tract of rats (full thickness, including muscles and nerves) has been assessed and the study found evidence of NMDA receptor expression in the lower urogenital tract[69], although the specific cell type that expressed NMDA receptors was not identified. This raises the question whether the damage induced by ketamine exposure in the urinary bladder is a consequence of apoptosis mediated via NMDA receptor expression in the urothelium. Recent unpublished work by the Jack Birch Unit at the University of York using an in vitro human urothelial cell model has revealed ketamine-induced cell death in urothelial cells but showed that urothelial cells do not express the NMDA receptor. These findings suggest that ketamine must be acting in an NMDA receptor independent manner, with an alternative and as yet poorly understood mechanism causing damage to the urinary bladder. One can only speculate whether it was rat urothelial cells that expressed NMDA receptors, or that a difference exists between the human and the rat model.

Braun et al[70] suggested that ketamine induces apoptosis in human lymphocytes and neuronal cells independently of the NMDA receptor based on their findings from in vitro studies and the recognition that the apoptosis-inducing effect of ketamine is not stereospecific, thus providing further evidence that the mechanism of damage from ketamine in the urinary tract is independent of the NMDA receptor.

2. Aims

In summary, ketamine abuse has been shown to lead to cystitis with long-term and potentially irreversible damage to the bladder. NMDA receptors have been shown not to be expressed by the urothelium in humans, thus requiring an alternative explanation for the damage seen. Preliminary observations have suggested upregulation of p75NGFR expression in ketamine cystitis tissue specimens, and a loss of cKit expression in the urothelium. Based on the hypothesis that ketamine is inducing these changes either directly or indirectly, the aims of this study are:

i) To explore the neurogenic effects of ketamine, and test the hypothesis that NHU cells can be induced to produce neurogenic factors when exposed to ketamine

ii) To analyse the histological features of ketamine-cystitis specimens compared with normal urinary tract specimens, and relate these findings to the hypothesis that neurogenic stimulation is a contributory factor to the symptoms in ketamine-cystitis

iii) To extend preliminary observations that a sub-population of urothelial cells that express cKit are potentially absent from ketamine-cystitis specimens by examining the expression of c-kit on ketamine-treated NHU cells cultured in vitro

3. Objectives

Experimental approach

Previous studies have used neuronal cell lines such as LAN-5 and PC12 to assess the response to factors such as NGF [71,72]. Cell types such as differentiated adipose derived stem cells (ASCs), regardless of fat source, have been shown to secrete higher levels of NGF and BDNF than undifferentiated ASCs and cause increased neurite outgrowth in co-culture bioassays with dorsal root ganglia neurons[73]. Other studies have used similar bioassays with co-culture of neuronal cells, with the measurement of neurite outgrowth as a marker for neurogenic stimulation; elevated levels of BDNF and NGF were shown suggesting that these neurotrophic factors secreted from the co-culture cells (mesenchymal stem cells) that were stimulating neurite outgrowth[74]. PC12 is a cell line derived from a phaeochromocytoma of the rat adrenal medulla and cells terminally differentiate when treated with nerve growth factor[72,75]. Certain neuroblastoma cell lines would appear therefore to provide a suitable model for measuring response to neurotrophin factors. Retinoic acid and cAMP are established as neuronal inducing agents[76,77], and could feasibly be used as controlling factors for testing against other agents, such as ketamine. Therefore, the first objective of this study was to establish an in vitro cell model to study the effects of ketamine on neuronal cells, and in the second instance to consider a co-culture bioassay model with urothelial cells and neuronal cells that would aim to test the hypothesis that urothelial cells can be induced to produce neurotrophic factors after exposure to ketamine.

The experimental objectives were:

i) Use neuronal cells as a measure of potential neurogenesis to test the effects of a conditioned medium removed from ketamine-treated NHU cells

ii) Examine both normal and ketamine-cystitis specimens to identify and describe the expression of specific biomarkers. These will include nerve growth factor receptor (NGFR), nerve filament protein (NFP), smooth muscle actin (SMA), vimentin, and cKit

iii) Culture NHU cells in vitro to study the effects of ketamine on the expression of cKit, and compare results with the immunohistological findings

4. *Methodology*

4.1 Histology

4.1.1 Obtaining tissue for research purposes

Research Ethics Committee (REC) approval was obtained to remove specimens of bladder. All patients gave written fully informed consent for their tissues to be used for research. In most cases the specimens of bladder were taken at the time of cystoscopy as part of their investigations, having presented with urinary tract symptoms and a history of ketamine abuse. These samples of bladder were fixed in 10% (v/v) formalin in PBS, dehydrated through graded alcohols and embedded in paraffin. Normal ureter or bladder specimens were used as control tissue. Five micro-meter sections were cut using a microtome and tissue sections were de-waxed in xylene and rehydrated through graded alcohol to water.

4.1.2 Immunohistochemistry

Immunohistochemistry was performed on the tissue sections using the streptavidin/biotin Complex/Horseradish Peroxidase system from Dako Cytomation (Ely, UK. Endogenous peroxidase activity was blocked with hydrogen peroxidase for 10 minutes before washing under running water for a further 10 minutes. Sections underwent an antigen retrieval process to retrieve antigens lost during primary tissue processing. This involved one of the following processes depending on the optimal conditions for that specific antigen: 1. boiled for 13 minutes in 10mM citric acid buffer, pH 6.0, 2. Incubated at 37^0C for 10mins with 0.1% trypsin solution (0.1g Trypsin dissolved in 0.1% $CaCl_2$ pH 7.8(w/v), 3. Combined trypsinisation followed by microwave in 10mM citric acid buffer pH6.0 for 13 minutes. A 10% serum from the host secondary antibody used was added for 5 minutes to block any non-specific reaction. The primary antibody was added to the slides and left to incubate at 4^0 Celsius overnight (see table 1 for list of primary antibodies used). Slides were then washed, incubated in biotinylated secondary antibodies and a streptavidin biotin horseradish peroxidase complex (Dako Cytomation) and visualized using a diaminobenzidine substrate reaction (Sigma-Aldrich Ltd). After sections were counterstained with

haematoxylin, they were dehydrated and mounted in DPX (Sigma-Aldrich Ltd). Cover slips were applied and the slides allowed to dry. Negative and positive antibody specific controls were included in all experiments. Positive control specimens used same host antibodies, for example Cytokeratin 7 was used as a positive control for mouse antibodies, HNF 3α was used for goat antibodies, and laminin was used for rabbit antibodies. Normal human ureter specimens were used as comparison for pattern of immunolabelling. Slides were visualised under brightfield conditions using an Olympus microscope with x10, x20, x40, and x60 objectives.

Antibody	**Clone**	**Storage**	**Host**	**Titration and Antigen retrieval method**	**Manufacturer**
NGFR	7F10	Tray 23 Number 494	Mouse	1:100 MW	Novocastra
NFP	2F11	Tray 23 Number 546	Mouse	1:200 MW (CA)	Dako
SMA	1A4	Tray 1 Number 119	Mouse	1:4000 Trypsin	Sigma
cKit	Polyclonal	Tray 19 Number 500	Goat	1:250 MW	Dako
Vimentin	Monoclonal	Tray 19 Number 502	Rabbit	1:100 MW	Sigma

Table 1: A list of antibodies used for immunohistochemical analysis. MW=microwave antigen retrieval. CA=citric acid buffer.

4.2 Urothelial cell culture

4.2.1 Normal human urothelial cell culture

NHU cells were established and maintained as finite cell lines in keratinocyte serum-free medium (KSFM), containing recombinant epidermal growth factor and bovine pituitary extract at the manufacturer's recommended concentrations (Invitrogen Ltd, Paisley, UK). NHU cell lines were propagated in Primaria tissue culture flasks (Becton Dickinson, Cowley, UK), maintained at 37^0 Celsius in a humidified atmosphere of 5% CO_2 in air. At approximately 90% confluency, the culture medium was aspirated and 5ml 0.1% EDTA in PBS was added for 5 minutes at 37^0C. The EDTA was aspirated and replaced with 1ml trypsin versene (0.25% trypsin in PBS containing 0.02% EDTA) for a further 3 minutes at 37^0C until the cells had become detached from the base of the flask. Trypsin activity was inhibited by addition of 1ml trypsin inhibitor (TI) with KSFMc (KSFM containing growth factor and bovine pituitary extract as described above). The cells were aspirated from the flask and pipetted into a universal tube and centrifuged at 1200rpm for 4 minutes. The supernatant fluid was extracted and the pellet of cells resuspended in KSFMc. 20µl of cell suspension was placed on a Neubauer haemocytometer. The slide was placed under an inverted Nikon microscope and cells in the outer squares of the grid were counted and then divided by four to provide a cell count of $1x10^4$/ml. Cells were reseeded at the desired cell density, calculated from the cell count after resuspension [(78)]. Ketamine treatments were prepared by dissolving pure ketamine powder into media of the same type that was used to culture cells - serial dilutions were performed to obtain the desired ketamine concentration. The media was then added to cultured cells at the desired timepoints.

4.3 Neuronal cell culture

4.3.1 B104 rat neuroblastoma cell culture

The B104 rat neuroblastoma cells were kindly donated by Dr Gareth Evans for use in the neuronal cell experiments.

Cells were cultured in medium that consisted of DMEM with pyruvate, high glucose and glutamine (GIBCO:41966-029), and 10% foetal calf serum (FCS). Antibiotics were not routinely added to the culture medium. Cells were seeded on to 25cm^2 Primaria tissue culture flasks (Becton Dickinson, Cowley, UK), maintained at 37^0 Celsius in a humidified atmosphere of 5% CO_2 in air and passaged when approaching 90% confluency.

4.3.2 Cell passaging

Cells were passaged every 48 hours and this process followed the same steps as for the urothelial cells described above with exception of the different growth medium used for culture. Cells were counted using the Neubauer haemocytometer and seeding density for plating was calculated from this. 24 well plates were used and cells were seeded onto 13mm No 1 thickness glass coverslips that had been sterilised in the oven at 160^{0}C for at least 2 hours. 1ml of suspension with 1x10^4 cells was added per well and added to this was either 10ng/ml nerve growth factor (NGF), or 50ng/ml cyclic adenosine monophosphate (cAMP). A no treatment control was used for comparison. The cells were not treated with ketamine – the reason for this was that having not worked with B104 cells before, it was important to establish neurite outgrowth in response to positive controls to ensure correct characterisation of the cell line inorder to test the hypothesis that they were a suitable cell line to assess neurotrophic stimulation.

4.3.3 Fixation, permeabilisation, and fluorescent phallotoxin staining

After 4 days the cells washed twice with pre-warmed phosphate-buffered saline, pH 7.4 (PBS) to remove any dead cells. The cells were then fixed in 10% formalin for 20 minutes. After washing with PBS, 0.1% Triton X-100 with 1% bovine serum albumin (BSA) in PBS was added for 5 minutes. Two washes with PBS followed this. Texas Red-X Phalloidin (Invitrogen) in methanolic stock solution was added to PBS to obtain a 1:10 dilution. 1% BSA was added to the staining solution to minimise non-specific

background staining. The staining solution was placed on the coverslips for 20 minutes at room temperature.

4.3.4 Hoechst stain

Cells were washed in PBS for 5 minutes and aspirated off. Hoechst 33258 (1:10000 dilution) was added to the cells for 10 minutes at room temperature. Slides were mounted in N-propyl gallate antifade and left to dry in the dark. Slides were visualised under epifluorescent conditions using an Olympus microscope with barrier fluorescent filters for Texas Red and Hoechst 33258. Images were captured and transferred to Neuron J software (Image J plugin).

4.3.5 Neurite measurement

A neurite was defined as the distance from the tip of the neurite to the junction between the neurite base and neural body.

4.3.6 LAN-5 neuroblastoma cells

A contamination problem with *Mycoplasma spp.* affecting the B104 cells meant they had to be discarded and an alternative cell line had to be found. Dr Gareth Evans kindly donated LAN-5 human neuroblastoma cells that had been freshly isolated and I maintained these cells in a quarantined environment to reduce the chances of repeat contamination. Cells were cultured in RPMI 1640 supplemented with 10% FCS and 1% glutamine. Cells were maintained as described above, with passaging of cells every 3-4 days. Cells were seeded onto 12 well glass slides at a density of $1x10^4$ per well and treated with one of 1µM retinoic acid, 10ng/ml NGF, or 1µM ketamine. A control with normal culture medium was also used.

After 72 hours, cells were fixed, permeabilised and stained with a fluorescent phallotoxin and Hoechst 33258 as described above. The slides were mounted in N-propyl gallate antifade and allowed to dry before visualisation using the Olympus microscope. Images were transferred to NeuronJ software and neurite lengths were measured. Statistical analysis was performed using tests specified in the results section.

4.3.7 Freezing down LAN-5 cells

After passaging, cells were centrifuged at 1200rpm for 4 minutes. Cells were re-suspended in a Falcon tube in the freezing mix that consisted of 10% DMSO (dimethylsulphoxide), 10% serum, RPMI, 1% glutamine. 1ml of each cell suspension was placed in a cryo vial and labelled appropriately. Vials were stored overnight at -80^{0}C before transfer to liquid nitrogen for longer term storage.

4.4 RNA extraction

RNA extraction was kindly performed by Dr Simon Baker. 10ml polypropylene tubes and caps (Starstedt) were incubated overnight with 0.1% diethyl pyrocarbonate (DEPC) to inhibit RNAase activity. Following this, the tubes were autoclaved to destroy DEPC. An area of the laboratory designated solely for RNA work was prepared and cleaned. 5ml Trizol^{TM} reagent (Invitrogen) was added to urothelial cell cultures grown in 75cm^2 flasks with different treatments over specified time points. The culture medium was removed before addition of Trizol. Cells became solubilised and the cell lysates were collected in the polypropylene tubes. 1ml chloroform (0.2ml/ml of Trizol) was added , the tubes were vortexed and then placed on ice for 5 minutes. Lysates were centrifuged at 12 000g for 15 minutes at 4^{0}C. The upper aqueous phase containing the RNA was carefully removed and transferred to a new polypropylene tube. An equal volume isopropanol was added at ambient temperature for a period of 10 minutes to allow RNA precipitation. This was centrifuged at 12 000g for 10 minutes at 4^{0}C. The RNA pellet was washed with 75% ethanol, air-dried, and resuspended in 30µl dH_20. Storage of RNA was at -80^{0}C.

