THE ROLE OF THE RHO–ROCK PATHWAY IN THE REGULATION OF MICTURITION

PhD thesis

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Abbreviations

ACh – acetylcholine

ATP – adenosine triphosphate

B2m – beta-2 microglobulin

CaM – calmodulin

cAMP – cyclic adenosine monophosphate

CCh – carbamoylcholine

cDNA – complementary deoxyribonucleic acid

Chrm1 – M_1 receptor gene

Chrm2 – M_2 receptor gene

Chrm3 – M_3 receptor gene

Chrm4 – M_4 receptor gene

Chrm5 – M_5 receptor gene

Cre-ERT2 – fusion protein of Cre recombinase with mutated human estrogen receptor ligand binding domain

CTRL – control

G proteins – heterotrimeric guanine nucleotide-binding regulatory proteins

GAP – GTPase-activating protein

GDI – guanine nucleotide dissociation inhibitor

GDP – guanosine diphosphate

GEF – guanine nucleotide exchange factor

GPCR – G protein-coupled receptor

GTP – guanosine-5'-triphosphate

IP_3 – inositol 1,4,5-trisphosphate

IP_3R – inositol trisphosphate receptor

KO – knockout

LUT – lower urinary tract

mAChR – muscarinic acetylcholine receptor

MLC – myosin light-chain

MLCK – myosin light-chain kinase

MLCP – myosin light-chain phosphatase

mRNA – messenger ribonucleic acid

NA – norepinephrine

OAB – overactive bladder syndrome

PKC – protein kinase C

PLC – phospholipase C

PTX – pertussis toxin

qPCR – quantitative polymerase chain reaction

RBD – Rho binding domain

RNA – ribonucleic acid

ROCK – Rho-associated coiled-coil-containing protein kinase

Rock1 – Rho-associated coiled-coil-containing protein kinase 1 gene

Rock2 – Rho-associated coiled-coil-containing protein kinase 2 gene

RyR – ryanodine receptor

SMC – smooth muscle cell

SMMHC – smooth muscle myosin heavy chain

SR – sarcoplasmic reticulum

UB – urinary bladder

UBSM – urinary bladder smooth muscle

WT – wild type

α_1-AR – α_1 adrenergic receptor

α_2-AR – α_2 adrenergic receptor

β_3-AR – β_3 adrenergic receptor

1. Introduction

1.1. Regulation of micturition

The urinary bladder (UB) is a highly flexible, hollow smooth muscle organ with two main functions: storage of urine and expulsion. It features a layered smooth muscle structure with urothelium and lamina propria. The storage space of the urine during the filling phase is maintained by low intravesical pressure sustained by the relaxed urinary bladder smooth muscle (UBSM), especially the detrusor muscle. At the time of voiding the detrusor muscle rapidly contracts which attracts the increase of the intravesical pressure and afterwards expulsion of the urine can occur simultaneously with the opening of the external urethral sphincter. The functions of the urothelium, afferent nerves, spinal and hypothalamic centers, efferent pathways, detrusor and sphincter muscles have to be coordinated during both the storage and voiding phase (1). Disturbances of the filling phase may result in lower urinary tract (LUT) symptoms, like urgency, frequency, urge incontinence and overactive bladder syndrome (OAB). Moreover, the dysregulation of the voiding phase may result in symptoms of hesitancy, weak urine stream, incomplete bladder emptying and post-micturition dribble (2).

Integration of these functions is mediated by a complex neural control system. Most importantly, the bladder is under the control of the parasympathetic and the sympathetic nervous system (3, 4).

1.2. Adrenergic mechanisms of the urinary bladder

The sympathetic nervous system plays the predominant role in the physiological regulation of the UB during the filling phase, since sympathetic nerves activate smooth muscle contraction in the urethra and the bladder neck along with the relaxation of the detrusor muscle (5). Moreover, the inhibition of the micturition stimulating pathways and the activation of inhibitory pathways also contribute to the low and relatively constant bladder pressure (5, 6). The contraction of the urethra and bladder neck and the relaxation of the detrusor muscle are mediated by different adrenergic receptors as a part of the sympathetic system. It has been demonstrated that α_1 and β_3 adrenergic receptors (α_1-ARs, β_3-ARs) are expressed in the human UB. However, the low density of the α_1-ARs has also been confirmed at messenger ribonucleic acid (mRNA) and

protein levels suggesting the predominant role of β_3-ARs in the regulation of detrusor muscle during the storage phase (7). In contrast, the α_1-ARs are important in maintaining outlet resistance (8). The activation of the sympathetic innervation causes the release of norepinephrine (NA) to activate the adrenergic receptors. Therefore, NA causes contraction in the urethral and bladder neck smooth muscle via α_1-ARs in concert with the activation of the β_3-ARs inducing detrusor muscle relaxation (9). Activation of β_3-ARs results in elevation of cyclic adenosine monophosphate (cAMP) production within the cells resulting in relaxation of the UBSM. Moreover, it has been proposed that the β_3-ARs can interact with K^+ channels as well, suggesting a cAMP-independent effect of the β_3-ARs on the detrusor muscle relaxation (10, 11).

Previously, α_2 adrenergic receptors (α_2-ARs) were considered to be the major presynaptic regulators of the release of NA as part of a negative feedback loop. Recently, their role have been reported at postsynaptic locations to regulate the effects induced by catecholamines released from sympathetic nerve terminals (12). However, according to Michel *et al*. (2006), there is no study reporting the expression of α_2-ARs mRNA in the bladder tissue (7), it has been demonstrated that α_2-ARs can be detected in rabbit, pig and human UB at the protein level (13-16). α_2-ARs regulate the activity of the parasympathetic nerves triggering bladder contraction in cats and rabbits by the inhibition of neurotransmitter release from the parasympathetic ganglion (17-20). These findings support the key function of adrenoreceptors, the promotion of the urinary continence. Furthermore, the reduction of volume-induced bladder contractions has been established *in vivo* in anesthetized rats by the stimulation of α_2-ARs (18, 21).

It has been suggested that the contribution of the α_1-ARs to the regulation of the UBSM may change under pathophysiological conditions related to detrusor overactivity (22, 23). In addition, the imbalance between α_1-ARs and β_3-ARs functions has been suggested in bladder outflow obstruction (24, 25).

1.3. Cholinergic neurotransmission regulating voiding mechanism

Under physiological conditions, parasympathetic cholinergic neurotransmission seems to play the dominant role in the regulation of the UB compared to that of the adrenergic mechanisms (1). The parasympathetic nervous system is the key regulator for the UBSM contraction with the aim of urine expulsion at the end of the filling phase.

When the volume of urine reaches a critical threshold within the bladder, contractions of UBSM cells will occur in concert with the opening of the bladder neck and relaxation of the external urethral sphincter resulting in the expulsion of the urine (5). The main neurotransmitter of the parasympathetic neurons is acetylcholine (ACh) (26) which transmits its effects via muscarinic acetylcholine receptors (mAChRs). Moreover, it has been demonstrated that the release of adenosine triphosphate (ATP) also occurs as a co-transmitter after parasympathetic stimulation in some species indicating that the two neurotransmitters maintain the normal bladder function simultaneously (26, 27).

It has been revealed that the mAChRs comprise five subtypes, M_1-M_5 (28). Although they show a high degree of homology, their function in physiological responses and G protein binding affinity differ significantly (29). According to the literature, M_1, M_3 and M_5 receptors prefer to couple to $G_{q/11}$ proteins, whereas M_2 and M_4 receptors show selectivity to $G_{i/o}$ proteins (28, 30-32). To date, it has been demonstrated that the M_1 receptors enhance, whereas M_2 receptors inhibit the release of ACh prejunctionally in rat and rabbit bladders (27, 33). Moreover, it has been suggested that the release of ACh is inhibited by M_4 receptors in both human and murine UBs (34, 35).

It has been established that the mAChRs are expressed in the bladder urothelium and the suburothelial area (36-39). The entire amount of the M_1 receptors is located in basal cells of the urothelial layer (40), whereas M_2 and M_3 receptor expressions were described in detrusor muscle, nerve fibers, urothelium and also in myofibroblast-like cells within the suburothelial layer in the human UB (41). There is conflicting literature data about the expression of the muscarinic receptors in the detrusor muscle. Abrams *et al.* (2006) and Sigala *et al.* (2002) suggested that all mAChR subtypes are expressed in the UBSM (42, 43). However, in other studies, no evidence was demonstrated of the presence of the M_5 receptors at mRNA and protein levels in the detrusor muscle (44-46). Furthermore, Bahadory *et al.* (2013) reported that M_5 receptors are expressed in the porcine and human UB (47), but not necessarily in the UBSM. On the other hand, several studies in human, rat, guinea pig and rabbit bladders have ambiguously demonstrated that only M_2 and M_3 receptors are expressed in the detrusor (27, 45) and principally mediate UBSM contractions, which observation is supported by the predominance of mRNAs encoding M_2 and M_3 receptors in the human UB (43, 44). M_3

receptors were previously regarded as the primary mediators of UBSM contraction and M_2 receptors were believed to play only a supportive role in bladder physiology via antagonizing smooth muscle relaxation induced by G_i-coupled receptors (48). Nonetheless, the significant role of M_2 receptors in the contraction of detrusor muscle was clearly demonstrated by investigations in M_2 receptor-deficient mice (49). Interestingly, the expression of M_2 receptors is outnumbered by that of the M_3 receptors in most species, as the expression density of M_2 receptors is 3-fold higher (50); however, the reason for the abundance of M_2 receptors in the bladder remains to be clarified.

