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Published in:
European Journal of Pharmacology

DOI:
10.1016/j.ejphar.2015.05.037

2015

Link to publication

Citation for published version (APA):

Total number of authors:
5

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Non-uniform changes in membrane receptors in the rat urinary bladder following outlet obstruction

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ABSTRACT

The aim of the present study was to investigate the expression and distribution of membrane receptors after bladder outlet obstruction (BOO). Partial bladder outlet obstruction (BOO) was induced in female rats and bladders were harvested after either 10 days or 6 weeks of BOO. The expression of different receptors was surveyed by microarrays and corroborated by immunohistochemistry and western blotting. A microarray experiment identified 10 membrane receptors that were differentially expressed compared to sham-operated rats including both upregulated and downregulated receptors. Four of these were selected for functional experiments on the basis of magnitude of change and relevance to bladder physiology. At 6 weeks of BOO, maximal contraction was reduced for neuromedin B and vasopressin (AVP), consistent with reductions of receptor mRNA levels. Glycine receptor-induced contraction on the other hand was increased and receptor mRNA expression was accordingly upregulated. Maximal relaxation by the β3-adrenergic receptor agonist CL316243 was reduced as was the receptor mRNA level. Immunohistochemistry supported reduced expression of neuromedin B receptors, V1a receptors and β3-adrenergic receptors, but glycine receptor expression appeared unchanged. Western blotting confirmed repression of V1a receptors and induction of glycine receptors in BOO. mRNA for vasopressin was detectable in the bladder, suggesting local AVP production. We conclude that changes in receptor expression following bladder outlet obstruction are non-uniform. Some receptors are upregulated, conferring increased responsiveness to agonist, whereas others are downregulated, leading to decreased agonist-induced responses. This study might help to select pharmacological agents that are effective in modulating lower urinary tract symptoms in BOO.
Key Words: membrane receptor; bladder outlet obstruction; detrusor; Glycine receptor; Neuromedin B receptor; AVP; V1a receptor; β3-adrenoceptor; thrombin receptor; Tachykinin receptor1; endothelin type A receptor; Lgr5; Lgr6; purinergic receptors; P2x5

Abbreviations and Acronyms: bladder outlet obstruction; BOO; Benign prostatic hyperplasia, BPH; Neuromedin B receptor, Nmbr; Glycine receptor, Glra1; β3-adrenergic receptor, Adrb3; thrombin receptor, F2r; tachykinin receptor 1, Tacr1; endothelin type A receptor, Ednra

1. Introduction

Benign prostatic hyperplasia (BPH) is a common urological problem in elderly male patients, affecting 30% of men over 60 (Boyle et al., 2003). Clinical manifestations of BPH include increased micturition frequency, urgency, hesitancy, and incomplete bladder emptying. This may be followed by urinary retention or incontinence (Barry et al., 2008). Bladder outlet obstruction (BOO) is a common pathological consequence of BPH, resulting in an increased urethral resistance and an increased bladder wall tension (Greenland et al., 2001). This leads to growth and remodeling of the bladder, resulting in an increased bladder wall thickness and fibrosis, and a variety of other morphological, contractile and biochemical changes within the bladder wall (Gabella and Uvelius, 1990, O’Connor et al., 1999, Schröder et al., 2001).

Membrane receptors, including ion channel-linked receptors, enzyme-linked receptors, and G protein-coupled receptors, are specialized integral membrane proteins responsible for signal transduction. It has been reported that several membrane receptors are involved in the pathophysiology of BOO. Endothelin A receptor upregulation for example plays a role in modulation of voiding after BOO (Ukai et al., 2006). Angiotensin II receptors, on the other hand, are downregulated, and this is of possible relevance for urinary tract dysfunction in rats with
BOO (Yamada et al., 2009). Accordingly, current therapies for lower urinary tract symptoms target membrane receptors, including M₃ muscarinic receptors (Andersson 2001) and β₃-adrenergic receptors (Andersson et al., 2013, Kuo et al., 2014). Our knowledge regarding effects of BOO on membrane receptor expression is still limited however, and an unbiased survey of receptor expression in BOO may disclose novel targets for therapy.