4.5 cDNA synthesis

Isolated RNA extracts were transcribed to cDNA using the Superscript first-strand synthesis System^{TM} (Invitrogen). To control for DNA or polymerase chain reaction (PCR) product contamination, samples were incubated with DEPC-treated water to provide the 'RT negative' reaction, and replicate samples were incubated with reverse transcriptase to provide the 'RT positive' reaction.

4.6 Primers

Primer pairs for PCR were designed by Felix Wezel using published sequences from NCBI/Ensembl and checked for specificity using databases that included Ensembl project genome (http://www.ensembl.org/), the NCBI Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and UCSC Genome Browser (http://genome.ucsc.edu/). See table 2 below for the primer sequences used.

The primers used were specific to cKit and GAPDH genes, the sequences of which can be seen below in Table 2.

Gene	Forward 5' - 3'	Reverse 5' – 3'	Size (bp)	NCBI Ensembl code	Intron	Control
cKit	CTTGTTGACC GCTCCTTGTA	GGATGAATTT TTCCGACAGC	250	ENSG000 00157404	No	Genomic DNA
GAPDH	ACCCAGAAGA CTGTGGATGG	TTCTAGACGG CAGGTCAGGT	201	ENSG000 00111640	No	Genomic DNA

Table 2: The above table shows the primers used for the RT-PCR experiments. GAPDH was used as a housekeeping gene to test cDNA integrity and act as a method control.

4.7 Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was performed on a PCR Express Thermal Cycler (Hybaid). 19µl of PCR mastermix was prepared for each cDNA sample formed from the cDNA synthesis described above. The PCR mastermix contained 2µl Surestart Taq Buffer (Stratagene), 0.4µl (10mM) dNTP, 2µl (10µM) forward primer, 2µl (10µM) reverse primer, 0.1µl Surestart Taq Polymerase (Stratagene) and 12.5µl of DEPC-treated water. The total volume of mastermix was dependent on the number of cDNA samples used. 1µl of cDNA was added to the 19µl of mastermix to provide a total volume of 20µl. GAPDH was used as a housekeeping gene – this is a constitutive gene that is required for the maintenance of basic cellular function, is expressed in all cells of an organism, and acted as the method control for RT-PCR and tested cDNA integrity. 1µl of genomic DNA was used as a positive control in order to demonstrate successful experimental

methods and effective reagents. 1µl of no-template H_20 was added to the mastermix as a negative control in order to demonstrate no contamination of product.

The cDNA samples were denatured in the PCR Express Thermal Cycler for 5 minutes at 95^0C followed by 30 cycles at 95^0C for 30 seconds, annealing for 30 seconds at 57^0C and 40 seconds at 72^0C to allow extension. A final elongation phase of 10 minutes at 72^0C was followed by incubation at 4^0C.

The PCR products were visualised using gel electrophoresis. Agarose gel was prepared by boiling agarose in 1x TBE to provide a 2% agarose concentration. After cooling to approximately 50^0C, 8µl GelRed (Cambridge Bioscience) was added and the product was allowed to cool further to form a gel. Any bubbles were removed carefully. 2µl loading dye (6x Blue/orange(Promega)) were added to each PCR product tube and each sample was loaded in to the wells. 6µl of 100bp DNA ladder (Hyperladder IV, Invitrogen), was run in each experiment to measure the size of DNA. The gel was covered in approximately 200mls 1xTBE and run at 110 Volts for 60-120 minutes. Once the loading dye showed a good degree of separation from the wells, the gel was transferred to a transilluminator with Genesnap software (Syngene) and the images were downloaded.

4.8 Western blotting

Protein lysates had been prepared by Dr Simon Baker at the Jack Birch Unit, University of York.

Proteins were resolved by electrophoresis through NuPAGE gels using the Novex system(Invitrogen). The pre-cast gels and running buffers used were based upon the molecular weight of the target protein (145kDa), with 4-12% Bis-Tris used as the Gel and MOPS used as the running buffer. Combs were removed, the wells washed with running buffer, and the gels were assembled in the Novex tank according to manufacturer's instructions. 10-20µg of each protein lysate was made up to 18µl with dH_20 and had 4µl NuPAGE LDS, which incorporates a loading dye, added. Samples were incubated at 70^0C for 10 minutes. Gels were also loaded with 5µl of Kaleidoscope and All-Blue prestained molecular weight markerladders (Bio-Rad). Gels were

electrophoresed at 125V for 60 minutes or until the dye front ran to the bottom of the gel.

Total protein separated by SDS-PAGE was transferred to PVDF membranes at 30V for 2 hours at 4^0C using the Novex system (Invitrogen). PVDF membranes were pre-wet with methanol, rinsed in distilled water and then equilibrated in transfer buffer for 5 minutes. Transfer buffer was a solution of 12mM Tris and 96mM glycine in dH_20 with 20% (v/v) methanol. The membranes were stained with ponceau red for a few seconds to test transfer efficiency and concentration of protein across the lanes on the membrane. The ponceau red stain was washed off with dH_2O.

Membranes were blocked using a 50% Odyssey Blocking Buffer (Li-Cor) in TBS for 1 hour at room temperature on an orbital shaker. The membranes were probed with the primary antibody diluted in 50% Odyssey blocking buffer in TBS containing 0.2% Tween-20 overnight (for 18 hours) in the fridge at 4^0C on an orbital shaker. The primary antibody used was cKit (R&D systems, polyclonal goat antibody) at 1:2000 concentration. Negative control lanes were used alongside using the same conditions but without addition of the primary antibody. To achieve this, the negative control lane membranes were cut off and incubated in TBS separately without the primary antibody. The membranes underwent four five-minute washes with TBS-Tween 0.1%. Membranes were then probed with the secondary antibody (Alexa fluor donkey anti-goat 1:2000 concentration) diluted in 50% Odyssey blocking buffer in TBS-Tween 0.2% for 1 hour at room temperature on an orbital shaker and covered in foil to protect from light. Membranes were washed four times with TBS-Tween 0.1% and once with TBS for five minutes each protected from the light. Proteins were visualised by epifluorescent illumination at 800nm using the Odyssey system (Li-Cor). Scans were analysed using Odyssey software (Li-Cor), which was used to measure band intensity and perform background subtraction. Band intensities were measured by drawing boxes around the bands and using the Li-Cor Odyssey software to calculate integrated intensity by measuring box height, by length, by pixel intensity. Membranes were stripped to allow re-probing by incubation in Western Blot Recycling Kit reagent (Autogen Bioclear) for 30 minutes at room temperature on an orbital shaker. Membranes were then washed in TBS, re-blocked and re-probed as described. Negative control lanes were included which contained protein but no primary antibody to ensure there was no non-specific secondary antibody binding.

5. Results

5.1 Immunohistochemistry

5.1.1 Nerve Growth Factor Receptor (NGFR)

In normal human urothelial tissue, NGFR expression was confined to the basal layer of the urothelium and was not typically seen in the stroma (Fig 1).

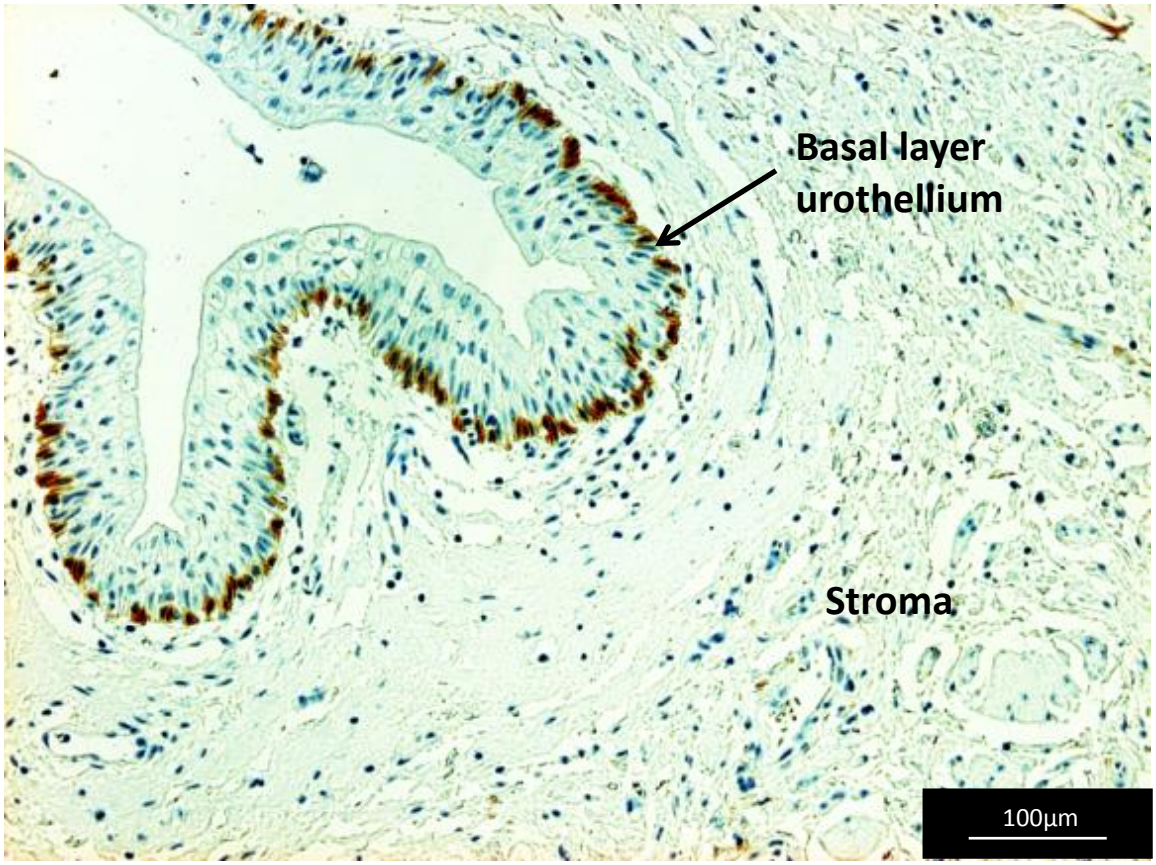


Figure 1: image of normal human urothelium in a sample of ureter labelled with anti-human p75NGFR antibody. The dark brown stain in the basal layer of the urothelium shows NGFR expression which is normal. The stroma does not show NGFR expression.

NGFR expression in ketamine-induced cystitis (KIC) specimens varied from the normal pattern, with suprabasal expansion of NGFR into the intermediate urothelium, and in some specimens NGFR positive cells were seen throughout the full thickness of the urothelium. Of the 20 KIC samples examined, only three specimens had urothelium that appeared intact after immunolabelling, and of these, one had full thickness urothelial NGFR expression, one had suprabasal expansion, and the other had a normal pattern of expression (Fig 4).

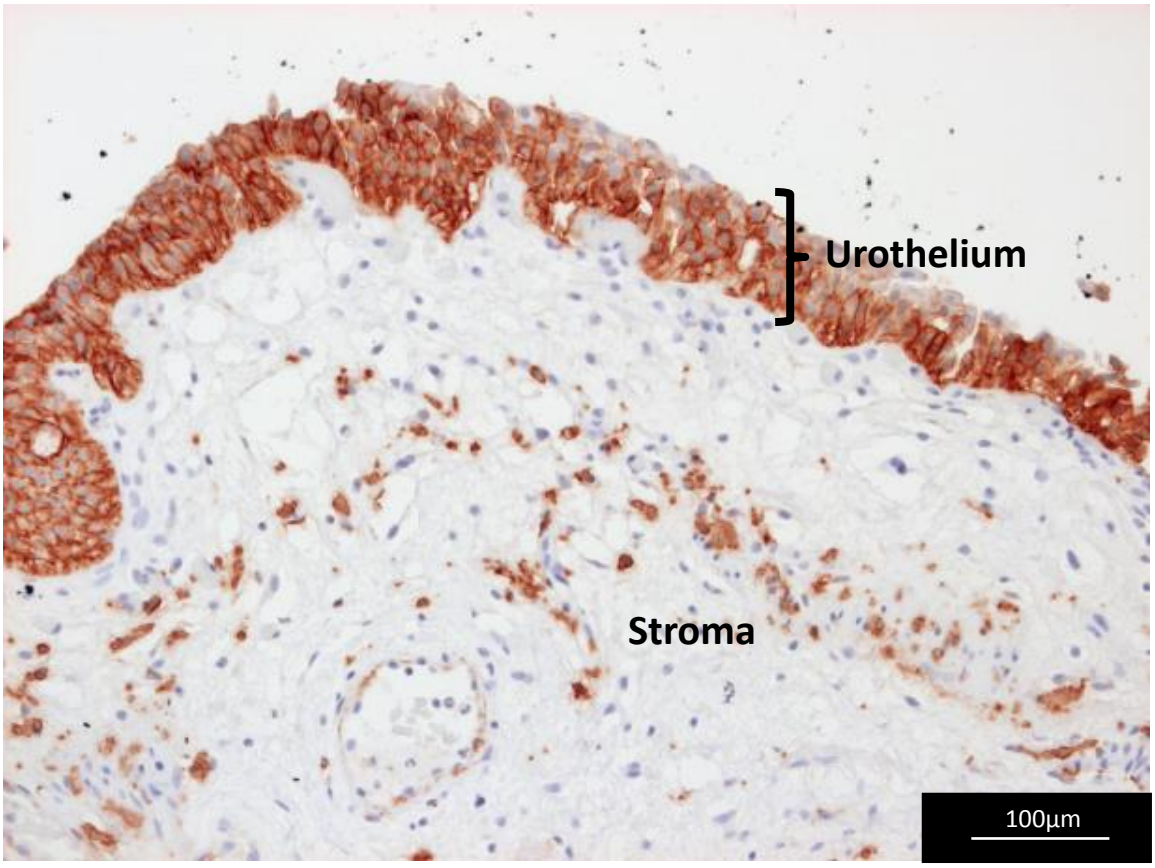


Figure 2: This shows a sample of KIC bladder tissue labelled with anti-human p75NGFR antibody. It shows NGFR expression in all layers of the urothelium (basal, intermediate, and superficial). In normal urothelium, NGFR is confined to the basal urothelium, thus indicating an increase in NGFR expression in KIC tissue.