There is good evidence that the function and the expression level of the muscarinic receptors can change in the UB under pathophysiological conditions. The correlation between increased expression levels of M_2 and M_3 receptors in painful bladder syndrome and idiopathic detrusor overactivity suggested their significance in these disorders (40). Furthermore, the impairment of the detrusor function as a result of human prenatal mutation of the M_3 muscarinic receptor has also been reported (51). The expression level changes of the muscarinic receptors in OAB are rather conflicting as there are studies that reported the increased expression of M_2 and M_3 receptors in ovariectomy-induced bladder dysfunction as an animal model of post-menopausal overactive bladder (52); however, other groups demonstrated that there is no alteration of muscarinic receptors in association with OAB symptoms (53, 54). Mukerji *et al.* (2006) revealed the increase of M_2 and M_3 receptors by immunohistochemistry in bladder syndromes and their positive correlation with clinical scores (41). In addition, it has been reported that the M_2 receptors play a pivotal role in UB contraction in neurogenic bladder dysfunctions (55). The promising results of studies about the role of mAChRs under pathophysiological conditions initiated drug development for LUT disorders. Unfortunately; however, the development of small molecule antagonists with clear mAChR selectivity for treating UB disorders turned out to be extremely challenging because of the high degree sequence similarity of these receptors (56) explaining the wide range of adverse effects and low patient adherence associated with the usage of these medicines (57).

1.4. G protein-coupled signaling pathways

The contraction of detrusor muscle cells can be triggered by the release of neurotransmitters from the nerve terminals. The main neurotransmitter to regulate contraction is ACh, which transmits its effects via muscarinic receptors. Muscarinic receptors belong to the family of G protein-coupled receptors (GPCRs) with seven-transmembrane domains with the role of the transmission of external stimuli into intracellular signaling cascades (56, 58). According to the literature, the signal through the GPCR is relayed directly by activating heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) or arrestin (59, 60). The signaling follows the classical paradigm of UBSM contraction with the activation of G proteins that are composed of α, β and γ subunits (61). The binding of the excitatory transmitter promotes the exchange of guanosine diphosphate (GDP) to guanosine-5'-triphosphate (GTP) on the GTPase binding domain of Gα resulting in the dissociation of Gα from Gβγ subunits, each can mediate intracellular signaling on their own (61).

G proteins are classified into four families according to their Gα subunit: G_i, G_s, $G_{12/13}$ and G_q. The G_q family consists of five members: G_q, G_{11}, G_{14}, G_{15} and G_{16} (62, 63). The genes of the G_q and G_{11} proteins are linked explaining their almost identical functionality; however, their distribution is diverse in different tissues (62, 64). As mentioned previously, in the detrusor muscle, M_2 and M_3 receptors are considered to be the principal members of the muscarinic receptor family regulating smooth muscle contraction. M_2 and M_3 receptors are preferentially coupled to G_i and $G_{q/11}$ proteins, respectively (29, 65). The G_q-mediated intracellular signaling involves the activation of the phospholipase C (PLC) enzyme, especially the PLCβ isoform, by the $G\alpha_q$ subunit (66). Although G_i-mediated signaling is typically considered to act via inhibition of the adenylyl cyclase activity, recently it has become clear that G_i is also capable of Rho activation in HEK 293 cells and bronchial smooth muscle (58, 67). In addition, PLCβ can also be activated by Gβγ dissociated from G_i-coupled GPCRs (68). Interestingly, according to Pfeil *et al.* (2020), the G_i-mediated Ca^{2+} signal cannot occur in the absence of G_q (69).

1.5. Intracellular signaling of smooth muscle contraction

The excitation and contraction process of the smooth muscle cells (SMCs) are mediated by two main mechanisms. The activation of the SMCs is regulated by Ca^{2+}-dependent and Ca^{2+}-independent, Ca^{2+} sensitizing signaling pathways. Recently, it has become clear that the Ca^{2+} sensitizing pathway also plays a pivotal role in the regulation of the excitation-contraction coupling and under physiological conditions these two mechanisms act simultaneously. The increase in intracellular Ca^{2+} levels can occur from the extracellular space or intracellular sources. To maintain the increased Ca^{2+} concentration in the intracellular space Ca^{2+} binds to the regulatory protein calmodulin (CaM) forming the $Ca^{2+}-CaM$ complex (70-72). The consequent conformation change of CaM ensures the interaction with different molecular targets; for instance, myosin light-chain kinase (MLCK) (73). The activated MLCK increases myosin ATPase activity thereby phosphorylating the myosin light-chain (MLC) (74) leading to the interaction of actin and myosin chains, initiating the cross-bridge cycle and contraction (75). The decreasing concentration of intracellular Ca^{2+} leads to the dephosphorylation of MLC by myosin light-chain phosphatase (MLCP) resulting in smooth muscle relaxation. The inhibition of the MLCP strengthens smooth muscle contraction by increasing MLC phosphorylation resulting in hypercontractility (76).

a) Calcium-dependent pathway of smooth muscle contraction

Among its peculiar coordination chemistry, Ca^{2+} plays an important role in the intracellular signaling of all cell types, including SMCs (71). Ca^{2+} can arise from the extracellular space through influx via various Ca^{2+} channels. Most importantly, voltage-gated Ca^{2+} channels open after the depolarization of the plasma membrane. In addition, the appearing Ca^{2+} triggers the activation of ryanodine (RyR) and inositol trisphosphate receptors (IP_3R) in the sarcoplasmic reticulum (SR) consequently causing the efflux of Ca^{2+} from the SR into the cytoplasm (77, 78). However, IP_3R activation is predominantly induced by binding inositol 1,4,5-trisphosphate (IP_3) which is a signaling molecule derived from the hydrolysis of phosphoinositol 4,5-bisphosphate by PLCβ (66). Based on the literature, both the Ca^{2+} influx and the release of Ca^{2+} from SR are necessary for detrusor muscle contraction (79).

b) Calcium sensitizing pathway of detrusor contraction

Ca^{2+} sensitization means the inhibition of the MLCP consequently inducing increased contraction force while Ca^{2+} is maintained at a constant level with MLCK activity (79, 80). Nevertheless, the modulation of Ca^{2+} sensitivity is not limited to the phosphorylation of myosin, but it can be achieved by changing the Ca^{2+} sensitivity of IP_3Rs or also by the phosphorylation of RyRs (81-83). Additionally, Ca^{2+} sensitivity does not seem to be affected by IP_3 and it is also under examination whether diacylglycerol, the other product of PLC, has a considerable role in the regulation of Ca^{2+} sensitivity (84). Moreover, it was previously described, that both protein kinase C (PKC) and Rho proteins may induce increases in Ca^{2+} sensitivity (85, 86). However, other studies show that the sensitization mechanism is not augmented by PKC (87-89). In consideration of previous studies, the MLCK:MLCP ratio and the RhoA–Rho associated coiled-coil-containing protein kinase (ROCK) pathway are believed to be the dominant regulator of Ca^{2+} sensitivity (75, 84, 90-99).

The RhoA protein is a small, monomeric G protein belonging to the family of Ras, a protein family of small GTPases. Although Rho proteins have certain differences, similar to the above-mentioned heterotrimeric G proteins, they can also switch between their inactive and active states by binding GDP or GTP, respectively.

The regulation of Rho protein activation is implemented in three levels by guanine nucleotide dissociation inhibitor (GDI), GTPase-activating protein (GAP) and guanine nucleotide exchange factor (GEF). Furthermore, it has been noted that high protein kinase G expression level is associated with the regulation of Rho transcription, suggesting that the overall activity of Rho proteins can also be regulated transcriptionally (100). By default, GDI binds by its lipid tails to the Rho protein forming a soluble complex in the cytosol. Because of the high affinity of GDP and the very slow hydrolysis of GTP, the switch between the active and inactive phase of Rho proteins needs the support of GEFs and GAPs to accelerate GDP dissociation and the GTP hydrolysis, respectively (101). Posttranslational modification on the C terminal may lead to the activation of Rho by the dissociation of GDI by the GDI displacement factor, which is the first step of the activation process. It is necessary to provide an appropriate phospholipid microenvironment for the function of Rho GTPases in order to alter signal molecules' activities and roles (e.g.: p115-RhoGEF needs to bind with the

activated $G\alpha_{12/13}$ protein leading to RhoA activation) (102). Modification of the nucleotide binding site of Rho by GEF eventuates the release of Rho proteins from GDI-bound complexes resulting in an increased amount of GTP-bound Rho proteins by the discharge of GDP (101).