The aim of the current study was to explore expression of different membrane receptors in BOO using our recent microarray analysis (Ekman et al., 2013), to confirm any changes using immunohistochemistry and western blotting and to examine the consequences for bladder contraction of altered receptor expression.

2. Materials and methods

2.1. Ethics statement and animals

Animal experiments were conducted in conformity with national and international guidelines and were approved by the Malmö/Lund animal ethics committee (M260-11, M113-13). A total of 56 female Sprague-Dawley rats (200–300 g, ≈12w old at surgery) were used for the study. 32 rats received a standardized partial outflow obstruction and 24 were sham-operated. All control rats were matched for age. Rats had free access to tap water and standard pellet food.

2.2. Partial bladder outlet obstruction surgery

Partial outflow obstruction was induced as described (Mattiasson and Uvelius, 1982). Rats were anesthetized with intramuscular injection of 100 mg/kg ketamine (Ketalar, Parke-Davis, Barcelona, Spain) and 15 mg/kg xylazin (Rompun: Bayer AG, Leverkusen, Germany). The bladder and proximal urethra were exposed through a midline abdominal incision and the urethra
was dissected free from surrounding tissue. A stainless steel rod (ø=1mm) was placed along the urethra and a 4/0 Prolene ligature was tightened around the rod and the urethra. The abdomen was closed following removal of the rod. Sham-operated animals went through the same procedure except for the urethral ligation.

2.3. Tissue preparation

The experiment was terminated by killing rats using increasing CO₂ at 10 days or 6 weeks after surgery. These time points represent the end of the early rapid growth phase and chronic outlet obstruction, respectively. The abdomen was opened and the bladder was removed en bloc. Detrusor preparations for western blotting, immunohistochemistry, and mechanical experiments were then prepared by micro-dissection in pre-cooled HEPES buffered Krebs solution (composition in mM: NaCl 135.5, KCl 5.9, MgCl₂ 1.2, glucose 11.6, HEPES 11.6, pH 7.4).

2.4. Microarray data

Microarray data generated in our recent study on miR-29 (Ekman et al., 2013, GEO accession number GSE47080) was surveyed for differentially (q=0 at either 10d or 6w versus sham, by significance analysis of microarrays) expressed membrane receptors by searching for “receptor”. Hits were manually curated and sorted according to fold change (sham versus 6w obstructed) and the five most upregulated receptors and the five most repressed receptors are presented. Absolute signal on the microarray was taken as an approximation of receptor mRNA level in Fig. 1B. Official gene symbols are used for receptors as follows (IUPHAR nomenclature is given first followed in parentheses by official gene names): glycine receptor α₁ subunit (Glra1), leucine-rich repeat-containing G protein-coupled receptor 6 (Lgr6), P₂X₅ receptor (P2rx5), ETₐ receptor (Ednra), PAR1 (thrombin receptor, F2r), NK₁ receptor (Tacr1), β₃-adrenoceptor (Adrb3), V₁ₐ
receptor (Avpr1a), leucine rich repeat containing G protein coupled receptor 5 (Lgr5), and BB1 receptor (neuromedin B receptor, Nmbr).