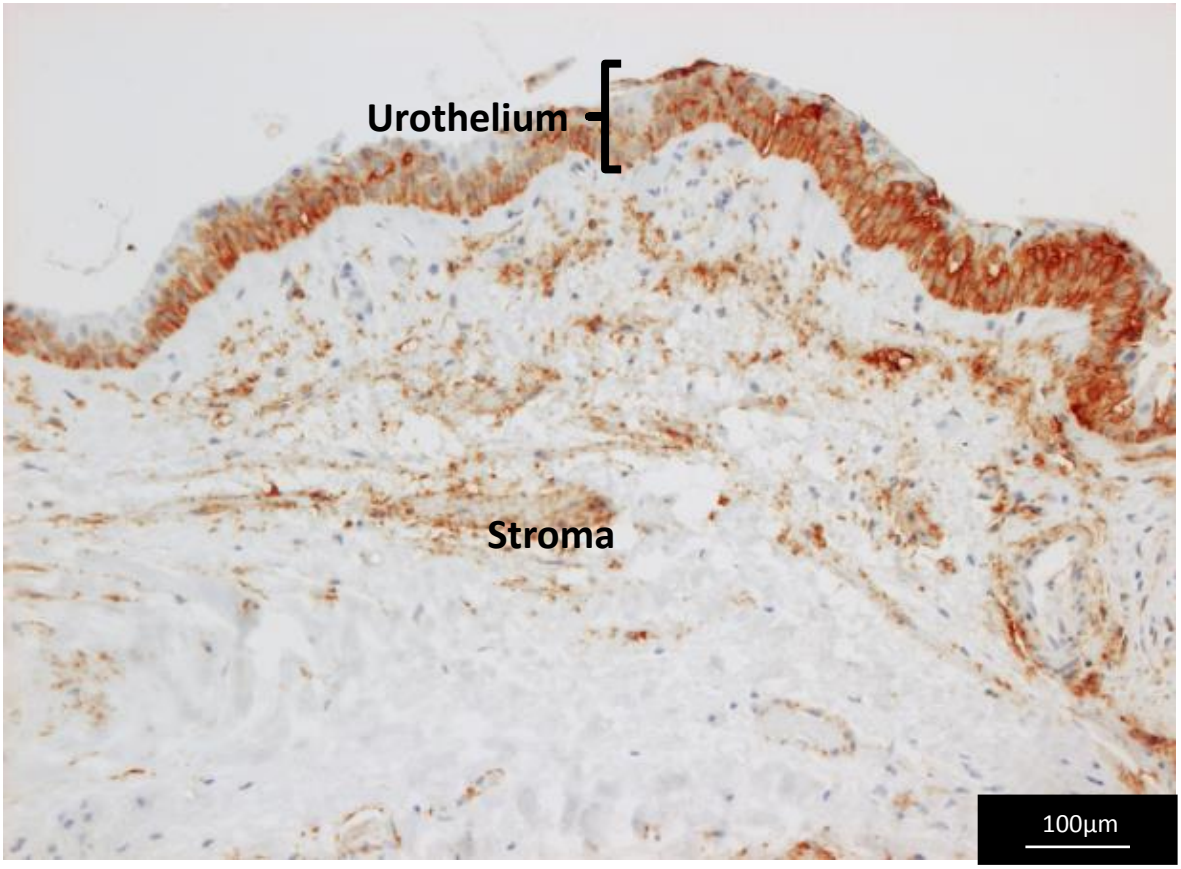


Figure 3: This KIC sample is labelled with anti-human p75NGFR. It shows NGFR expression in the basal layer of the urothelium with expansion of NGFR expression into the intermediate urothelial layer. NGFR is largely absent from the superficial urothelial cells.

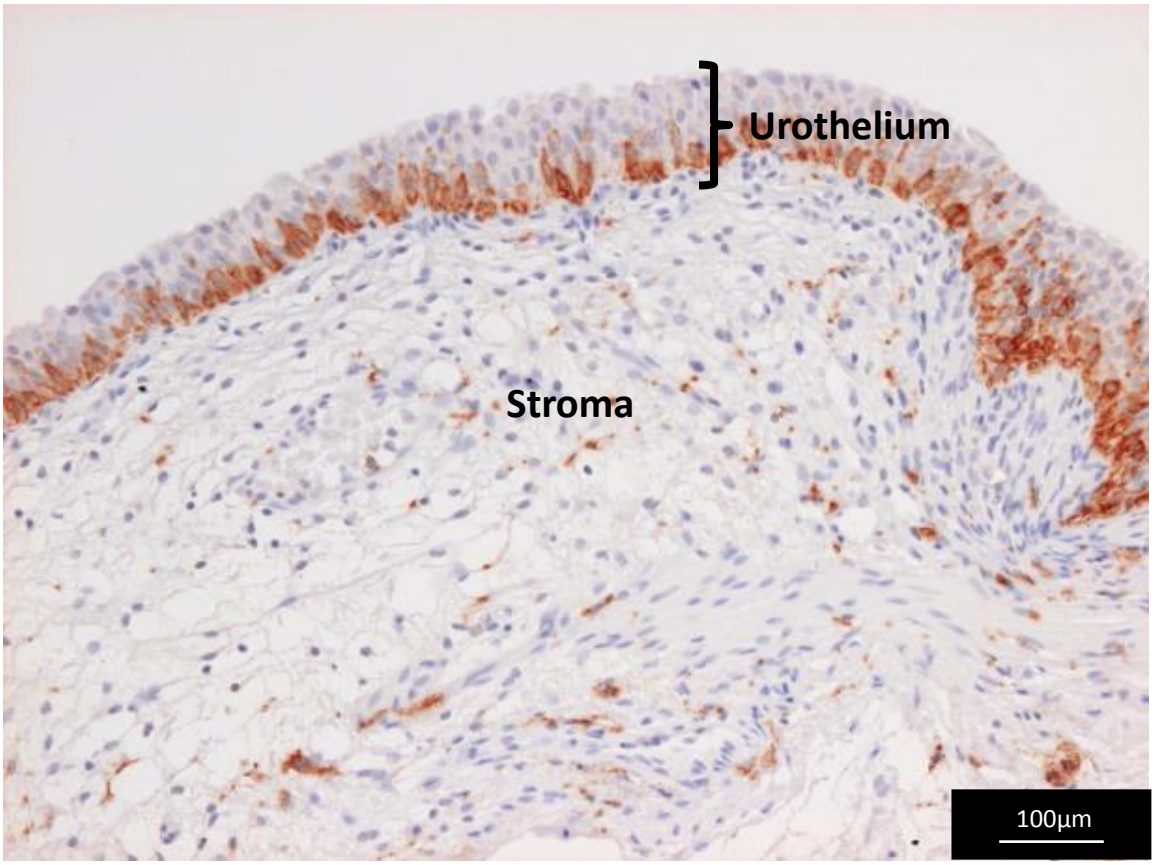


Figure 4: This shows a further KIC bladder sample labelled with anti-human p75NGFR antibody. NGFR expression is localised to the basal urothelium, as is seen in normal urothelium. There is no evidence of suprabsal expansion in this sample thus indicating a normal pattern of NGFR expression.

Seven of the remaining KIC samples showed a partially-denuded urothelium and a mixed pattern of NGFR expression, with three samples displaying full thickness expression, two samples displaying suprabasal expansion, and two samples showing a normal pattern of expression. The denudation of the urothelium in one sample was such that the superficial and intermediate layers were absent; therefore comments were only made upon the condition of the urothelium, and not about whether there was suprabasal expression.

The normal control tissue (ureter) lacked NGFR expression in the stroma. In the KIC samples, 16 out of 20 of these showed stromal NGFR expression, and in 13 of the samples, large NGFR positive nerve fibres were seen. These were identified as a collection of NGFR positive cells that formed an organised structure within the stroma with the appearances of a nerve fibre (Fig 5) as confirmed by the clinical histo-pathologist Dr Jens Stahlschmidt at Leeds Teaching Hospital NHS Trust.

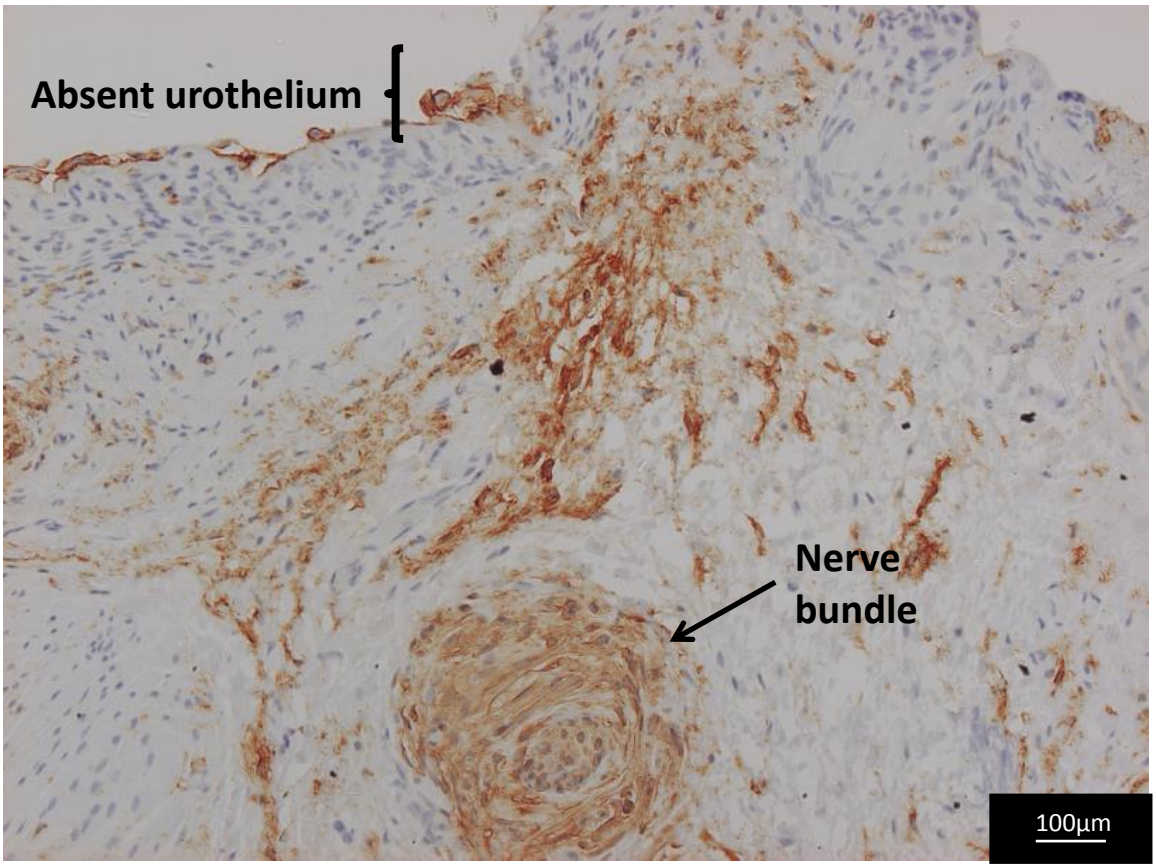


Figure 5: This image shows a KIC bladder sample labelled with anti-human p75NGFR antibody. Note the dense red/brown area within the stroma that is positive for NGFR. The swirly appearance of this is not seen in normal tissue and resembles a large nerve fibre. These observations were made with the help of Dr Jens Stahlschmidt, a clinical pathologist at Leeds Teaching Hospitals NHS Trust.

5.1.2 *Nerve filament protein (NFP)*

In normal human tissue of the urinary tract, there was no evidence of NFP expression within either the stroma or the urothelium (Fig 6). The majority of the KIC samples (18/20) showed expression of NFP in the stroma, with three of these samples being strongly positive (Fig 7). One KIC sample showed a normal pattern of expression of NFP, and the other KIC sample could not be interpreted due to the quality of the specimen having degraded significantly at the time of biopsy.