Although more than 30 effector proteins have been identified that interact with the activated Rho, the first established and most studied effector proteins are the ROCKs with diverse biological effects (103-106). Interestingly, ROCKs are key regulators of the Ca^{2+} sensitization mechanism in the smooth muscle. The two protein isoforms encoded by the ROCK genes (*Rock1* and *Rock2*) share 62 % sequence identity and their distribution throughout the body differs markedly. The mRNA of the $ROCK_2$ enzyme is principally expressed in the brain and heart, whereas the $ROCK_1$ is mostly presented in other tissues. Both the $ROCK_1$ and $ROCK_2$ enzymes are found in the UB at both mRNA and protein levels (107-109). ROCK enzymes are members of the AGC kinase family (110) with three major domains: a Rho binding domain (RBD), a kinase domain and a cysteine-rich domain (103, 111). With regard to the observation that their kinase domains are 83 % identical, it has been suggested that they may exhibit similar substrate specificity (112). Both ROCK isoforms are activated by the binding of the Rho protein to the RBD region (104, 106) resulting in the loss of the negative regulatory role of RBD and activation of ROCK (112). Subsequently, ROCK will be able to phosphorylate its target proteins such as MLCP at the myosin phosphatase-targeting subunit 1 causing a reduction of MLCP activity and consequently an increase of phosphorylated MLC (113). Furthermore, it has been suggested that the ROCK can directly phosphorylate MLC *in vitro* (114, 115) and GAP proteins can also be phosphorylated by ROCK resulting in a prolonged Rho activation suggesting the formation of a positive feedback loop in the regulation of the Rho–ROCK pathway (116). Taken together, the activation of the Rho–ROCK pathway goes hand in with decreased MLCP activity and increased phosphorylation of MLC; therefore, the activation of smooth muscle contraction.

It has been reported that the inhibition of Ca^{2+} sensitization by bacterial toxins shows agonist, tissue and species specificity indicating that other pathways may also lead to Ca^{2+} sensitization (91, 97, 117, 118). Further investigations of these pathways would accelerate the understanding of smooth muscle contraction at the molecular level

and may provide pharmacological targets in diseases related to smooth muscle hyper-reactivity.

1.6. ROCK as a pharmacological treatment target for the disorders of the urinary bladder

It has been reported that the Rho–ROCK pathway is involved in a variety of diseases in different species and tissues. The involvement of the ROCK has been established in the regulation of neuronal cell function as well (119). Furthermore, ROCK inhibitors showed potential therapeutic effects in Alzheimer's disease, Parkinson's disease and epilepsy (120-124). In addition, it has also been demonstrated to play an important role in smooth muscle-related diseases/disorders. Inhibition of Rho–ROCK is proposed to be a promising therapeutic target in the treatment of hypertension as well as coronary and cerebral artery vasospasm via the inhibition of smooth muscle contraction (125, 126). Moreover, Mayra *et al.* (2020) indicated that ROCK has a pivotal role in airway smooth muscle hyper-contractility (76). Furthermore, diabetes-associated upregulation of Rho–ROCK and increased contractility of gastric smooth muscle were demonstrated in a study by Mahavadi *et al*. (2017) (127).

Moreover, increased Ca^{2+} sensitivity of the regulatory and contractile proteins appears to maintain spontaneous detrusor muscle contractile activity in pathophysiological conditions (75). The elevation of the Rho and ROCK expression levels was found in UB hypertrophy allowing detrusor contractions at low Ca^{2+} concentration, thereby explaining the sustained contraction response under this condition (128, 129). Furthermore, the role of the ROCK in diabetes-associated bladder dysfunction was suggested previously (108, 130). In addition, an increased expression of RhoA protein has been reported in urinary bladder tissues isolated from spontaneous hypertensive rats with OAB symptoms; however, the expression level of ROCK has not been elucidated (131). Braverman *et al.* (2006) suggested an enhanced role of ROCK in mediating UB contraction in denervated bladder compared to normal bladders (132). In addition, ROCK inhibition had a significant reducing effect on muscarinic receptor-mediated bladder contractions in a rat model of cyclophosphamide-induced cystitis (133, 134). Moreover, the importance of the Rho–ROCK pathway in the

pathophysiology of bladder dysfunctions is also emphasized by the fact that a ROCK inhibitor, fasudil, can alleviate detrusor overactivity in a rat OAB model (135). Interestingly, it has also been reported that ROCK activity increases with age, as does the occurrence of voiding disorders (135-137). Furthermore, the impairment of estrogen deficiency on the expression and function of ROCK has been demonstrated after ovariectomy which might explain the greater incidence of OAB after menopause in women (138-140).

2. Objectives

Although ACh plays an essential role in the regulation of micturition and the alterations of cholinergic neurotransmission have been implicated in the pathogenesis of urinary bladder dysfunctions, our knowledge of the signaling pathways involved in ACh-induced detrusor contraction still remains incomplete.

Antimuscarinics are widely used in the management of OAB, yet several adverse effects limit their application and patients' compliance. Moreover, it has been demonstrated that the Rho–ROCK pathway plays a pivotal role in the regulation of detrusor contraction. Thus, we aimed to better understand the signal transduction of mAChRs and the role of the RhoA–ROCK pathway in murine and human UBSM. Our final goal is to provide a theoretical basis for the development of a more specific medication for OAB with fewer adverse effects.

Specifically, our main objectives were

- to determine which mAChRs mediate carbamoylcholine (CCh)-induced UBSM contractions,
- to identify the G proteins involved in signaling and
- to reveal the intracellular signaling pathways of mAChRs in UBSM, focusing on the role of the RhoA–ROCK pathway.

3. Methods

3.1. Animals

All procedures were carried out according to the guidelines of the Hungarian Law of Animal Protection (28/1998) and were approved by the Government Office of Pest County (Permission numbers: PEI/001/2709-13/2014, PE/EA/925-7/2021).

UBs were obtained from adult male (90–120 days old, 30–35 g), C57BL/6N wild type (WT) mice (from Charles River Laboratories, Isaszeg, Hungary) and from animals deficient in the mAChRs [M_2-KO, M_3-KO, M_2/M_3-KO] (141) developed in the Molecular Signaling Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, USA. In an additional group of mice, $G\alpha_{q/11}$ signaling was conditionally inactivated in a smooth muscle-specific manner ($G\alpha_{q/11}$-KO). The mouse line with smooth muscle-specific inducible deletion of the $G\alpha_{q/11}$ signaling pathway was generated on a $G\alpha_{11}$-deficient (*Gna11*$^{-/-}$) background (142) with floxed alleles of the genes coding $G\alpha_q$ (*Gnaq*$^{flox/flox}$) and expressing a fusion protein of Cre recombinase mutated human estrogen receptor ligand binding domain (Cre-ERT2) (143) under the control of the smooth muscle myosin heavy chain (SMMHC) promoter. Deletion of *Gnaq* was induced by intraperitoneal tamoxifen injection (1 mg/day for 5 consecutive days) in SMMHC-CreERT2$^{+/-}$;*Gnaq*$^{flox/flox}$;*Gna11*$^{-/-}$ mice as described (144). Tamoxifen-treated SMMHC-CreERT2$^{-/-}$;*Gnaq*$^{flox/flox}$;*Gna11*$^{+/+}$ animals served as controls and are referred to as $G\alpha_{q/11}$-CTRL.

Inactivation of $G\alpha_i$ proteins was achieved by intraperitoneal administration of pertussis toxin (PTX) (145) every 24 hours for six times. The control (CTRL) animals received vehicle treatment in the same way as PTX-treated ones. The effectiveness of the PTX treatment was monitored in Langendorff-perfused heart preparations with the methodology described previously (146). Briefly, the lack of the $G\alpha_i$-mediated heart-rate-reducing effect of ACh verified the inactivation of $G\alpha_i$ proteins.

3.2. Human urinary bladder tissues

All procedures involving human UB tissues have been approved by the Scientific and Research Committee of the Medical Research Council of Hungary (License No.: 21545-2/2019/EKU). Human UB tissues were obtained from 19 patients (15 males, 4

females; mean age of 65.5 ± 9.3 years, range between 44 and 78 years) undergoing open radical cystectomy due to muscle-invasive bladder malignancy after having obtained written patient consent. None of the patients had any urodynamic disorders, symptoms of OAB syndrome, or were taking drugs for OAB.

Following the surgical removal of the bladders, they were immediately placed in physiological saline solution and transported to the Department of Pathology, Forensic and Insurance Medicine of Semmelweis University, Budapest. Here, an approximately 3 × 2 cm piece of healthy, tumor-free whole bladder wall tissue was excised by a uro-pathologist within approximately 15–20 min following removal of the bladders from patients. The healthy bladder tissue was immediately placed into room temperature Hank's Balanced Salt Solution and transported to the myograph laboratory, where preparation of the smooth muscle strips was performed immediately. Overall, myographic experiments started within 45–60 min following bladder removal from the patients.

3.3. Preparation of smooth muscle strips

After the euthanasia of the mice by carbon dioxide (CO_2) asphyxiation, the UBs were removed and placed into Krebs solution (119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2 \cdot H_2O$, 1.2 mM $MgSO_4 \cdot 7H_2O$, 20 mM $NaHCO_3$, 0.03 mM EDTA and 10 mM glucose, pH 7.4) at room temperature. Under a dissection microscope (M3Z; Wild Heerbrugg AG, Gais, Switzerland) the serosal surface was cleaned from adipose and connective tissues. Bladders were cut longitudinally into two or four equal-length strips for RhoA activity assay and measurement of bladder contractility, respectively. The whole mucosal layer (urothelium + submucosa) was also gently removed to prevent the potential release of paracrine factors from the urothelium or submucosal tissue and to avoid tension changes related to the contraction of myofibroblasts (147, 148).