2.5. Western blotting

Bladders were frozen in liquid nitrogen, pulverized in a mortar and dissolved using lysis buffer (62.5 mM Tris-HCL PH6.8, 2% SDS and 10% glycerol, with protease and phosphatase inhibitor cocktails added) (Shakirova et al., 2006). 25 μg of protein was loaded in the wells of TGX Criterion gels (Any KD, BioRad). Protein was transferred to nitrocellulose membranes using the Trans-Blot Turbo system (BioRad). Membranes were incubated with primary antibodies against β3 adrenergic receptors (ab59685, Abcam, 1:2000), Avpr1a (sc-18096, Santa Cruz, 1:100), Glra1 (OAPC00117, Aviva systems biology, 1:100), Nmbr (HPA026665, Sigma life science, 1:100) and Gapdh (ab9485, Abcam, 1:1000). Bands were visualized using horseradish peroxidase-conjugated secondary antibodies (SAB3700180, Sigma Aldrich, 1:5000, #7074, Cell Signaling, 1:5000, AB7120, Abcam, 1:5000) and West Femto chemiluminescence reagent (Pierce, Rockford, IL). Images were acquired using the LI-COR Odyssey Fc equipment (LI-COR Biosciences).

2.6. Immunohistochemistry

Sham-operated and obstructed bladders were fixed in 4% paraformaldehyde in phosphate buffered saline for 4 hours. Specimens were embedded in paraffin and 5 μm sections were cut using a microtome (Thermo Scientific, HM340E). Sections were incubated in an oven for 2 h at 60°C, deparaffinized, gradient dehydrated and trypsin- or citrate buffer-treated for antigen retrieval. Cross-sections were permeabilized with 0.025% Triton X-100 and unspecific binding was blocked with 2% BSA. Sections were incubated with primary antibody against Glra1
(OAPC00117, Aviva systems biology, 1:1000), Adb3 (TA323760, Origene technologies, 1:20), Nmbr (HPA026665, Sigma life scienc, 1:200), Avpr1a (sc-18096, Santa Cruz, 1:100) overnight at 4°C. After washing in Phosphate-Buffered Saline (PBS), sections were incubated with secondary antibodies (SAB3700840 anti-rabbit, Sigma Aldrich, 1:200, #7074, Cell Signaling, 1:200, AB7120, Abcam, 1:200) for 2h at room temperature. After further washing in PBS, sections were incubated with DAB + Substrate (K346811, Dako). After washing with distilled water, slides were incubated with Mayer’s Hematoxyline for 30 seconds, washed with tap water and incubated in gradient ethanol and xylene followed by mounting with Pertex (00840, Histolab). Images were acquired using an Olympus DP72 microscope equipped with a digital camera.

2.7. Myograph experiments

2 mm wide equatorial detrusor strips from sham-operated rats and rats obstructed for 6 weeks were prepared as described (Sadegh et al., 2011). Strips with intact mucosa were mounted horizontally in myograph chambers (610 M, Danish MyoTechnology, Aarhus, Denmark) containing aerated HEPES buffered Krebs solution with 2.5 mM Ca^{2+}. Following slow stretching to a basal tension of 10 mN and subsequent equilibration for 45 min at 37 °C, preparations were contracted three times with K^{+}-high solution (60 mM KCl, obtained by exchange of NaCl for KCl). Neuromedin B and the β3-adrenoceptor agonist CL316243, which is 10-fold selective over β2 receptors and 130-fold selective over β1 receptors (Baker, 2005), were added in a cumulative manner, the latter following precontraction with 40 mM KCl. AVP was applied at a single supramaximal (Uvelius et al., 1990) concentration (10^{-6} M). No apparent contractile effect was observed when the Glra1 agonist glycine was added to relaxed preparations. Based on prior work with Glra1 in airway smooth muscle (Yim et al., 2011) we predicted that glycine would cause
relaxation and therefore pre-contracted bladder strips with 1 µM carbachol. Thrombin receptors were activated using 10 µM of the TRAP-6 agonist peptide (SFLLRN), which shows little selectivity for PAR1 (F2r) over PAR2 (Lerner et al., 1996). Agonist responses were normalized to the third reference contraction. CL316243 and glycine are the only exceptions, where force is given as a percentage of the force reached on pre-contraction. Each concentration was maintained for 7 minutes. To generate concentration-response curves, force was integrated over the entire stimulation period. For AVP the maximum responses were measured.