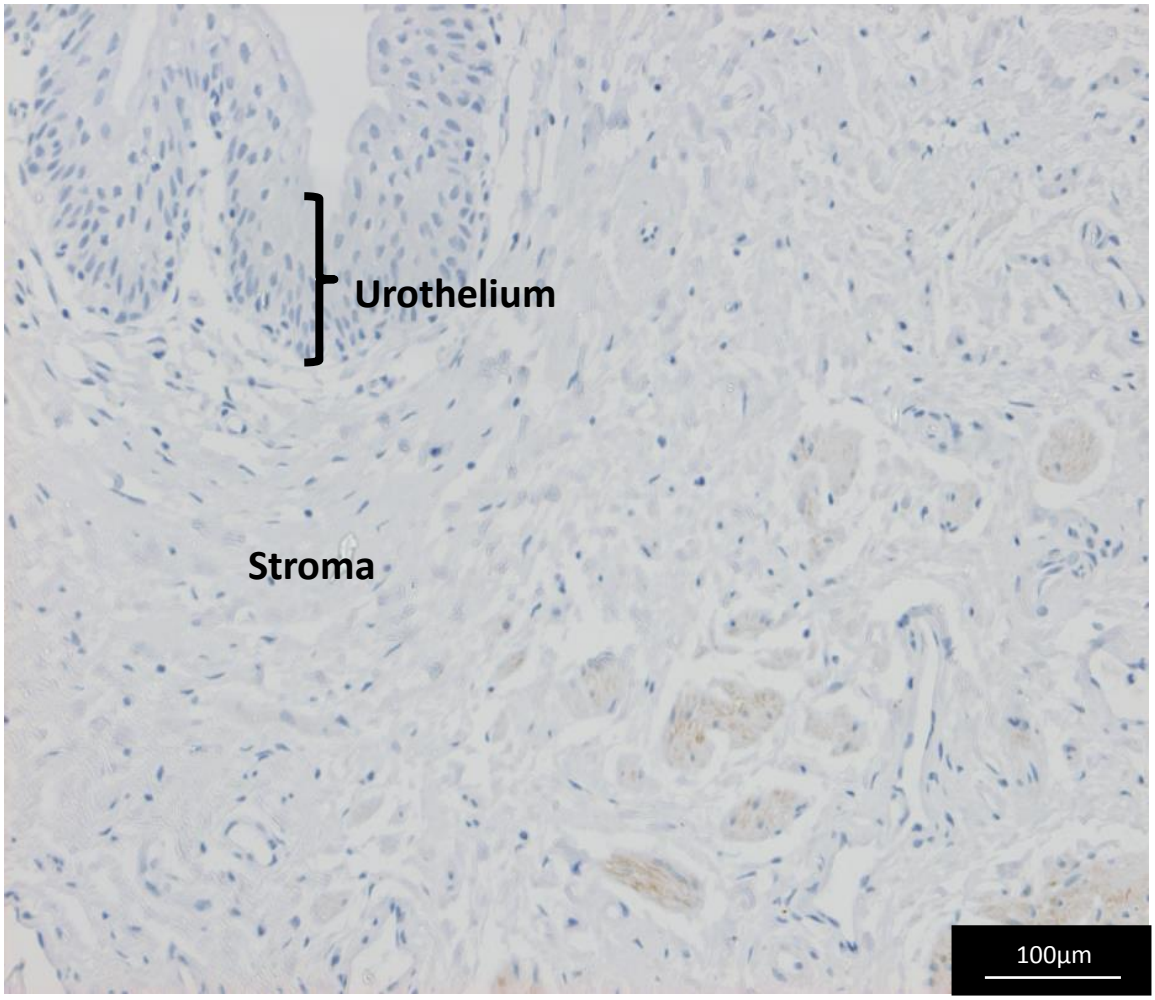


Figure 6: This image shows normal human ureter labelled with neurofilament protein (NFP) antibody. There was no evidence of NFP in the normal tissue.

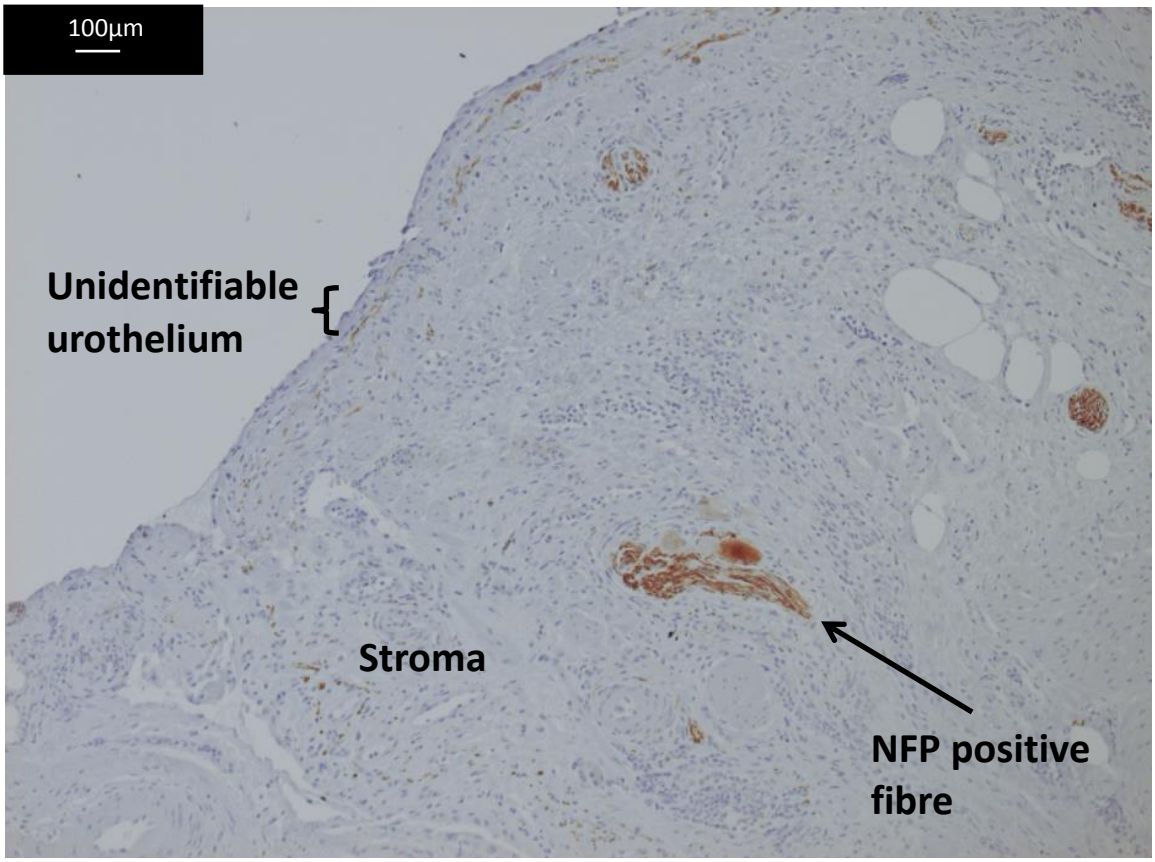


Figure 7: This image shows a KIC sample labelled with NFP. Note the positive NFP expression within the stroma. This could represent nerve fibres within the stroma that are not seen in normal tissue.

5.1.3 Smooth muscle actin (SMA)

As expected in normal urothelial tissue, SMA expression was absent from the urothelium, as illustrated in a human ureter specimen (Fig 8).

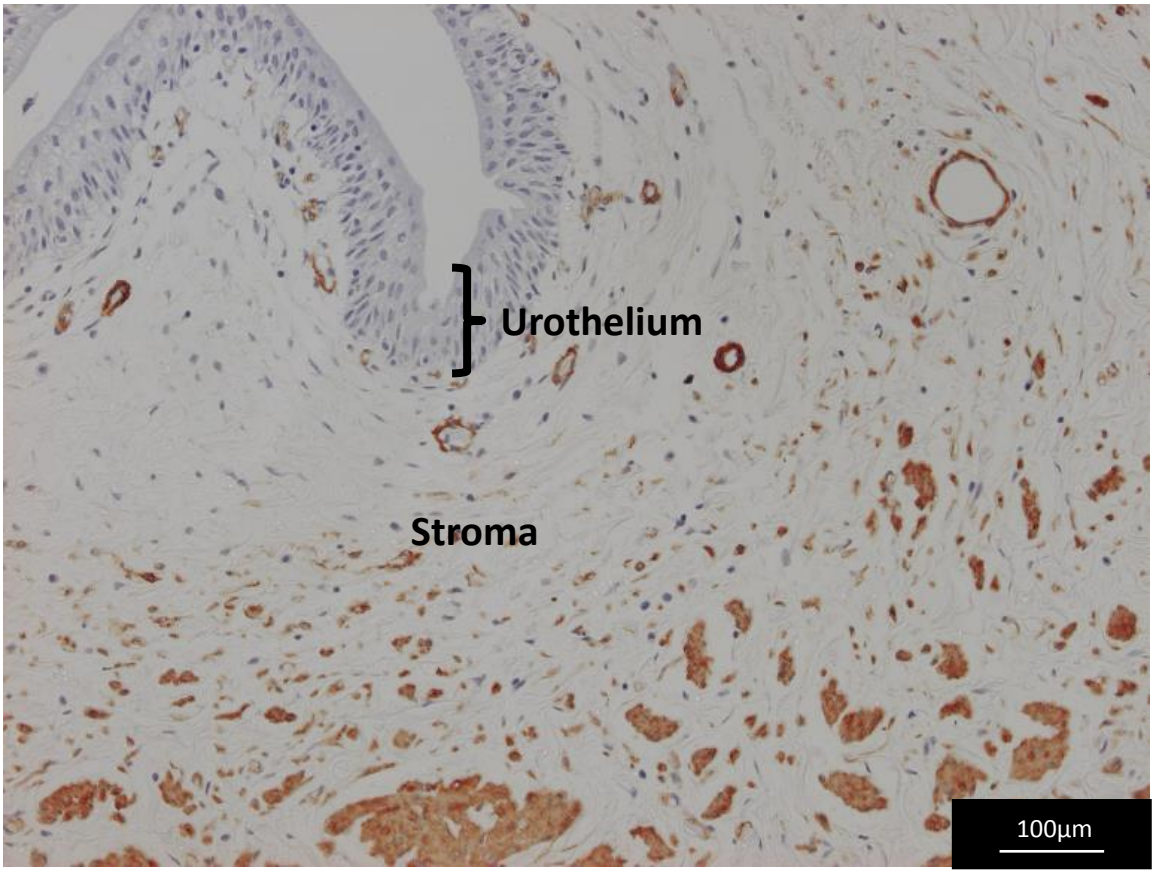


Figure 8: This image shows normal human ureter labelled with smooth muscle actin (SMA). The urothelium did not express SMA. The stroma expressed SMA confined to smooth muscle fibres as seen.

13 KIC samples displayed identifiable urothelium and of these SMA was not seen in 10, however the other three KIC samples showed urothelial expression. Of the three KIC samples that showed urothelial expression, one sample showed SMA expressed throughout all layers of the urothelium, one sample displayed SMA confined to the basal layer, and one sample showed SMA in the intermediate and superficial layers of the urothelium (Fig 9). No comment could be made on urothelial expression in those 7 KIC samples that lacked identifiable urothelium.

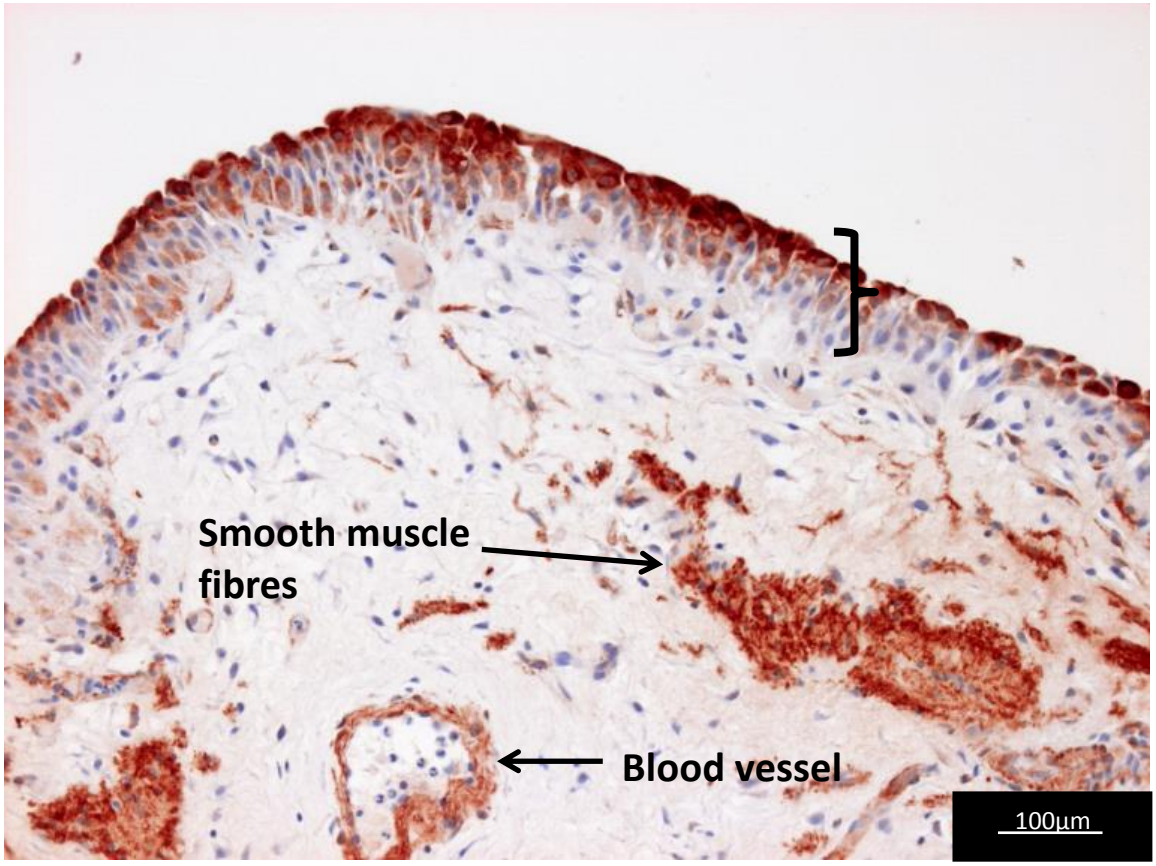


Figure 9: This KIC sample showed SMA expression in the superficial urothelium that extended down into the the intermediate urothelial layer. As expected, SMA was also seen in the stroma confined to smooth muscle fibres.

5.1.4 Vimentin

In normal urothelial tissue, vimentin was typically seen in the suburothelial layer (Fig 10). Although no double labelling experiments were performed, the pattern of vimentin expression in the suburothelial layer appeared on observation to be similar to smooth muscle actin expression. Further work is obviously required to confirm this and double labelling could be a viable method of determining co-localisation of vimentin with smooth muscle actin. All KIC samples displayed vimentin positive cells in the suburothelial layer in keeping with a normal pattern of expression (Fig 11). The pattern of vimentin did not mimic the urothelial expression of SMA seen in some KIC specimens.