Human UB specimens were also placed into Krebs solution at room temperature during the preparation. Under a dissection microscope, the serosal tissue and the mucosal layer were removed. The isolated detrusor muscle specimens were cut into equal, approximately 3 × 2 × 1 mm strips for myography.

3.4. Myography, determination of concentration-response curves

Detrusor muscle strips were mounted perpendicularly on two parallel tissue-holding needles of a myograph (200-µm needles, 610 M Multi Wire Myograph System, Danish Myo Technology A/S, Aarhus, Denmark). Chambers were filled with 6 mL of 37 °C Krebs solution aerated with carbogen (mixture of 5 % CO_2 and 95 % O_2). Detrusor muscle contractions were registered under isometric conditions. The emerging contraction force was measured along the longitudinal axis of the samples parallel with the axis of the force transducer.

Every experiment started with a 60-minute resting period while the UB strips were stretched to and stabilized at a resting tension of 5 mN. After the equilibration period, the UBSM was challenged twice with 124 mM K^+-containing Krebs solution (made by iso-osmolar replacement of Na^+ by K^+) to test the viability of the tissues. To investigate the contractility of UBSM strips from mice of different experimental groups, we compared their responses to the 124 mM KCl solution. The contractile forces did not show statistically significant differences in different mouse strains, implying that they show a similar ability to generate UBSM contraction. However, according to the recommendation of Erdogan *et al.* 2020 (149) in the case of using K^+-induced contraction as a reference, we are presenting quantitative KCl responses for each experimental group in Figures 1 and 2.

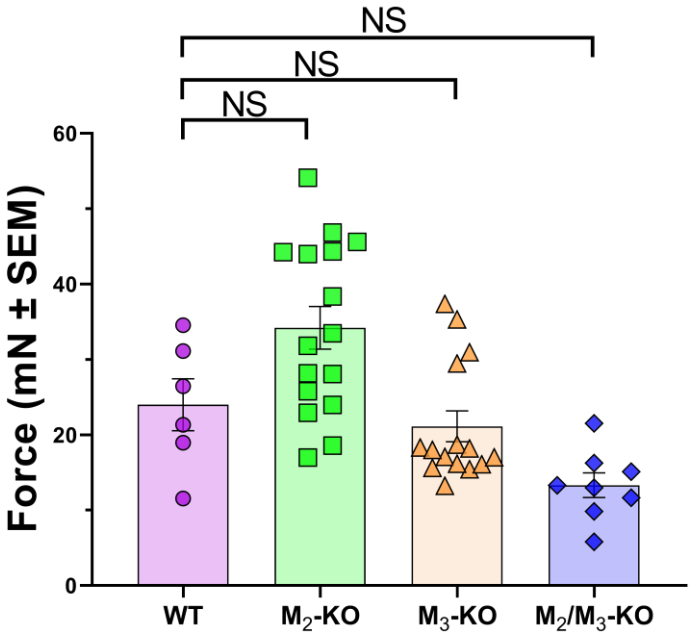

Figure 1 | Comparison of urinary bladder smooth muscle (UBSM) contractions induced by 124 mM KCl solution in wild type (WT) and muscarinic receptor M_2-, M_3-, M_2/M_3-knockout (KO) mice[1]. Contractions induced by 124 mM KCl solution did not differ significantly in UBSM strips prepared from WT or KO animals. WT: n = 6, M_2-KO: n = 16, M_3-KO: n = 15, M_2/M_3-KO: n = 8. Kruskal-Wallis test, $P < 0.05$ was considered as significant difference.

[1] Balla, Helga; Borsodi, Kinga; Örsy, Petra; Horváth, Béla; Molnar, Peter J.; Lénárt, Ádám; et al. (2023). Supplemental Material Intracellular signaling pathways of muscarinic acetylcholine receptor-mediated detrusor muscle contractions. figshare. Figure. https://doi.org/10.6084/m9.figshare.23859108.v1 (150)

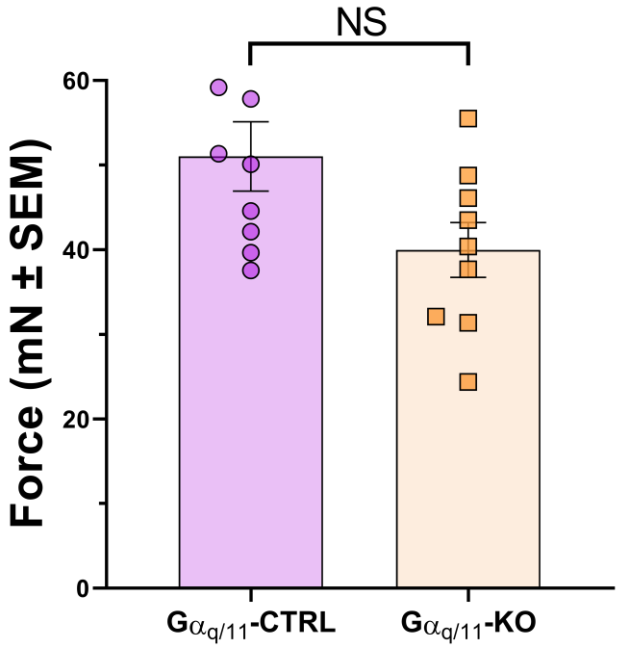

Figure 2 | Comparison of detrusor contractions induced by 124 mM KCl solution in $G\alpha_{q/11}$ control (CTRL) and knockout (KO) mice[2]. Contractions induced by 124 mM KCl solution did not differ significantly in detrusor strips prepared from CTRL or KO animals. $G\alpha_{q/11}$-CTRL: n = 9, $G\alpha_{q/11}$-KO: n = 9. Mann-Whitney test, $P < 0.05$ was considered as significant difference.

After determining the K^+-induced responses, the chambers were washed several times with normal Krebs solution and a 25-30 min resting period was allowed before obtaining cumulative concentration-response curves of CCh (10^{-4}-10^{-8} M). Some of the strips were incubated with the ROCK inhibitor Y-27632 (1 μM or 10 μM, 20 min) before the administration of the agonist. The concentration of Y-27632 that was used in the present study reportedly inhibits both $ROCK_1$ and $ROCK_2$ without any effects on other kinases in the detrusor muscle (107). Finally, bladder strips were exposed to 124 mM K^+-containing Krebs solution to retest the viability of the detrusor strips at the end of the experiment. CCh-induced contractions were expressed as the percentage of the contraction induced by 124 mM K^+ (second administration) in the same preparation.

[2] Balla, Helga; Borsodi, Kinga; Örsy, Petra; Horváth, Béla; Molnar, Peter J.; Lénárt, Ádám; et al. (2023). Supplemental Material Intracellular signaling pathways of muscarinic acetylcholine receptor-mediated detrusor muscle contractions. figshare. Figure. https://doi.org/10.6084/m9.figshare.23859108.v1 (150)

For the acquisition and analysis of myographic measurements, the MP100 system and AcqKnowledge 3.9.1 software from Biopac Systems (Goleta, CA) were used. The moving average smoothening function of the software was applied on recordings solely to eliminate the noises arising from the bubbling of the medium and to reduce the high-frequency, low-amplitude spontaneous tension oscillations. The parameters of the smoothening filter were carefully chosen in order to eliminate only the noises but not to alter the amplitude of the CCh-induced responses; therefore, the baseline and peak values of contractions were always compared before and after the smoothing. The sample rate of the recordings was 10 samples/s (10 Hz) and the smoothing factor was between 10 and 40 samples.

3.5. Quantification of RhoA activation

Mouse UBs were cut into half and the detrusor muscle was prepared as described above. After 60 min of resting, bladder strips were pre-stretched to 5 mN in myograph chambers containing normal Krebs solution. Next, one-half of each bladder was challenged with a single dose of CCh (3×10^{-5} M, which induces submaximal contraction) or its vehicle. At 3 min after the CCh or vehicle administration, the strips were removed and rapidly snap frozen in liquid nitrogen to stop enzyme activity immediately.

To quantify the amount of activated and total RhoA protein, a RhoA G-LISA Activation Assay Kit and Total RhoA ELISA Assay Kit were used, respectively. In addition to the recommended assay protocols, the lysis buffer was supplemented with protease and phosphatase inhibitors (1 % Cytoskeleton protease inhibitor cocktail, 50 mM sodium fluoride, 1 % phosphatase inhibitor cocktail and 1 mM 4-nitrophenyl phosphate). Activated RhoA-GTP was normalized to the total RhoA content. Vehicle-treated baseline and its paired CCh-activated RhoA activity measurements were performed from the two halves of the same UB.

3.6. Evaluation of gene expression

UBSM strips of WT, M_2-, M_3-, M_2/M_3-KO mice were stored at −80 °C until quantitative polymerase chain reaction (qPCR) analysis. Detrusor samples were homogenized (BEL-19923-0000, Capitol Scientific, Austin, TX, USA) and subsequently lysed in 0.5 ml Tri Reagent. A total of 0.2 ml chloroform was added per

1 ml Tri Reagent and the samples were mixed for 15 seconds by vigorous shaking. Phase separation was allowed by placing the samples at room temperature for 15 min followed by centrifugation at 12,000 × *g* for 15 min at 4 °C. The upper aqueous phase was transferred to a fresh tube. 0.5 ml isopropanol/1 ml Tri Reagent was added, thoroughly mixed, incubated for 10 min and centrifuged at 12,000 × *g* for 10 min at 4 °C to precipitate the ribonucleic acid (RNA). The RNA pellet was washed with 2 ml ethanol per 1 ml Tri reagent and centrifugated at 12,000 × *g* for 8 min at 4 °C. The supernatant was aspirated and the sediment was air-dried. Total RNA was dissolved in UltraPure water and RNA quantity and purity of all samples were measured with a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Total RNA samples were stored at −80 °C until use.