2.8. Statistical analysis

Means ± S.E.M.s are shown. A two-tailed student's t-test for paired or unpaired data was used to test for differences using Prism (GraphPad Prism 5.0 Software). N indicates the number of animals. Full concentration response curves were compared using repeated measures ANOVAs. P<0.05 was considered significant. The array experiments were analyzed using Significance analysis of Microarrays and q=0 was considered significant. Significance is indicated by *P<0.05, **P<0.01, and ***P<0.001.

3. Results

3.1. Non-uniform changes of membrane receptor mRNA expression following outlet obstruction

We first surveyed microarrays from sham-operated and obstructed rat bladders (Ekman et al., 2013, GEO accession number GSE47080) for differentially expressed membrane receptors. Fig. 1A shows the five most upregulated receptors (Fig. 1Aa) and the five most downregulated receptors (Fig. 1Ab) at 10 days and 6 weeks of obstruction. Official gene symbols are used throughout. Ligand-gated ion channels, G protein-coupled receptors and orphan receptors were
represented. For example, a glycine receptor (Glra1), an ion channel-coupled receptor, increased 4-fold at 10 days and almost 6-fold at 6 weeks (n=6-8, q=0, Fig. 1Aa). Ednra and F2r, G protein-coupled receptors for endothelin and thrombin, respectively, shared the same pattern of change, being upregulated at 10 days and at 6 weeks (Fig. 1Aa). Tacr1, Adrb3, Avpr1a, Lgr5 and Nmbr on the other hand were reduced at both 10 days and 6 weeks (Fig. 1Ab). The decrease was most pronounced for Lgr5 and Nmbr. mRNAs for membrane receptors therefore change in a non-uniform manner following bladder outlet obstruction.

When mRNA levels change from a very low level the fold change may be large but the functional relevance limited. To compare expression levels between receptors we plotted the signal from the microarrays in sham-operated rats (Fig. 1B). Nmbr expression was highest followed, successively, by Avpr1a, F2r, Tacr1, Ednra, Lgr5, Adrb3 and P2rx5. Glra1 and Lgr6 receptors had the lowest expression levels (Fig. 1B). When expression levels after obstruction were considered (horizontal lines in Fig. 1B) it was clear that Nmbr lost several positions in this ranking whereas Glra1 gained several positions.

mRNA levels for some of the peptide agonists at these receptors were also examined. The AVP mRNA level was unchanged (not shown). Neuromedin B (Nmb) and thrombin were expressed at the level of negative controls, suggesting that they are not synthesized locally in the bladder.

### 3.2. Contractile effects induced by activation of membrane receptors after BOO

Detrusor muscle strips from control and obstructed bladders (6 weeks) were mounted in myographs and agonist-induced responses were measured. We first tested the effect of the Nmbr agonist Neuromedin B. Concentration-dependent contraction reaching saturation at 0.1 μM was seen in strips from sham-operated controls (Fig. 2A). In contrast, no neuromedin B-induced
contraction could be elicited in strips from obstructed bladders. We also examined the effect of the β3-adrenergic receptor agonist, CL316243 and found that concentration-dependent relaxation was elicited in control strips with little effect in strips from obstructed bladders (Fig. 2B). The contractile response on addition of AVP (1 μM) was similarly reduced by obstruction (Fig. 2C). Because we predicted that Glra1 stimulation would cause relaxation we stimulated preparations with glycine following pre-contraction. Contrary to our expectation, addition of glycine (1 mM) caused further contraction relative to the level of pre-contraction (100%, Fig. 2D) in obstructed bladders and had no additional effect in strips from sham-operated bladders. We finally tested the thrombin receptor agonist TRAP-6, but no significant contractile enhancement was seen (not shown).