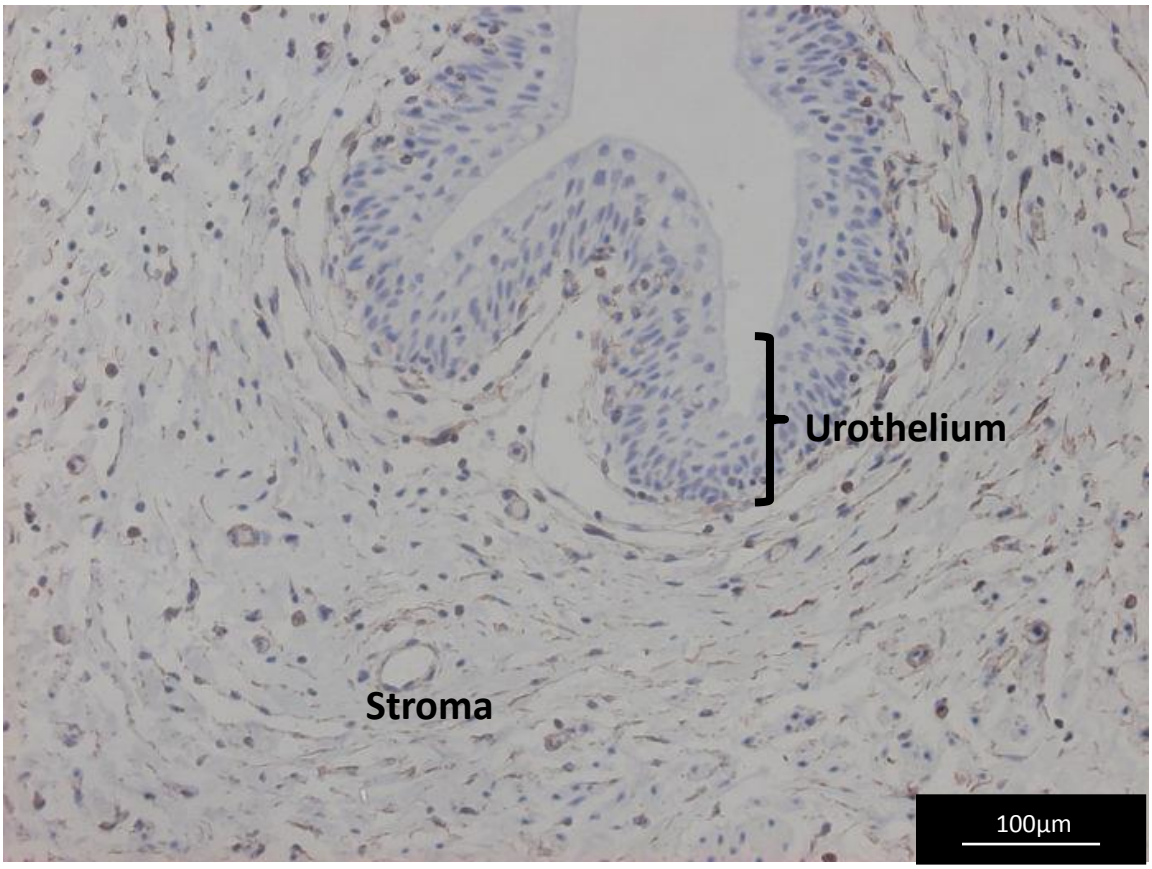


Figure 10: The image above shows normal ureter labelled with vimentin antibody. Expression seen as the dark red/brown cells was confined to the stroma. No urothelial expression was seen.

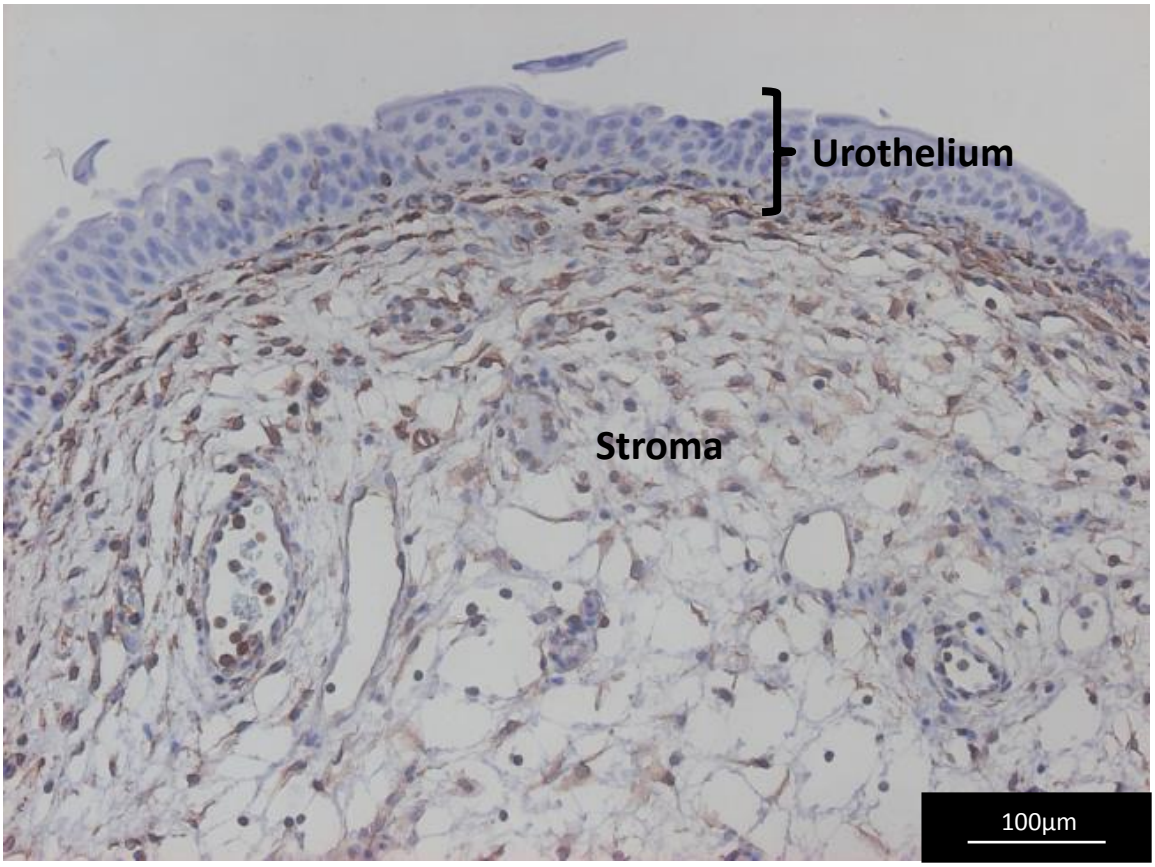


Figure 11: This shows a KIC sample labelled with vimentin antibody. Expression was confined mainly to the suburothelial layer. There was no evidence of urothelial expression.

5.1.5 cKit

In normal human urothelium, occasional cKit positive cells were seen (Fig 12).

Five of the KIC samples showed a similar pattern to normal, with the occasional positive cKit cell in the urothelium. The cells were tear-shaped in structure and appeared in the suprabasal urothelium with a pedicle extending down towards the basement membrane. Six of the KIC samples showed an absence of cKit positive cells in the urothelium (Fig 13). The remaining nine KIC samples did not have identifiable urothelium to comment on the presence or absence of cKit positive cells.

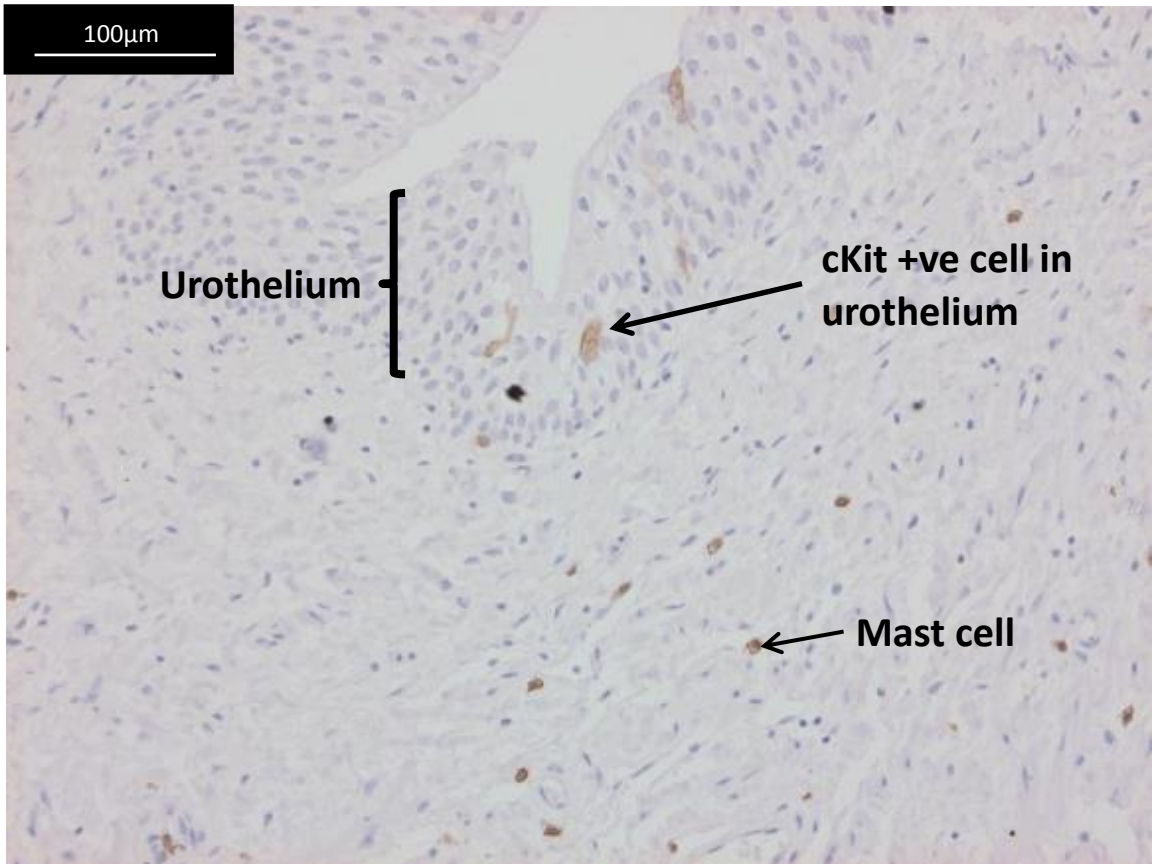


Figure 12: The image above shows normal ureter labelled for cKit. The red cells in the urothelium indicated cKit expression. These cells appeared to show a pedicle that was attached to the basement membrane. cKit positive cells seen in the stroma were thought to be mast cells based on their morphological appearance

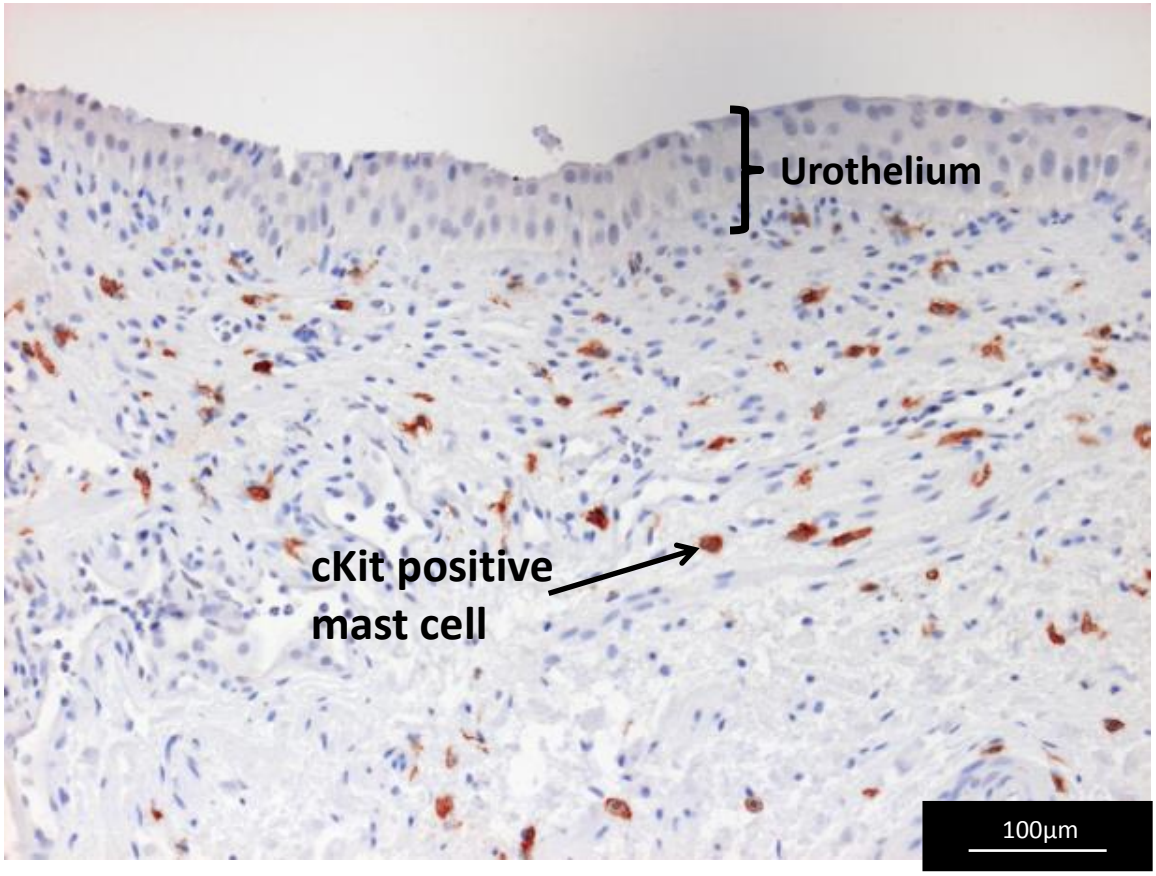


Figure 13: This shows a KIC sample labelled with cKit. The presence of cKit positive mast cells was seen in the stroma similarly to normal tissue. There was however an absence of cKit expressing cells within the urothelium.

5.2 Neuronal cell culture

5.2.1 B104 cells

After 4 days, B104 cultures exposed to 10 ng/ml NGF or 50 ng/ml cAMP had significantly extended neurites compared to the no treatment control (Fig 15). The initial experiment appeared to demonstrate a difference in neurite outgrowth between treatment groups that included 10ng/ml nerve growth factor (NGF), cyclic adenosine monophosphate (cAMP), versus a normal culture medium control (Figs 14 & 15).