First-strand complementary deoxyribonucleic acid (cDNA) was prepared from tissue RNA preparations using RevertAid First Strand cDNA Synthesis kit and Bio-Rad C1000 Touch PCR thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Reverse transcription was carried out according to the manufacturer's instructions using the following temperature cycles: 25 °C for 5 min, 42 °C for 60 min and 70 °C for 5 min. Thereafter, the cDNA was stored at −20 °C until further analysis.

Specific primer sets were designed by using Primer3Plus (https://www.primer3plus.com/) and/or Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and obtained from Sigma-Aldrich (151, 152). The PCR primer sequences are listed in Table 1.

qPCR reactions were performed on CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using SsoAdvanced Universal SYBR Green Supermix. The sample volume was 10 µl containing 2 µl diluted cDNA, 1 µl forward and 1 µl reverse primer (0.5-0.5 µM), 5 µl SsoAdvanced Universal SYBR Green Supermix and UltraPure water. All experiments were performed in triplicate. Temperature cycles were as follows: 95 °C for 60 s, 95 °C for 10 s and 58 °C for 30 s (40 cycles), followed by a melting curve analysis by heating from 65 °C to 95 °C with a rate of 0.5 °C/s and continuous fluorescence measurement. The specific PCR products were analyzed by gel electrophoresis to exclude the presence of primer dimers and verify the correct PCR product length.

Table 1 | The gene identities and forward (F) and reverse (R) primer sequences used for transcriptomic analysis of different genes.

Gene name	Primer sequence	NCBI reference sequence number	Reference
Target genes			
Chrm1 (cholinergic receptor, muscarinic 1)	F: AAGTGGCATTCATCGGGATCACCAC R: TGTTGACCTTGAAGGAGATGAGCAC	NM_007698.3	(153)
Chrm2 (cholinergic receptor, muscarinic 2)	F: GCTATTACCAGTCCTTACAAGACA R: CCAGAGGATGAAGGAAAGAACC	NM_203491.4	(154)
Chrm3 (cholinergic receptor, muscarinic 3)	F: AGGGCTGACTACTTAATCTTGGATA R: TGCAAGGTCATTGTGACTCTC	NM_033269.4	(155)
Chrm4 (cholinergic receptor, muscarinic 4)	F: TGAAGGTCTCCAGAGGTGGG R: TTTAGAGACCTGGGGTCCTTC	NM_007699.3	(152)
Chrm5 (cholinergic receptor, muscarinic 5)	F: TACTATTGCAGCTGTGACCG R: GCCAAGCTGAGCAGGTAATA	NM_205783.2	(152)
Rock1 (Rho-associated coiled-coil containing protein kinase 1)	F: ACCAGGGCATCCAATCCATC R: GCATCATGTCGACTGGGGAC	NM_009071.2	(152)
Rock2 (Rho-associated coiled-coil containing protein kinase 2)	F: GGCGAGAATGTGATTGGTGG R: GTTCCTACAAGTGAATCTGCGT	NM_009072.2	(152)
Reference gene			
B2m (beta-2 microglobulin)	F: CTTTCTGGTGCTTGTCTCACTG R: AGTATGTTCGGCTTCCCATTC	NM_009735.3	(152)

3.7. Drugs and solutions

Carbamoylcholine was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in saline to a stock solution of 2×10^{-1} M. Y-27632 was from Cayman Chemical (Ann Arbor, MI, USA) and it was dissolved in saline to 10^{-3} M stock solution. RhoA G-LISA Activation Assay Kit (Colorimetric format, catalog no. BK124), Total RhoA ELISA Assay (catalog no. BK150) and Cytoskeleton protease inhibitor cocktail (catalog no. PIC02) were purchased from Cytoskeleton (Denver, CO, USA). PTX was purchased from Cayman Chemical (Ann Arbor, MI, USA) and was dissolved in 20:80 glycerin:phosphate buffered saline (PBS) to 50 μg/500 ml stock solution. Before the administration, the stock solution was diluted 20-fold with saline. Isopropanol (catalog no. 59304), the RevertAid First Strand cDNA Synthesis kit (catalog no. K1621) and UltraPure water (Invitrogen) were from Thermo Scientific (Waltham, MA, USA). Tri Reagent (catalog no. R2050-1-50) was purchased from Zymo Research (Irvine, CA, USA) and chloroform was from Reanal Laborvegyszer Kft

(Budapest, Hungary). Ethanol and SsoAdvanced Universal SYBR Green Supermix were purchased from Molar Chemicals Kft (Budapest, Hungary) and Bio-Rad Laboratories (Hercules, CA, USA), respectively. All other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.8. Data analysis and statistics

The CCh-induced detrusor muscle contraction was defined as the peak value of tension developed after the addition of each concentration of CCh.

Raw qPCR data were analyzed using CFX Maestro Software 2.2 software (Bio-Rad Laboratories, Hercules, CA, USA). Data evaluation, statistical analysis and graphs were performed by GraphPad Prism software (v.6.07; GraphPad Software Inc., La Jolla, CA, USA).

The Pfaffl algorithm, also known as the delta–delta CT (ΔΔCT) method, with efficiency correction, was used to calculate the expression of the β-2 microglobulin (*B2m*), M_1 receptor gene (*Chrm1*), M_2 receptor gene (*Chrm2*), M_3 receptor gene (*Chrm3*), M_4 receptor gene (*Chrm4*), M_5 receptor gene (*Chrm5*) along with *Rock1* and *Rock2* genes (156, 157). *B2m* was considered as the housekeeping gene for normalizing gene expression. For B2m, efficiency was 1.13; for M_1: 1.1; for M_2: 0.9; for M_3: 1.05; for M_4: 1.06; for M_5: 0.97; for $ROCK_1$: 1.08; and for $ROCK_2$: 0.99. The minimum information for the publication of quantitative real-time PCR experiments (MIQE) guidelines was followed during the entire qPCR quantification workflow (157).

All experimental data are presented as means ± SEM and *n* represents the number of cases.

For statistical analysis, the Mann-Whitney test or Kruskal-Wallis test was used and $P < 0.05$ was considered a statistically significant difference. Statistical analysis and graph plotting were performed with GraphPad Prism software (v.6.07; GraphPad Software Inc., La Jolla, CA, USA).

Concentration-response curves were analyzed with nonlinear regression and the calculated E_{max} and EC_{50} values are presented on the figures as table inserts.

4. Results

4.1. The Rho–ROCK pathway has a major role in the regulation of carbamoylcholine (CCh)-induced smooth muscle contraction in murine and human urinary bladder

We first examined the role of the RhoA–ROCK pathway in CCh-induced contractile responses in murine UBSM strips (Figure 3). The concentration-dependent contractions were significantly reduced by the ROCK inhibitor Y-27632 (1 μM or 10 μM, 20 min incubation). At a concentration of 1 μM, Y-27632 reduced the E_{max} from 75.8 % to 63.9 %, whereas the EC_{50} values were not significantly different as compared to control (5.2×10^{-7} M vs. 4.3×10^{-7} M, respectively). In contrast, when the strips were incubated with 10 μM Y-27632, both the E_{max} and the EC_{50} values differed statistically from those of controls (E_{max} = 49.6 %, $EC_{50} = 9.0 \times 10^{-7}$ M). These results suggested that the ROCK enzyme plays a substantial role in mediating CCh-induced contractions in murine UBSM strips.

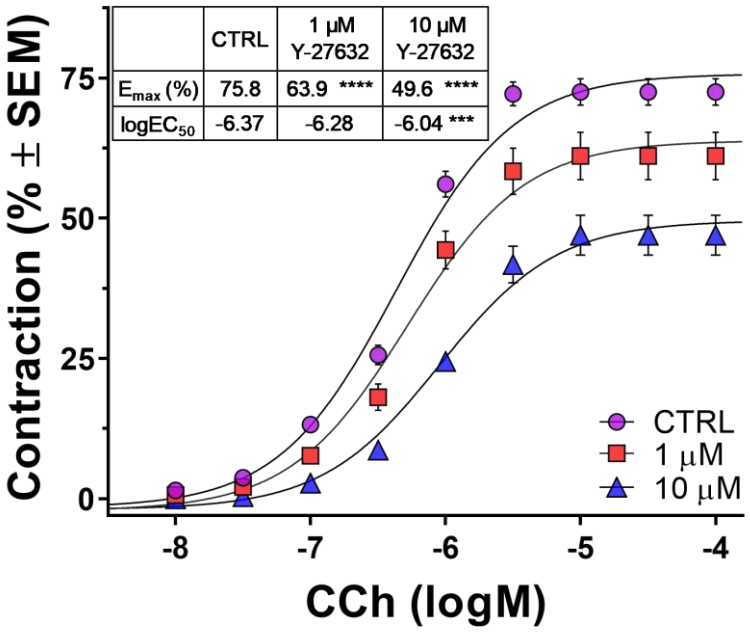

Figure 3 | Role of Rho kinase (ROCK) in mediating carbamoylcholine (CCh)-induced contraction in murine urinary bladder smooth muscle (150). In mouse detrusor muscle, contractile responses induced by CCh were reduced in the presence of

the ROCK inhibitor (Y-27632, 1 and 10 μM). At 1 μM, Y-27632 reduced the E_{max} of the concentration-response curve, whereas there was no statistical difference between the EC_{50} values. However, when Y-27632 was applied at 10 μM, both the EC_{50} and the E_{max} values were statistically different from control (CTRL). CTRL: n = 14, 1 μM Y-27632: n = 12, 10 μM Y-27632: n = 8. ***P < 0.001, ****P < 0.0001.