3.3. Immunohistochemical localization of membrane receptors in the bladder following BOO

To examine the cellular distribution of receptors we stained sham-operated and obstructed bladders (6 weeks) using antibodies. Avpr1a staining was seen inside muscle cells and overall staining was somewhat fainter after obstruction (Fig. 3, top panels). Neuromedin B receptor (Nmbr) staining was similarly seen in detrusor smooth muscle cells. In some preparations we observed fainter staining after obstruction (Fig. 3, middle panels), but this was not consistent throughout. Glra1 receptor staining was observed along the membranes of the detrusor muscle cells in both sham and obstructed bladders, but no change was observed after 6 weeks of obstruction (not shown). Adb3 staining, finally, was observed in detrusor muscle (Fig. 3, bottom panels). No staining was seen in the urothelium (not shown). In the obstructed group, staining for Adb3 was somewhat weaker, and in some specimens there was virtually no staining at all. In all,
these findings support the view that most of these receptors are expressed by detrusor smooth muscle cells and that staining intensity changes with obstruction.

3.4. Western blotting for membrane receptors in sham-operated and obstructed bladders.

We next examined receptor protein expression using western blotting. The Avpr1a antibody detected a band that changed in the expected manner at 10 days and 6 weeks of obstruction (Fig. 4A). Similarly, the Glra1 antibody detected a band that increased after 10 days and 6 weeks obstruction (Fig. 4B). In contrast, the Adrb3 receptor protein was difficult to detect and the Nmbr antibody detected several bands, some of which changed in the opposite direction (not shown). In summary, these findings further supported altered expression of Avpr1a and Glra1 proteins in obstructed bladders.

4. Discussion

In the present study we show that expression of several membrane receptors are affected by bladder outlet obstruction. We present multiple lines of evidence that Glra1 expression increases and that expression of Avpr1a, Adrb3 and Nmbr is reduced. The changes in expression of membrane receptors are thus nonuniform. An important consideration for translation of these changes into functional effects is that membrane area per volume cytoplasm drops dramatically in outlet obstruction (Shakirova et al., 2010). The membrane density of a receptor may therefore be maintained even if its expression level relative to a housekeeping protein in the cytoplasm is reduced. Conversely, an increase in receptor expression relative to a cytoplasmic housekeeping protein would be amplified at the level of membrane receptor density. One might therefore predict that increases in receptor mRNA expression should translate into an increased contractile response more readily compared to the opposite. This was not evident because the response to
Glra1 stimulation with glycine was only modestly increased and the response to thrombin receptor (F2r) stimulation unchanged. In contrast, the contractile responses to agonist for three downregulated receptors (Avpr1a, Adrb3 and Nmbr) were all clearly reduced. This suggests that hypertrophy-associated reductions in receptor expression may translate into functional differences more readily than increases do. If true, this may be due to increased diffusion distances in the enlarged smooth muscle cells (Gabella and Uvelius, 1990) and impaired coupling to downstream signaling pathways (Chang et al., 2009). A potential confounding factor is that expression changes in the mucosa may have little impact on contractility. This represents an alternative explanation for our failure to detect a functional impact of e.g. altered F2r expression. Another factor that must be considered is sex. We only studied female rats and cannot rule out with certainty that the oestrous cycle affect changes reported herein.

Previous studies have shown that glycine receptors, especially Glra1 receptors are localized in the brain stem, cerebellum, hippocampus, and retina (Wenthold et al., 1990, Webb and Lynch, 2007), but extraneuronal Glra1 expression and function has been less widely studied. Yim et al. (2011) identified expression of glycine Glra1 in human and guinea pig airway smooth muscle, and in cultured human airway smooth muscle cells. Here, we demonstrate expression of this receptor in the bladder wall. This, and our mechanical data, suggests that glycine could play a role in the detrusor function. Miyazato and colleagues (2008) found that the spinal and serum glycine levels were decreased in BOO, and suggested that BOO-induced detrusor overactivity might be due to a decreased spinal glycinergic inhibition of detrusor activity. Increased bladder glycine receptor expression after BOO may thus reflect compensation occurring in response to decreased levels of circulating ligand.
β-adrenergic receptors are thought to be involved in relaxation of the detrusor and to delay the urge to void (Andersson et al., 2013). There are differences between species with regard to the receptor subtype that contributes to bladder relaxation. In humans β3-adrenergic receptors are primarily responsible whereas in rats both β2 and β3 receptors seem to be involved (Michel et al., 2006). Previous PCR studies have shown that β3 receptors are located both in the urothelial layer and in the detrusor (Taygi et al., 2009). Our immunohistochemistry suggested that β3-adrenergic receptors were present in bladder smooth muscle, but not in the urothelium. It is important to note, however, that many antibodies for β-adrenoceptor subtypes lack specificity (Michel et al., 2011, Michel et al., 2014, Hamdani and van der Velden, 2009, Pradidarcheep et al., 2009). Our western blot results support this notion ruling out any firm conclusions regarding β3-receptor staining in the rat bladder.