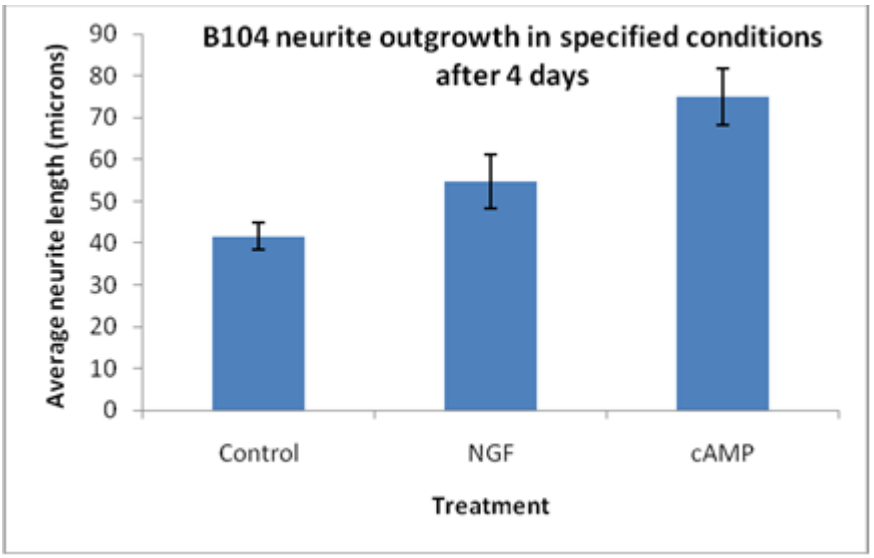


Figure 14: This graph shows the average neurite length for B104 cells in different treatment conditions. The greatest effect was seen in 50ng/ml cyclic adenosine monophosphate treatment (cAMP), followed by 10ng/ml nerve growth factor (NGF) treatment. The least neurite length was seen in the no treatment control group.

Statistical analysis was performed using the Kruskal-Wallis test and calculated a p-value of 0.001 (p-value<0.05 deemed to be significant). A Dunn's post-test was performed to compare groups. The difference between NGF treatment and the control was not statistically significant with a p-value>0.05, however a statistically significant difference was found between cAMP treatment versus the control group, and NGF treatment versus cAMP treatment with p-values<0.05. However, the cultures were subsequently screened for *Mycoplasma spp*. and found to be contaminated (see Fig 16), therefore this cell line was discarded and an alternative neuronal cell line was identified to use in future experiments (LAN-5 cells).

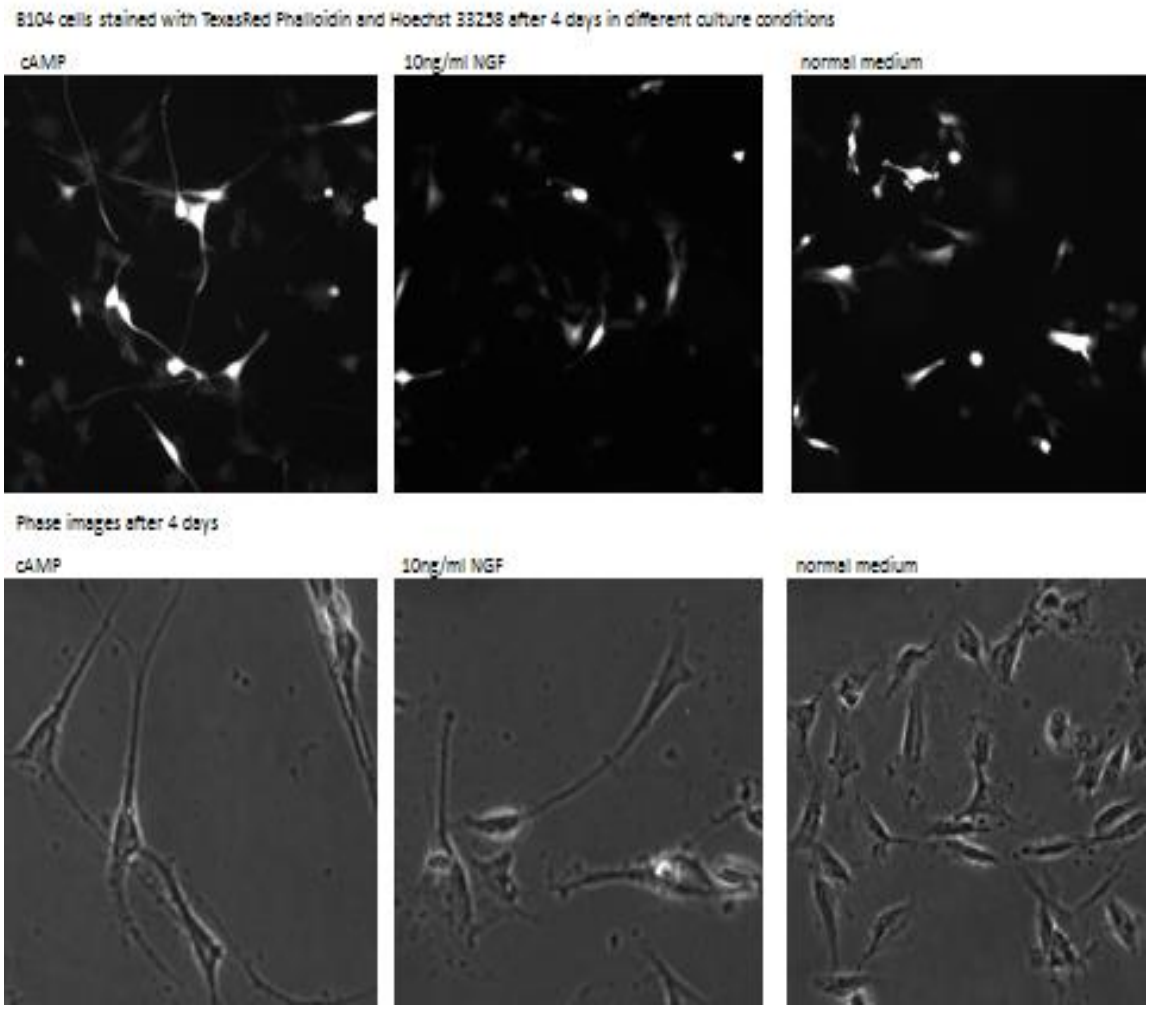
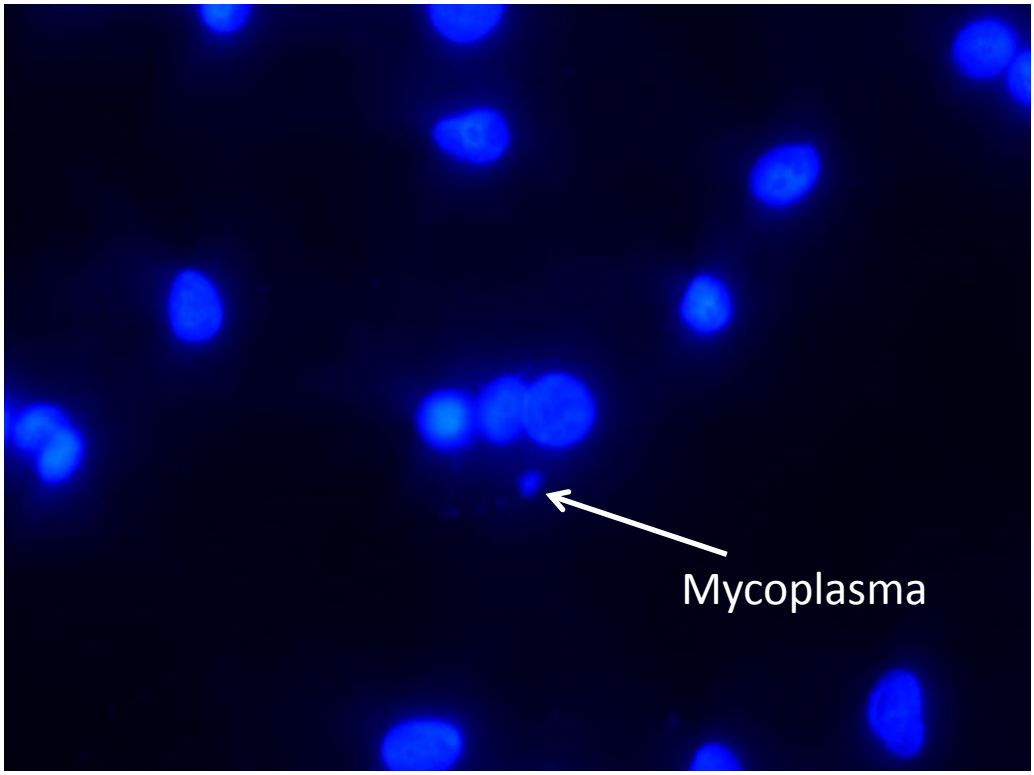


Figure 15: The images above show B104 cells treated with either 50ng/ml cAMP, or 10ng/ml NGF, or no treatment (control). After 4 days the cells were fixed and stained with TexasRed Phalloidin and Hoechst 33258. The images at the top show fluorescent images for each group and there appeared to be a difference in neurite length between treatment groups. Analysis of multiple images using NeuronJ software appeared to confirm these observations. Phase contrast images can also be seen and appear to show differences in neurite length with the greatest effect seen in the cAMP treatment group.



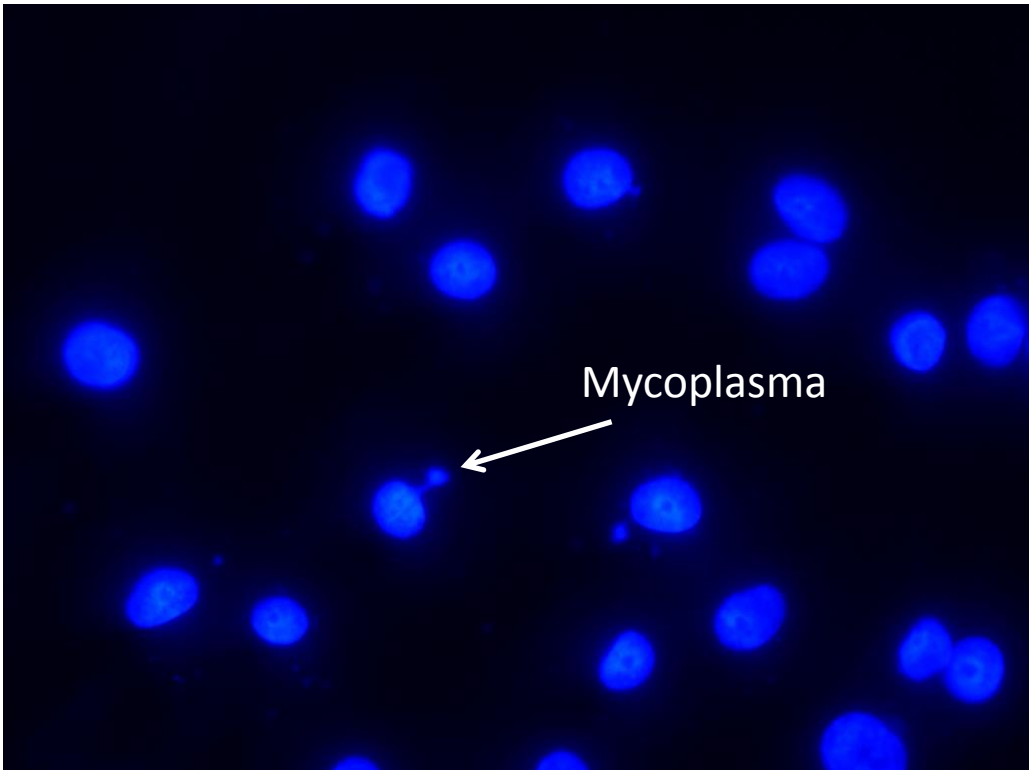


Figure 16: The fluorescent images above show B104 cells stained with Hoechst 33258. The cells appeared to be positive for *Mycoplasma spp.* The cells were therefore discarded and an alternative neuronal cell line was selected(LAN-5 cells).

5.2.2 LAN-5 cells

An initial experiment was performed to compare the effects of 1µM retinoic acid, 10ng/ml NGF and 1µM ketamine versus normal culture medium on LAN-5 neuroblastoma cells. Effects were measured by length of neurites sprouting from cells. Retinoic acid appeared to show the greatest effect on the length of neurite extensions at 72 hours, with NGF showing the next greatest effect, followed by ketamine (Fig 17).

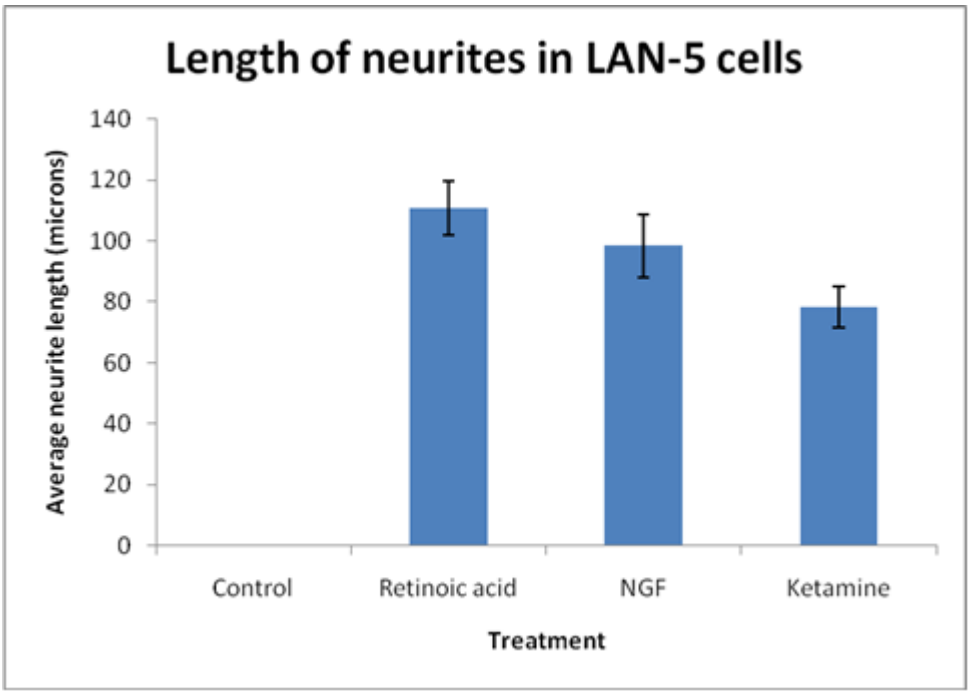


Figure 17: This graph displays average neurite length of LAN-5 neuroblastoma cells treated with either 1µM retinoic acid, 10ng/ml NGF, 1µM ketamine, or no treatment (control). Unfortunately the cells in the control group had poor adherence and were lost during washing. Amongst the treatment groups, the greatest effect on average neurite length was seen in retinoic acid treated cells, followed by NGF and then ketamine. The difference between treatment groups was found to be statistically significant ($p<0.001$).