Thereafter, we investigated the influence of the ROCK inhibitor Y-27632 (10 μM, 20 min incubation) on CCh-induced (10^{-6} M) detrusor contractions in the human UB (Figure 4). Similar to our murine results, the ROCK inhibitor decreased CCh-induced contractile responses of the human detrusor muscle as well. These results indicate that the RhoA–ROCK pathway plays an indispensable role in contractions induced by CCh both in murine and human UB.

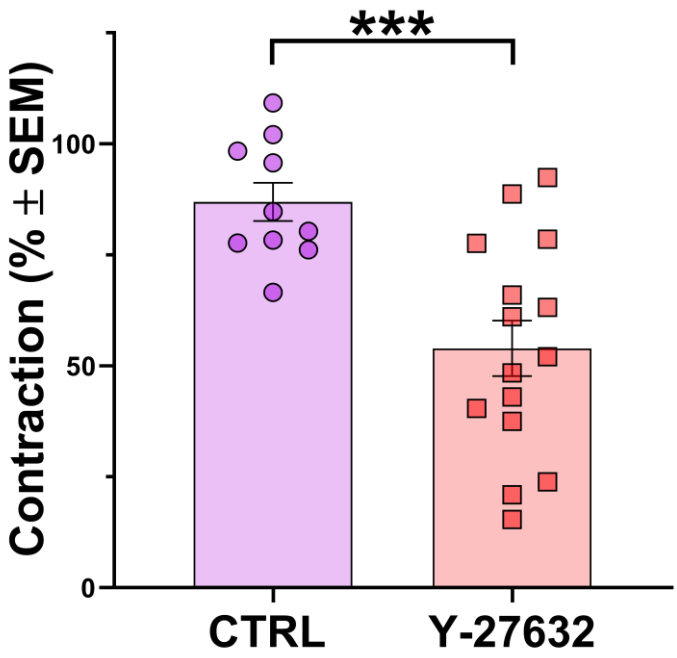

Figure 4 | The Rho kinase (ROCK) enzyme has a major role in carbamoylcholine (CCh)-induced detrusor contraction in the human urinary bladder[3]. Pretreatment of human urinary bladder smooth muscle strips with the ROCK inhibitor Y-27632

[3] Borsodi, Kinga; Balla, Helga; Molnar, Peter J.; Lénárt, Ádám; Kenessey, István; Horváth, András; et al. (2021). Supplementary figure 5B. The effect of Y-27632 pretreatment on 124 mM KCl-, CCh- and α,ß-meATP-induced contractions and on bladder tone in human detrusor muscle. figshare. Figure. https://doi.org/10.6084/m9.figshare.16815088.v5 (158)

(10 μM, 20 min) reduced contractions induced by CCh (10^{-6} M). CTRL: n = 10, Y-27632: n = 15. Mann-Whitney test, ***P < 0.001.

4.2. The role of the M_2 and M_3 receptors in the detrusor muscle contraction and RhoA activation induced by carbamoylcholine (CCh)

Our next aim was to identify the muscarinic receptor(s) mediating the activation of the RhoA–ROCK pathway and contraction in UBSM upon cholinergic stimulation. As UBSM principally expresses M_2 and M_3 muscarinic receptors (44, 159), we investigated CCh-induced contractions in UBSM preparations from M_2-KO, M_3-KO and M_2/M_3-KO double-knockout mice (Figure 5). The concentration-response curve of CCh was markedly changed in detrusor strips lacking either M_2 ($EC_{50} = 2.2 \times 10^{-6}$ M, $E_{max} = 97.5$ %) or M_3 receptors ($EC_{50} = 1.4 \times 10^{-6}$ M, $E_{max} = 42.2$ %), as compared to CTRL ($EC_{50} = 5.0 \times 10^{-7}$ M, $E_{max} = 103.3$ %) (Figure 5A). Moreover, the contractile responses were completely abolished in M_2/M_3-KO UBSM strips (Figure 5A), demonstrating that both M_2 and M_3 receptors, but no other muscarinic receptors contribute to CCh-induced UBSM contractions.

Since the main goal of this study was to investigate the RhoA–ROCK pathway, we also tested the effect of CCh (3×10^{-5} M, 3 min) on RhoA activation in WT and muscarinic receptor-deficient animals. CCh-induced RhoA activation was significantly decreased in M_2-KO and M_3-KO bladder strips, compared to that of WT preparations. Moreover, CCh-induced RhoA activation was completely abolished in M_2/M_3-KO UBSM (Figure 5B), indicating that both M_2 and M_3, but no other receptors, are involved in RhoA activation. Furthermore, Y-27632 (10 μM, 20 min) was able to induce an additional inhibition of CCh-induced contractions in both M_2-KO (Figure 5E) and M_3-KO (Figure 5F) bladders, indicating that neither M_2 nor M_3 receptors play an exclusive role in activating the RhoA–ROCK pathway.

Based on data gained by other research groups it seems to be implausible that the lack of one specific muscarinic receptor gene would alter the expression level of the other four muscarinic receptor subtypes (160-164). Therefore, we investigated the expression levels of the different muscarinic receptors in the UBSM from muscarinic receptor KO mice to confirm if there are any effects of the single gene deletion on other receptor subtypes. Our results show that there is no impact of the M_2 and M_3 gene deletion on mRNA expression levels of the other receptors in UBSM strips from M_2- and M_3-KO mice (Figure 5C, D). In addition, the qPCR analysis confirmed the lack of *Chrm2* and *Chrm3* in the M_2- and M_3-KO mice, respectively and in double KO animals.

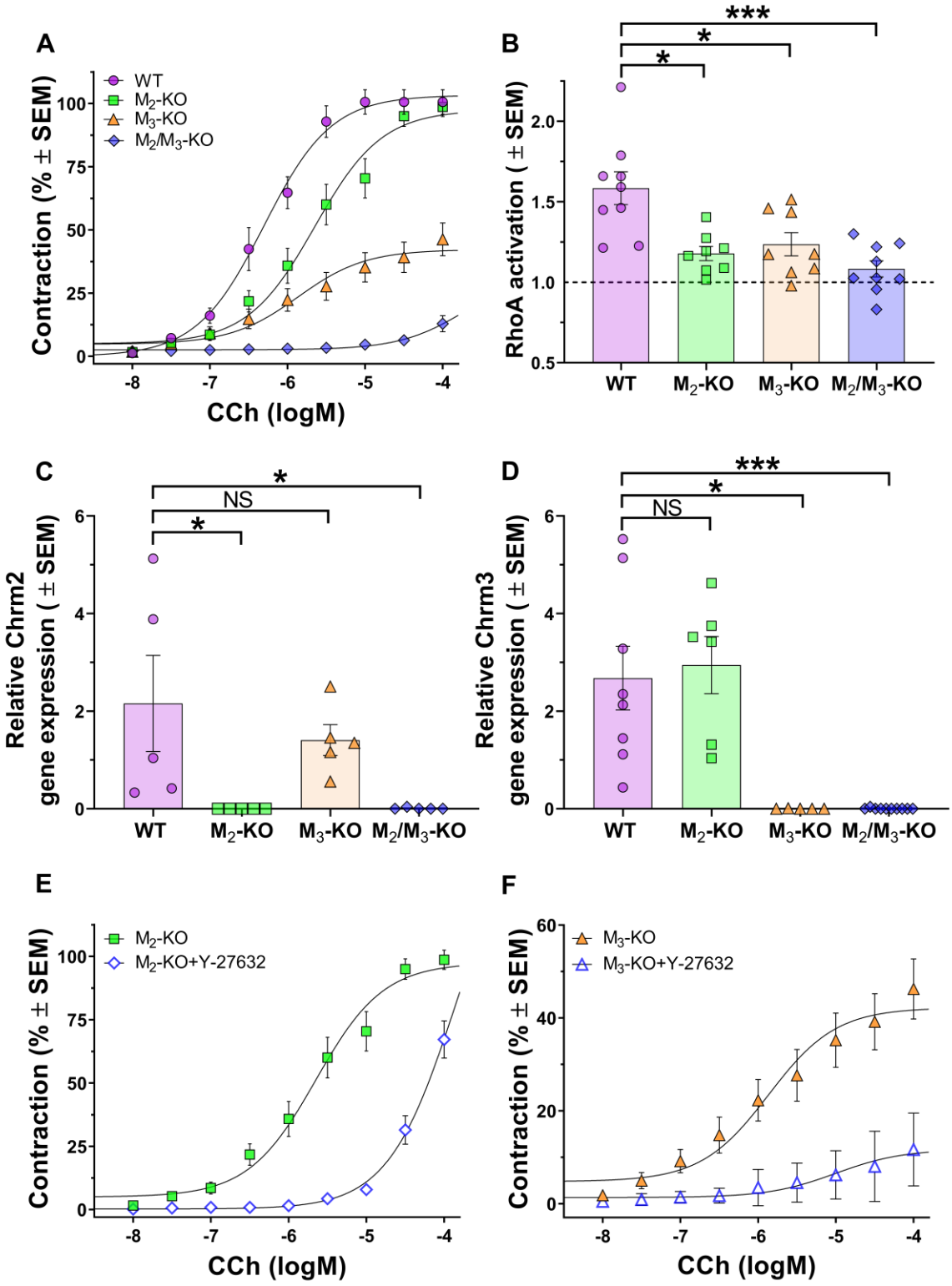

Figure 5 | Carbamoylcholine (CCh)-induced detrusor contraction and RhoA activation are mediated by both M_2 and M_3 muscarinic acetylcholine receptors (150). A: CCh-induced contractions were markedly impaired in detrusor strips lacking