Mirabegron, which is a β3-adrenoceptor agonist, has effects on urgency, urinary incontinence and frequency (Andersson et al., 2013, Kuo et al., 2014) in patients with the overactive bladder syndrome (OAB). Those studies did however not specifically analyze the effect of mirabegron in patients with BOO. In BOO patients, Nitti et al. (2013) found that mirabegron had little effect on OAB symptoms, detrusor pressure at maximum urinary flow, and post-void residual urine, suggesting that mirabegron may have little effect on detrusor function following outlet obstruction in man. Ichihara et al. (2015), on the other hand, found that adding mirabegron to a muscarinic receptor blocker improved symptoms and also increased the post-void residual, but the patients in that study, as judged by from maximum urinary flow rates, were less obstructed. It has been reported that β3-adrenoceptor agonists improve bladder storage function, inhibit smooth muscle contractility, experimental hyperreflexia and detrusor instability also in rat BOO (Woods et al., 2001, Michel et al., 2011). Here we found that the β3-receptor agonist CL316243 caused
less relaxation in BOO than in control detrusor. In all, our results are therefore consistent with prior studies (Svalø et al., 2013, Barendrecht et al., 2009) indicating that relaxation to $\beta_3$-adrenoceptor stimulation is attenuated following outlet obstruction.

It has been reported that the expression of $\beta_3$-adrenoceptor mRNA is significantly decreased in the bladder mucosa from patients with severe BOO compared to mild BOO and controls (Kurizaki et al., 2013). In contrast, Barendrecht et al. (2009) did not find any change in $\beta_3$-adrenoceptor mRNA in bladders from rats with BOO. They had however been obstructed for only 7 days, as compared with 10 days and 6 weeks in the present study.

Arginine-vasopressin (AVP), also known as antidiuretic hormone, is a peptide hormone secreted from the posterior pituitary gland. It has been reported that AVP and $V_1$ vasopressin receptors are present in the rat and rabbit urinary bladder (Uvelius et al., 1990, Holmquist et al., 1990). A decreased contractile effect of AVP has been reported in obstructed rat bladder (Berggren et al., 1993). We now demonstrate that this is associated with a reduced level of $V_{1a}$ receptor mRNA (Avpr1a) and protein. mRNA for vasopressin (Avp) on the other hand was unchanged, arguing that vasopressin is synthesized in the rat bladder but that its synthesis is unchanged after obstruction. Contrasting with vasopressin, neuromedin B (Nmb) and thrombin (F2) mRNAs were not detectable, suggesting that these proteins may be synthesized elsewhere. Our immunohistochemistry showed that $V_{1a}$ receptors were located in the detrusor smooth muscle, as well as in the submucosa adjacent to the urothelium. Taken together, our results indicate that repression of $V_{1a}$ mRNA and protein in BOO leads to reduced responsiveness to exogenous vasopressin.
Nmbr is a G protein-coupled receptor similar to Avpr1a. It regulates exocrine and endocrine secretions, cell growth, body temperature, blood pressure and glucose levels (e.g. Guo et al., 2015). Our array experiment showed that Nmbr was the receptor with the highest mRNA expression among the investigated membrane receptors. It also exhibited the largest relative fold repression in BOO. Immunohistochemistry supported a weaker staining in BOO, which is well in line with the reduced Nmbr mRNA level. It has been reported that Nmbr activation can induce bladder contraction in rat and man (Rouissi et al., 1991, Kullmann et al., 2013). Our myograph results confirm this and moreover demonstrate a striking reduction of neuromedin B-induced contraction in the obstructed bladder. It is presently unclear if neuromedin B plays any physiological role in bladder activation but its presence in pain- and itch-sensing somatosensory neurons has been reported (Fleming et al., 2012). One possibility, therefore, is that axonal reflexes are elicited by outlet obstruction, causing release of neuromedin B onto the detrusor and resultant repression of receptor expression.