Although there appeared to be a difference in neurite length between treatment groups, this observation was only comparable between treatment groups and could not be validated against the control due to poor cell adherence in the control group that did not permit analysis of neurite length. Statistical analysis could only show a statistical

difference between treatment groups and this was measured with a p value <0.001. A repeat experiment with the same treatment conditions showed the greatest response regarding neurite outgrowth in the 1µM ketamine treatment group, followed by NGF, retinoic acid, and the control group respectively (Fig 18). Statistical analysis was performed using the Kruskal-Wallis test with a p-value of 0.0015. A Dunn's post-test was performed to compare groups (a p-value<0.05 was deemed to be statistically significant). A statistically significant difference in neurite length was identified between the ketamine treatment group versus the control, and NGF treatment group versus the control (p-value<0.05). No significant difference was identified in the other groups that included retinoic acid treatment group versus the control, retinoic acid versus ketamine, retinoic acid versus NGF, and ketamine versus NGF (p-value>0.05).

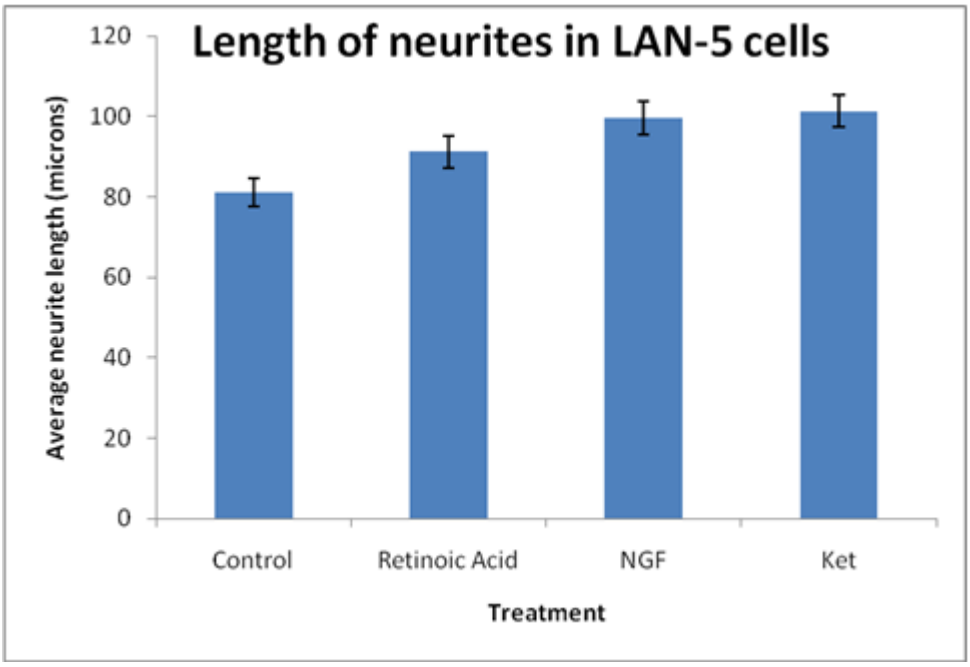


Figure 18: This graph displays the average neurite length of LAN-5 neuroblastoma cells treated with either 1µM retinoic acid, 10ng/ml NGF, 1µM ketamine, or no treatment (control). The greatest effect was seen in the ketamine treated cells, followed by NGF, retinoic acid, and no treatment control respectively. A statistically significant difference was only detected between the ketamine treated cells against the control, and the NGF treated cells and the control (p<0.05).

5.3 Western blot

5.3.1 Western blotting for cKit

cKit protein expression was examined in lysates prepared from cultured control NHU cells and following exposure to 1mM ketamine over a specified time-course. cKit expression was increased in both groups over the 14 day time course, with increased expression in the non-treated group compared to the ketamine-treated group (Fig 19). β actin was included as a loading control. These results indicated that treatment with ketamine may reduce cKit expression in NHU cells.

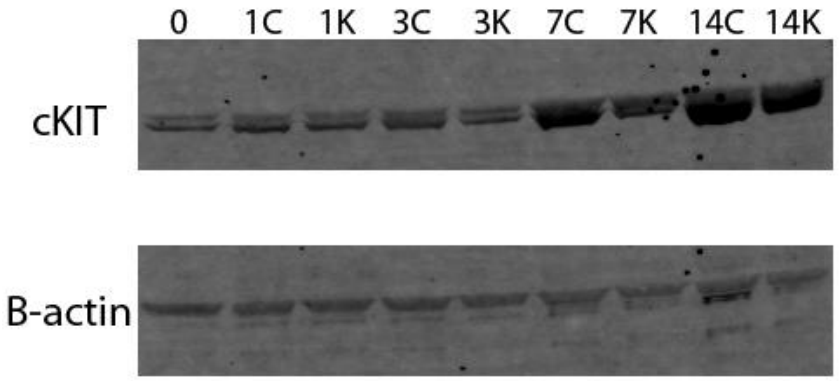


Figure 19: This image displays a Western blot for cKit protein expression over a specified time-course for ketamine treated cells against a no-treatment control (c=control, k=ketamine). B-actin was used as a method control and this was present across all samples. NHU cells were treated with 1nM ketamine over a 14 day time-point. Samples were taken at day 0, day 1, day 3, day 7, and day 14. cKit protein expression was seen to increase in samples for both cell lines (ketamine treated and no treatment control) over 14 days. Greater cKit protein expression was seen in the control samples (1C, 3C, 7C, 14C) compared to the ketamine treated cells (1K, 3K, 7K, 14K).

5.4 RT-PCR

5.4.1 RT-PCR for cKit

RT-PCR was performed on NHU cell lines in two experiments using independent donor cell lines. cKit expression was measured in proliferating cells, cells cultured for 7 and 14 days in normal culture medium, and cells treated with 1mM ketamine for 7 days and 14 days. B-actin was used as a loading control, with both RT+ve and RT-ve reactions. In all experiments, a genomic DNA positive template control was positive, and the dH_2O and the RT-ve controls were negative.

The first urothelial cell line (Y1064) showed cKit transcript expression in day 7 ketamine treated cells, and day 14 ketamine treated cells. A band was not visible in either the day 7 or day 14 control cells.

The control proliferating cells in the second cell line (Y1108) did not show any expression of cKit transcript, but it was detected in both the day 7 and day 14 ketamine treated cells (Fig 20).

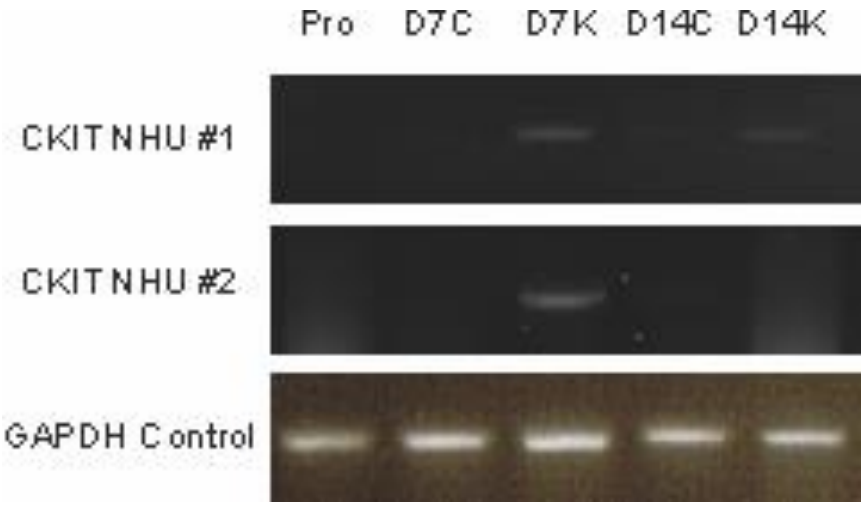


Figure 20: This displays RT-PCR images for expression of transcript in two urothelial cell lines in vitro. The two rows at the top show cKit transcript in Proliferating cell (Pro), Day 7 no treatment NHUs, Day 7 1µM ketamine treated NHUs, Day 14 no treatment NHUs, and Day 14 1µM ketamine treated NHUs. GAPDH was used as a housekeeping gene to ensure integrity of the cDNA. The results showed expression of cKit transcript in Day 7 and Day 14 ketamine treated NHUs. The no treatment NHUs did not appear to express cKit transcript.

6 Discussion

6.1 Nerve Growth Factor Receptor (NGFR)

6.1.1

This study demonstrated NGFR upregulation in KIC samples compared to control samples. Normal human urothelium displayed NGFR expression that was confined to the basal layer, however KIC samples showed a variation from the normal pattern, with NGFR expansion into the suprabasal urothelial layer and some samples displayed full thickness urothelial expression.

Previous studies have shown increased expression of NGFR in the urothelium of patients with neuropathic bladder [79] and also in animal studies with bladders that are inflamed after exposure to a chemical irritant (cyclophosphamide) [80,81]. It appears therefore that similarities exist between KIC samples and both neuropathic bladders and bladders exposed to chemical irritation with regard to the increased level of NGFR expression. The mechanism by which NGFR upregulation occurs remains unknown, however some of the possibilities may include:

i) Inflammatory response from the urothelium caused by chemical irritation such as cyclophosphamide or ketamine
ii) Direct inducing effects of a chemical on the urothelium to upregulate NGFR expression
iii) Indirect effects whereby induced endogenous factors cause NGFR upregulation
iv) NGFR is present as a bio-marker of tissue regeneration following injury

At cystoscopy, patients with ketamine cystitis have small capacity, contracted bladders with erythematous and often ulcerative appearances. Ketamine and its metabolites are excreted via the urinary tract and may be stored in the bladder for up to several hours. One of the possibilities therefore, is that ketamine in contact with the urothelium has a directly positive and neurostimulatory effect on the expression of NGFR in urothelial cells. One would expect though that if this were simply the case, superficial urothelial

cells would show NGFR expression in a greater number of KIC samples, however superficial expression was not seen in the majority of KIC sample but rather the general pattern seen was NGFR expansion from the basal layer into the suprabasal layer of the urothelium. An alternative possibility to the direct contact effects of ketamine as a cause of increased NGFR, is that ketamine is able to induce the production of factors such as NGF or BDNF that bind to NGFR. Whether these factors are secreted from urothelial cells, stromal cells or are part of a larger systemic or circulatory ketamine response has yet to be determined, however one study has shown that patients with a history of chronic ketamine use have higher levels of serum BDNF [(36)], although the mechanism of modification in the production and utilization of BDNF remains unexplained.

The stromal appearances in some KIC samples showed areas with dense NGFR expression that resembled nerve fibres. These were identified as a collection of NGFR positive cells that formed an organised structure within the stroma with the appearances of a nerve fibre as confirmed by a clinical histo-pathologist. These structures were not present in the control specimens and could explain why patients with a history of ketamine abuse develop painful bladders. Furthermore, the samples with a higher expression of stromal NGFR appear to have a more denuded urothelium than those with lower expression. This could simply be an incidental finding, or it could be significant that the loss of urothelium in KIC samples is a pre-disposing factor to increased stromal NGFR expression, and could determine severity of symptoms, although the clinical correlation has yet to be established. Future work will aim to address this by stratifying patients according to symptoms to better understand the relationship between symptoms severity and NGFR expression.

The identification of NGFR in other tissues such as oral mucosa and prostatic epithelium[(83,84)] suggests a role of NGFR that is integral to cells with distinct regenerative capacity. Urothelial tissue can be considered also as having excellent regenerative capacity, thus further emphasising the integral role of NGFR in regeneration. Other studies have demonstrated NGFR in bone marrow mesenchymal stem cells (MSCs) which are a population of multipotent cells that can proliferate and differentiate into multiple mesodermal tissues [(82)]. The increased NGFR expression in KIC samples could indicate the innate response of urothelial cells with their distinct regenerative capacity, reacting to insult and damage induced either directly or indirectly

by ketamine, and demonstrating a regenerative cellular response, with NGFR acting as a marker of progenitor cells in the urothelium.

6.1.2 Limitations and future work

The significance of using ureter as a model for the study of urothelial cellular processes rather than bladder tissue may have certain implications. Embryological differences exist between these two tissues, with the ureter and bladder trigone of mesodermal origin, whereas the bladder and urethra are derived from the endoderm [(85)]. Common procedures that have provided urothelial specimens include pyeloplasty for pelvi-ureteric junction obstruction, ureteric re-implantation for vesico-ureteric reflux, nephro-ureterectomy, and renal transplant surgery. The very nature of these procedures mean that the specimen provided is ureter rather than bladder, as there are very few procedures whereby a healthy bladder is removed. Normal bladder specimens are thereby extremely difficult to obtain for research purposes and for these experiments, normal ureter was used as a control. The same cellular processes are thought to occur in the urothelium derived from ureter as that derived from bladder, however this can not be verified in this study and may provide a limitation. Future work ideally would use normal bladder as the control. This study relied on the collection of KIC samples from hospitals in the UK. 20 KIC samples were included in this study. The clinical details of each case have yet to be stratified and correlated with the immunohistochemistry findings and this is an area for future work. A greater number of patients suffering with urinary symptoms secondary to ketamine abuse will be recruited to the study and this will provide more KIC samples for analysis and comparison.