either M_2 (M_2-KO) or M_3 receptors (M_3-KO). Furthermore, the contractile response to CCh was completely abolished in urinary bladder smooth muscle (UBSM) strips deficient in both M_2 and M_3 receptors (M_2/M_3-KO). B: CCh (3×10^{-5} M)-induced RhoA activation significantly decreased in both M_2-KO and M_3-KO mouse bladders and abolished in M_2/M_3-KO UBSM. C: No statistical difference was observed in M_2 receptor gene (*Chrm2*) expression in the M_3 receptor-deficient animals compared to wild type (WT). D: The expression of the M_3 receptor gene (*Chrm3*) was unaffected by the lack of M_2 receptors. E: ROCK inhibitor (Y-27632, 10 μM), induced a further marked decrease in contraction in M_2-KO mouse bladder strips. F: CCh-induced contractions were abolished completely by Y-27632 (10 μM) in UBSM strips deficient in M_3 receptors. A: WT: $n = 6$, M_2-KO: $n = 16$, M_3-KO: $n = 15$, M_2/M_3-KO: $n = 8$, B: WT: $n = 9$, M_2-KO $n = 8$, M_3-KO $n = 8$, M_2/M_3-KO = 9, C: WT: $n = 5$, M_2-KO: $n = 5$, M_3-KO: $n = 5$, M_2/M_3-KO: $n = 5$, D: WT: $n = 8$, M_2-KO: $n = 6$, M_3-KO: $n = 5$, M_2/M_3-KO: $n = 10$, E: M_2-KO $n = 16$, Y-27632: $n = 8$, F: M_3-KO: $n = 6$, Y-27632: $n = 8$. B, C, D: Kruskal-Wallis test, NS not significant, $^*P < 0.05$, $^{***}P < 0.001$.

Furthermore, there is no change in the expression levels of the other three muscarinic receptor subtypes (M_1, M_4, M_5) in detrusor strips deficient in M_2, M_3 or M_2/M_3 muscarinic receptors (Figure 6).

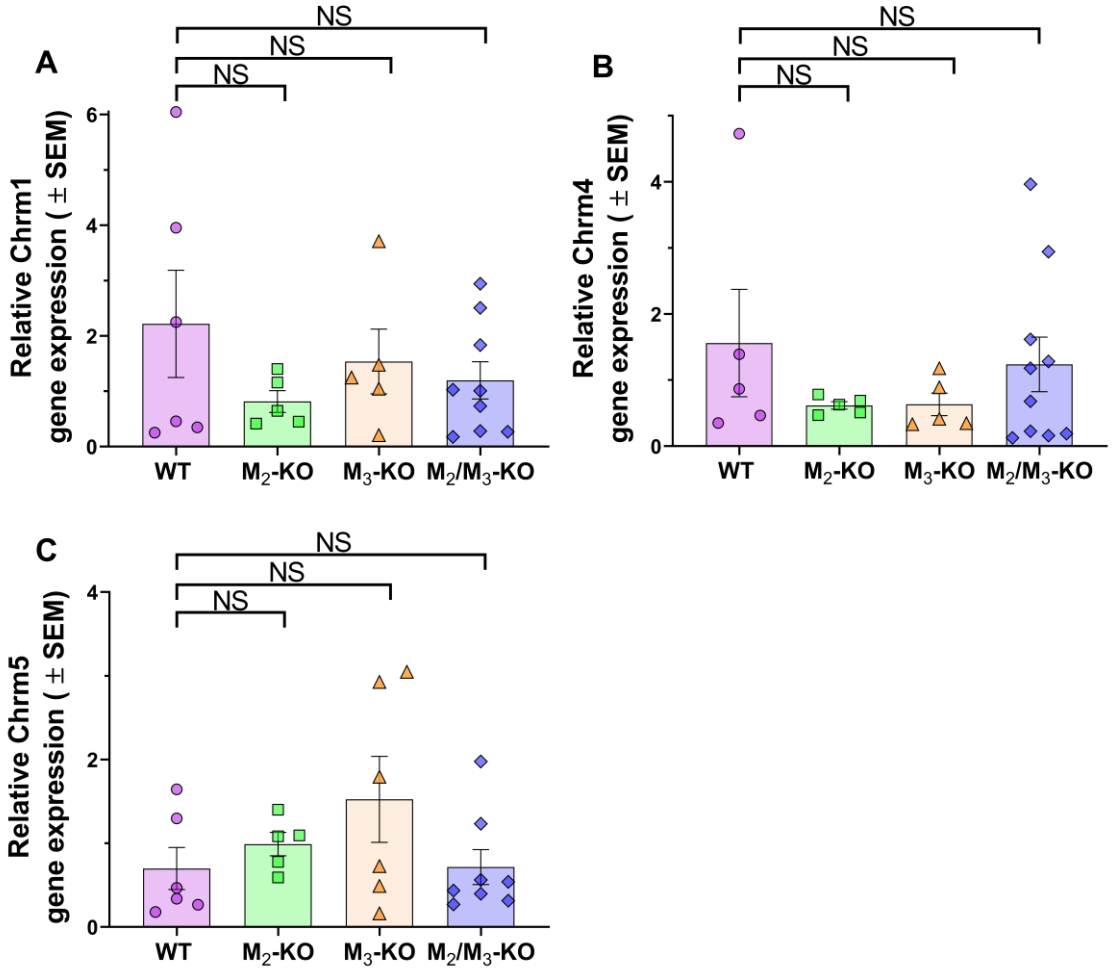

Figure 6 | Expression levels of M_1, M_4 and M_5 receptor genes (*Chrm1*, *Chrm4*, *Chrm5*) in detrusor strips from M_2-, M_3-, M_2/M_3-knockout (KO) mice[4]. A: The expression of *Chrm1* was unaffected by the lack of M_2 and M_3 receptors. B: No statistical difference was observed in M_4 receptor gene expression in the receptor-deficient animals compared to wild type (WT). C: The amount of the *Chrm5* did not change in M_2-, M_3- and M_2/M_3-KO mice compared to that of controls. A: WT: $n = 6$, M_2-KO: $n = 5$, M_3-KO: $n = 5$, M_2/M_3-KO: $n = 9$, B: WT: $n = 5$, M_2-KO: $n = 5$, M_3-KO:

[4] Balla, Helga; Borsodi, Kinga; Örsy, Petra; Horváth, Béla; Molnar, Peter J.; Lénárt, Ádám; et al. (2023). Supplemental Material Intracellular signaling pathways of muscarinic acetylcholine receptor-mediated detrusor muscle contractions. figshare. Figure. https://doi.org/10.6084/m9.figshare.23859108.v1 (150)

n = 5, M_2/M_3-KO: n = 10, C: WT: n = 6, M_2-KO: n = 5, M_3-KO: n = 6, M_2/M_3-KO: n = 8. Kruskal-Wallis test, $P < 0.05$ was considered as significant difference.

Interestingly, neither the deletion of M_2 nor that of M_3 induced any significant change in the mRNA expression of $ROCK_1$ or $ROCK_2$, whereas M_2/M_3 double KOs showed increased $ROCK_2$ expression, probably as a counter-regulation of diminished muscarinic receptor signaling in the UBSM (Figure 7). It is noteworthy that in contrast to M_2-KO preparations, the ROCK inhibitor was able to abolish the effect of CCh in M_3-KO bladders (Figure 5F). These results suggest that CCh induces UBSM contractions and RhoA–ROCK activation by both M_2 and M_3 muscarinic receptors and M_3 receptors are apparently able to stimulate UBSM contractions in a RhoA-ROCK-independent manner as well.