In conclusion, we have demonstrated non-uniform changes in membrane receptor expression after BOO. Changes in glycine-, neuromedin B-, CL316243- and AVP-induced bladder contraction and relaxation following outlet obstruction go hand in hand with changes in Glra1, Nmbr, Adrb3 and Avpr1a mRNA levels. These membrane receptor alterations may contribute to bladder dysfunction and could play a role in the pathophysiology of BOO. Attesting to the novelty of our findings, the fate in BOO of at least five of the receptors studied (Glra1, Lgr6, F2r, Lgr5, Nmbr) have not been previously reported.
Acknowledgements

This study was financially supported by The Swedish Research Council, The Greta and Johan Kock’s Foundation, The Crafoord Foundation, The Medical Faculty at Lund University, Gösta Jönsson’s foundation, Olle Engkvist Byggmästare’s foundation and a clinical grant from the Swedish National Health Service (ALF).

Conflict of interest

None

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Legends

Figure 1. Receptor mRNA expression in bladder outlet obstruction. Bladders from sham-operated and obstructed (10 days and 6 weeks) rats were harvested and RNA isolated and used for microarrays (Ekman et al., 2013, GEO accession number GSE47080). Arrays were surveyed for differentially expressed membrane receptors and the five most highly upregulated receptors (panel Aa) and the five most downregulated (panel Ab) receptors were plotted. Official gene symbols are used throughout; IUPHAR names for these receptors are listed in Materials and Methods. n=6-8. All differences between sham and BOO are significant by Significance Analysis of Microarrays (q=0 at either 10 days or 6 weeks versus sham). Panel B shows a comparison of the absolute signal on the arrays for the different receptors, showing an apparently much higher expression of Nmbr than of Lgr6 mRNA.

Figure 2. Membrane receptor alterations predict altered contractile effects in bladder outlet obstruction. Panel A and B show concentration-response relationships for neuromedin B and the β3-receptor agonist CL316243, respectively. Preparations were pre-contracted with 40 mM KCl in B. Panel C shows maximal (1 µM) AVP-induced contraction in sham and obstructed bladders, respectively. Panel D shows glycine-induced force. Glycine was added after pre-
contraction with 1 µM carbachol (=100%) and caused no additional contraction in bladders from sham-operated rats whereas significant additional contraction was seen after obstruction. n=6 throughout. *p<0.05, ***p<0.001.

**Figure 3. Localization of different membrane receptors in control and obstructed urinary bladders.** Staining for Avpr1a, Nmbr and Adrb3 from top to bottom in detrusor from sham-operated (left) and obstructed (right, 6 weeks) rats. Positive staining appears as brown and nuclei are blue. Scale bars indicate 20 µm. Micrographs are representative of results from three pairs of sham and obstructed rats. Arrow-heads point to muscle bundles. * in top left panel highlights a perivascular cell.

**Figure 4. Confirmation of reduced Avpr1a expression and increased Glra1 expression by western blotting.** Panels A and B show summarized data and western blots for Avpr1a and Glra1, respectively, in bladders from sham-operated and obstructed rats. Gapdh was used as loading control. n=8 for all.
Figure 1
Figure 2
Figure 3

*sham* 6w obstruction

Avpr1a

Nmbr

Adb3