6.2 Neurofilament protein (NFP)

6.2.1

Normal tissue (ureter) did not express NFP, however the majority of KIC samples displayed NFP in the stroma that appeared to be confined to nerve fibres, but these were not prolific. There was no evidence of NFP in the urothelium and thereby NFP did not appear to co-localise with NGFR positive urothelial cells. Three of the KIC samples showed large NGFR positive structures forming around NFP positive fibres. This suggests a neuronal response within the stroma that is consistent with the hypothesis that these changes are driven either directly or indirectly by ketamine.

6.2.2 Future work

Further work is needed to assess whether these findings correlate to the clinical symptoms of ketamine abuser. Serial sectioning of KIC samples would also be useful to look at co-localisation of nerve fibre staining antibodies.

6.3 cKit

6.3.1

This study demonstrated the absence of cKit positive cells from a number of KIC samples. Normal urothelium demonstrated occasional tear-shaped cKit positive cells with a pedicle that extended to the basement membrane. The role of cKit in the urothelium remains poorly understood, however the context of these findings can be explored.

6.3.2 What is cKit?

c-Kit (CD 117) is a tyrosine kinase receptor that is bound to by stem cell factor (SCF) that acts as a cKit ligand. c-Kit is expressed in various cell types including haematopoietic stem cells (HSCs), mast cells, melanocytes, and germ cells. It is also expressed in hematopoietic progenitor cells including erythroblasts, myeloblasts, and megakaryocytes. However, with the exception of mast cells, expression decreases as these hematopoietic cells mature and c-Kit is not present when these cells are fully differentiated [(86)]. The activation of c-Kit leads to the activation of multiple signalling cascades, including the RAS/ERK, PI3-Kinase, and Src kinase pathways. These are important pathways in cellular proliferation, differentiation and survival, and inappropriate activation is common in human cancers such as melanoma and sarcoma [(87,88)]. Studies have shown that malignant melanocytes lose cKit expression during melanoma progression[(89)] thus suggesting an alteration in the cKit pathway that may influence progression to malignant disease.

6.3.3 cKit expression in the gastro-intestinal tract

Previous studies have described interstitial cells of Cajal (ICC) that express cKit. ICC are thought to be pacemaker cells that are important in regulating neurotransmission and smooth muscle activity within the gastro-intestinal tract [(90)]. Mutations in c-kit or stem cell factor have been shown to result in the absence of ICC, and without these cells, electrical slow waves are absent and peristalsis becomes impaired.

6.3.4 cKit expression in the urinary tract

Animal studies have described the presence of ICC in the urothelial, suburothelial and muscular layers of the urinary tract [(91,92,93)]. ICC in the suburothelial layers were found

to be more prevalent in rats with bladder outflow obstruction, however the study that included urothelium, showed a decrease in ICCs in the urothelial area and detrusor smooth muscle in menopausal rat urinary bladders which exhibited bladder overactivity.

One study in humans investigated the expression of c-kit positive cells in the muscle layer of congenital pelvi-uretric obstruction in children and found decreased expression in the muscle layers compared to normal ureter[(94)]. Although there is little evidence from previous studies regarding the role of cKit within the urothelium, decreased expression in the urothelium of rat bladders with bladder overactivity could indicate a mechanico-sensory role of cKit expressing cells within normal urothelium, acting in a similar role to interstitial cells of Cajal found in the submucosal layers of both the urinary and gastrointestinal tract, and that can become down-regulated in disease. Evidence to support this in this study can be found in the immunohistochemistry performed on KIC samples that demonstrated the absence of cKit in the urothelium. Furthermore, ketamine cystitis patients often have the clinical symptoms of bladder overactivity with urinary frequency, urgency, and nocturia. The correlation of symptom severity with absence of cKit on immunohistochemistry has yet to be established and will provide an area for future work, with stratification of patients to the respective KIC specimens. The presence of cKit within the stroma was no different in the comparison of normal tissue with KIC samples - expression of cKit and cellular structure were consistent with mast cells and therefore no further inferences were made regarding cKit presence within the stromal layer.

Another possible role of cKit within the urothelium is as a marker of regulation. As discussed previously, cKit is important in regulatory pathways that determine proliferation, differentiation, and survival, therefore absence of cKit could indicate a disruption in the normal pathways that lead to tissue damage. This could lead to an impaired ability for the urothelium to regenerate and this would correlate with both the macroscopic and microscopic appearances of the bladder in ketamine cystitis patients with evidence of tissue denudation.

Western blot analysis of NHU cells treated with ketamine showed reduced cKit protein expression compared with the control NHUs without treatment. This in vitro experiment supports the theory that ketamine down-regulates cKit protein expression in NHU cells. However, RT-PCR performed on two NHU cell lines treated with ketamine versus the

normal control detected cKit transcript in both day 7 and day 14 of the ketamine treated cell samples. This suggests that cKit transcript expression increases in the presence of ketamine, but cKit protein expression is reduced as shown using the Western blot. The reasons for this remain unclear. One possibility is experimental differences and the first thing to do will be to repeat these experiments to ascertain consistent reproducibility. There are many processes between transcription and translation - the stability of cKIT protein has yet to be established and how ketamine may directly or indirectly influence this stability.

6.3.5 Limitations and future work

A relatively small number of KIC samples were studied (20) and correlation to the clinical symptoms was not possible at this stage. Future work will aim to stratify patients to KIC samples to identify whether symptom severity affects cKit expression. The results that showed increased expression of cKit transcript, but reduced cKit protein in ketamine treated cells provides a very interesting area for further work to explore the potential mechanisms that are involved.

6.4 Smooth muscle actin (SMA) and Vimentin

6.4.1 Smooth muscle actin

This study identified SMA expression in the urothelium of two KIC samples and was absent in normal urothelium. Submucosal SMA expression was also increased in the KIC samples, particularly in the stroma. The implication of SMA expression in urothelial cells raises questions about the cell phenotype and its role in KIC.

SMA is predominantly expressed in smooth muscle cells, of which smooth muscle α-actin is the predominate isoform. Cell types such as myofibroblasts usually stain for smooth muscle actin and the intermediate filament vimentin which is a general mesenchymal marker, and are found subepithelially in many mucosal surfaces such as the gastrointestinal and genitourinary tracts [(95)]. One possibility is that tissue damage form ketamine is stimulating a regenerative response, with the appearance of myofibroblasts in the urothelium. To test this hypothesis, immunolabelling with vimentin was performed on all KIC samples but with a particular focus on the samples that showed urothelial SMA expression in order to support the theory that SMA positive cells were myofibroblasts. The implication of identifying myofibroblasts in the urothelium would be highly significant given the role of myofibroblasts. Their role in wound contraction and healing by using smooth muscle type actin-myosin complexes would certainly fit with the overall clinical picture of ketamine cystitis patients with contracted and small capacity bladders.

6.4.2 Vimentin

Vimentin is a type III intermediate filament (IF) protein that is expressed in mesenchymal cells and is often used as a marker of mesenchymally-derived cells or cells undergoing an epithelial-to-mesenchymal transition (EMT) during both normal development and metastatic progression[(96)]. Mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate into a variety of cell types and exhibit two main characteristics that define stem cells: self-renewal and differentiation. MSCs can also migrate to sites of injury, inflammation, tumour, and can differentiate to form myofibroblasts with increased expression of SMA[(97,98)]. This study identified SMA in the urothelium, however these cells did not express vimentin. Uncertainty remains therefore over the cell phenotype of the SMA positive cells. One of the possibilities is

that the anti-SMA rabbit antibody was non-specific in labelling for SMA, and that the positive cells were artefacts.

6.4.3 Future work

Future work will use alternative SMA antibodies to compare the results of the immunolabelling. Another possibility is that SMA expressing cells are not myofibroblasts, but have an alternative cell phenotype that expresses SMA but not vimentin, and this provides an area for future work.

6.5 Neuronal bioassay

6.5.1 B104 and LAN-5 neuroblastoma cells

This study successfully established neuronal cell bio-assays with two individual types of neuronal cells: B104 rat neuroblastoma cells, and LAN-5 human neuroblastoma cells.

Experimentations with B104 cells used treatments that included nerve growth factor (NGF), and cyclic adenosine monophosphate (cAMP), with comparison of neurite length against a no treatment control. Despite a statistically significant difference in average neurite length being identified between the cAMP treated cells and the no treatment control, and a significant difference being detected between the NGF treatment group and the cAMP treatment group, it was unfortunate that these results were invalid due to contamination with *Mycoplasma spp*.

The LAN-5 neuroblastoma cells were used for experimentation as an alternate neuronal cell line. Treatments included retinoic acid, NGF, and ketamine versus a no treatment control. One of the experiments encountered a problem with cell adherence to the glass slide with loss of cells in the control sample at the time of washing. This was thought to be due to inadequate preparation of the slide with ethanol cleaning prior to cell seeding. On repeat experimentation, there were no problems with cells adherence to the glass slides. Loss of the control group in the first experiment meant that interpretation of data was limited to only being able to assess for differences between treatment groups. There appeared to be a difference between treatment groups, with the greatest effect on neurite length seen in the retinoic acid group, followed by NGF and then ketamine. The second experiment showed the greatest effect on neurite outgrowth in the ketamine treated cells, followed by NGF, retinoic acid, and the control group respectively. Retinoic acid and cAMP were chosen as treatment groups because of their neuronal inducing properties; these treatments acted as useful markers with positive agonistic behaviour against which ketamine and NGF could be compared [(76,77)].

Previous studies have used neuronal cell lines such as LAN-5 and PC12 to assess the response to factors such as NGF [(71,72)]. PC12 is a cell line derived from a phaeochromocytoma of the rat adrenal medulla and cells terminally differentiate when treated with nerve growth factor[(72,75)]. This makes PC12 cells useful as a model system for neuronal differentiation. For this study however, PC12 cells were not readily

available and therefore alternate cell lines were chosen as a means to assess whether these could be used as a viable model for neuronal differentiation. B104 cells appeared to show positive effects from NGF regarding neurite outgrowth and similar findings were shown in LAN-5 cells, suggesting that these cell lines could be useful model for neuronal differentiation. Furthermore, the potentiation of neuronal differentiation that was seen in this study in ketamine treated cells has also been shown in another study that performed neurite outgrowth assays with PC12 cells treated with ketamine[99]. The hypothesis remains that ketamine is able to stimulate a neuronal response either directly through the effects as seen on neuronal cell lines, or indirectly via the stimulation of the release of factors that could include factors such as NGF which has also been shown to have a positive agonistic effect on neuronal differentiation. The correlation of these effects, with the development of painful bladder symptoms reported by ketamine abusers provides an interesting area for further work. From this platform, it is reasonable to suggest that LAN-5 neuroblastoma cells in culture can provide a useful model for assessing neuronal differentiation induced by both ketamine, and NGF.

6.5.2 Limitations and future work

Contamination with *Mycoplasma spp.* was unfortunate as it prevented accurate interpretation of the data from the B104 cells which may have been a good model for assessing neuronal differentiation. To encounter this problem was however a useful lesson in working with cells in culture and understanding the potential pitfalls to laboratory work and data gathering.

Future work will use the model described with LAN-5 neuroblastoma to establish a co-culture system whereby conditioned medium from ketamine treated NHU cells is added to LAN-5 cells, with subsequent measurement of neurite outgrowth.

7. Conclusions

Ketamine induced bladder dysfunction, or ketamine cystitis, has become a more recognised condition amongst urologists within the last five years and continues to cause significant and disabling symptoms for patients who are usually of a young age group. The use of ketamine as a party drug is on the increase, and it is therefore inevitable that more patients will present to specialists with this condition over the coming years, thus highlighting the need for research into the potential mechanisms by which damage to the urinary tract occurs. Once we have a better understanding of the pathophysiological mechanisms at work, we are in a better position to provide and develop more focussed treatments for a condition which is unresponsive to conventional pharmacological treatments, and can often result in the need for major surgery such as cystectomy.

This study has identified the upregulation of NGFR expression in the urothelium of KIC samples, and the appearance of large follicle-like NGFR positive areas within the stroma that are likely to represent large nerve fibres. It is hypothesised that ketamine is either directly or indirectly stimulating NGFR upregulation in both the urothelium and the stroma, and that the role of NGFR is important in the pathogenesis of pain symptoms in ketamine cystitis as seen in other studies that have revealed NGFR upregulation in painful bladders after chemical irritation. The immunohistochemical findings in KIC samples correlate with this hypothesis, and furthermore, the neuronal cell bioassays have shown a neurogenic response in the presence of ketamine. The implication of ketamine acting as a stimulator of neurite outgrowth in neuronal cells supports the theory for a direct role of ketamine in a neurogenic response within the urinary tract. However, the potential indirect role of ketamine causing NGFR upregulation, and the role of urothelial cells in this mechanism has yet to be established. Future work will involve the now established bioassay with LAN-5 neuroblastoma cells to create a co-culture system whereby conditioned medium from ketamine treated urothelial cells in vitro is added to neuronal cells in culture to study possible neurite potentiation. The theory remains therefore that urothelial cells could, in the presence of ketamine, produce factors that are important in the upregulation of NGFR and thus the development of painful bladder symptoms. Alternatively, other indirect effects of

ketamine could be the induction of centrally produced circulatory neurotrophic factors in vivo, such as BDNF, and this poses further questions about possible systemic effects of ketamine on the urinary tract, providing an interesting area for future work.

This study showed the absence of a sub-population of cells that expressed cKit in KIC specimens. The role of cKit in the urothelium remains poorly understood and the mechanism for the loss of these cells remains unknown, however it has been shown in vitro that cKit is present in ketamine treated NHU cells at the transcript level but becomes reduced at the level of protein expression. The mechanism by which this occurs will provide an area for further research. It is possible that cKit expressing cells in the urothelium play a role in mechanico-sensory stimulation, and that the absence of this cell-type could contribute to the dysfunction of the bladder and the development of the typical urinary symptoms seen in ketamine cystitis.

8. Suppliers

Abcam	330 Cambridge Science Park Cambridge CB4 0FL
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