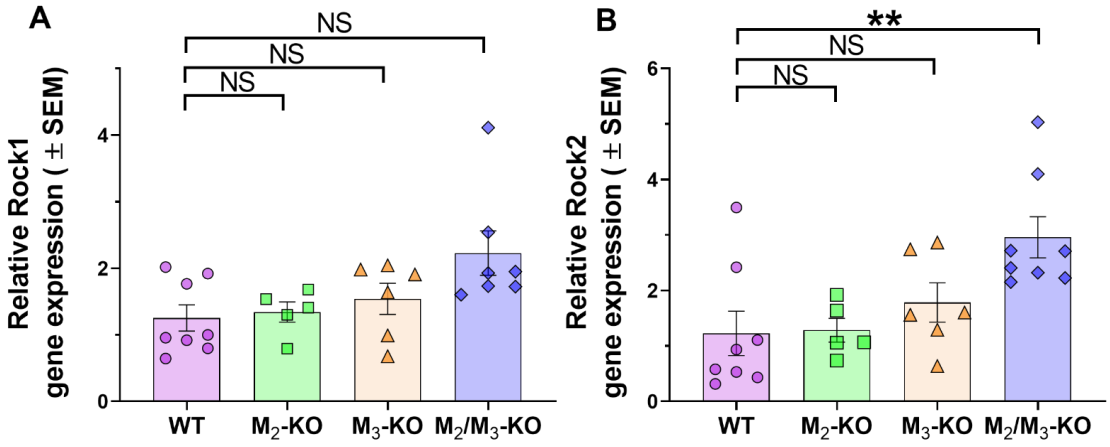

Figure 7 | Gene expression of Rho kinase 1 and 2 (*Rock1*, *Rock2*) in M_2-, M_3- and M_2/M_3-knockout (KO) mice[5]. A: There was no significant difference in the expression of *Rock1* in wild type (WT) and receptor-deficient animals. B: The mRNA expression of the $ROCK_2$ was elevated in M_2/M_3-KO animals compared to that of WT. A: WT: n = 8, M_2-KO: n = 5, M_3-KO: n = 6, M_2/M_3-KO: n = 7, B: WT: n = 8, M_2-KO: n = 5, M_3-KO: n = 6, M_2/M_3-KO: n = 8. Kruskal-Wallis test, $^{**}P < 0.01$.

[5] Balla, Helga; Borsodi, Kinga; Örsy, Petra; Horváth, Béla; Molnar, Peter J.; Lénárt, Ádám; et al. (2023) Supplemental Material Intracellular signaling pathways of muscarinic acetylcholine receptor-mediated detrusor muscle contractions. figshare. Figure. https://doi.org/10.6084/m9.figshare.23859108.v1 (150)

4.3. Inhibitory effect of pertussis toxin (PTX) on carbamoylcholine (CCh)-induced urinary bladder smooth muscle contraction and RhoA activation

Next, we analyzed the contribution of different heterotrimeric G proteins to CCh-induced RhoA–ROCK activation and UBSM contractions. As M_2 receptors preferentially signal through $G\alpha_i$-proteins, we first investigated the effect of PTX, a specific inhibitor of $G\alpha_i$, on the UBSM contractions and RhoA activation induced by CCh. The concentration-response curve of CCh was shifted to the right in UBSM strips from WT mice treated with PTX evidenced by the increase of CCh EC_{50} from 3.9 to 6.5×10^{-7} M (Figure 8A). After the demonstration of $G\alpha_i$-dependent contraction of CCh, we investigated whether CCh-induced RhoA activation was dependent on $G\alpha_i$ signaling. In UBSM strips of PTX-treated mice, the RhoA activation induced by CCh was significantly reduced (Figure 8B). Taken together, these results indicate that $G\alpha_i$-proteins play an important role in mediating muscarinic agonist-induced RhoA activation and contraction in UBSM.

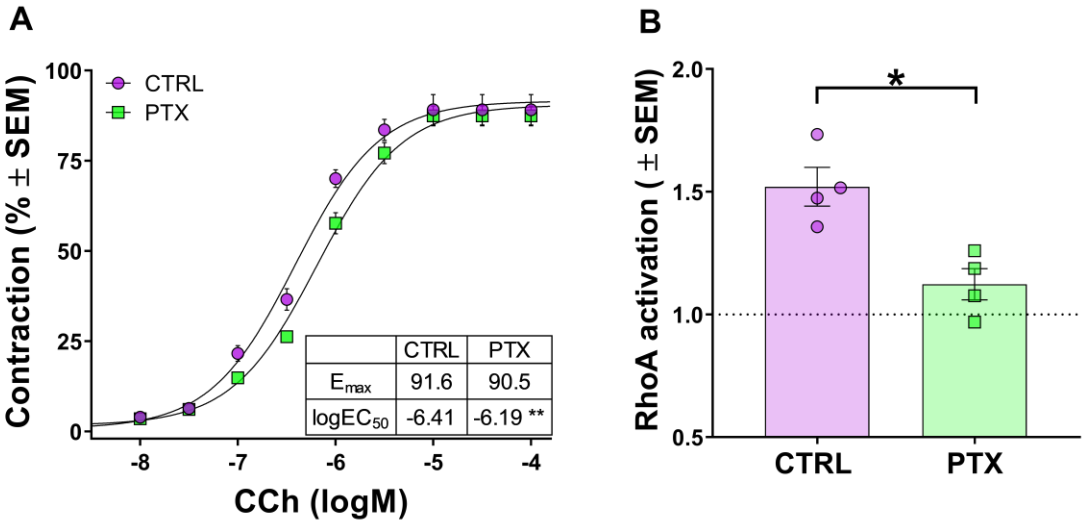

Figure 8 | Inhibition of carbamoylcholine (CCh)-induced detrusor muscle contraction and RhoA activation by pertussis toxin (PTX) (150). A: The concentration-response curve of CCh was shifted to the right in urinary bladder smooth muscle (UBSM) strips from mice treated with PTX without changing the E_{max} value. B: The CCh (3×10^{-5} M)-induced RhoA activation was diminished in UBSM strips

from PTX-treated mice. A: CTRL: *n* = 19, PTX: *n* = 26, B: CTRL: *n* = 4, PTX: *n* = 4. **$P < 0.01$. B: Mann-Whitney test, *$P < 0.05$.

4.4. $G\alpha_{q/11}$-coupled signaling pathway plays a key role in urinary bladder contractions but not in RhoA activation induced by carbamoylcholine (CCh)

Considering our results gained from M_3-KO animals and the fact that M_3 receptors preferentially couple to $G\alpha_{q/11}$ (132), we studied the potential involvement of $G\alpha_{q/11}$-proteins along with the role of the ROCK enzyme in UBSM contractions and RhoA activation induced by CCh. The concentration-dependent contractions induced by CCh were markedly reduced in UBSM preparations from mice lacking $G\alpha_{q/11}$ protein ($EC_{50} = 5.2 \times 10^{-7}$ M vs. 2.9×10^{-6} M, $E_{max} = 85.3$ % vs. 34.3 %) and were further abolished in the presence of the ROCK inhibitor Y-27632 (10 μM, 20 min) in UBSM deficient in $G\alpha_{q/11}$ ($EC_{50} = 6.5 \times 10^{-6}$ M, $E_{max} = 18.0$ %) (Figure 9A). This observation indicates that $G\alpha_{q/11}$-coupled signaling plays a dominant role in muscarinic agonist-induced UBSM contractions. Surprisingly; however, the magnitude of CCh-induced RhoA activation remained unaltered in the absence of $G\alpha_{q/11}$ signaling (Figure 9B). In summary, these data support the concept that although M_3-receptor-induced activation of the RhoA–ROCK pathway is involved in UBSM contraction, it is not mediated by $G\alpha_{q/11}$, but rather by $G\alpha_i$.

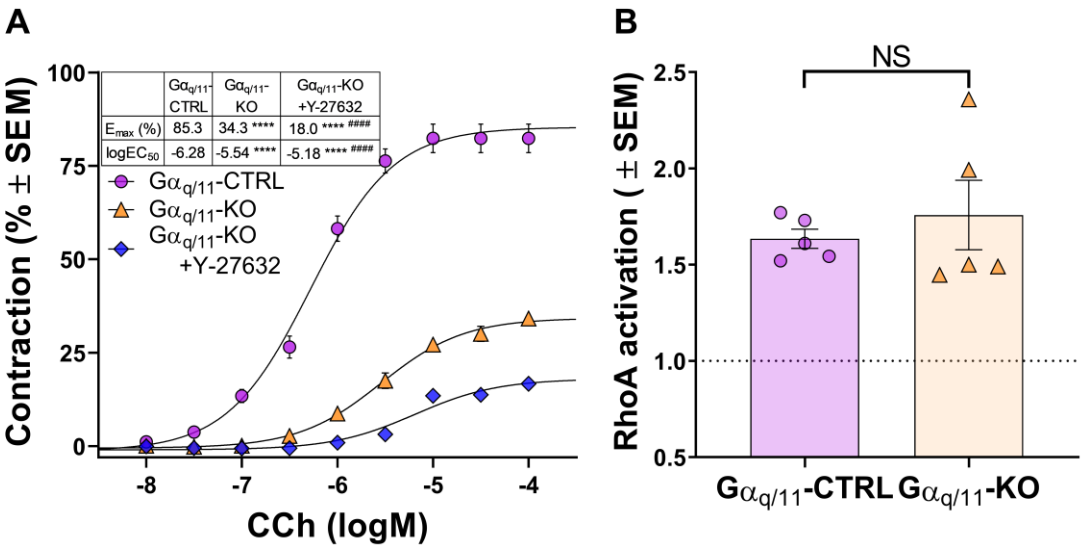

Figure 9 | Gα$_{q/11}$ signaling plays a key role in mediating carbamoylcholine (CCh)-induced bladder contractions but not in RhoA activation (150). A: Detrusor muscle contractions evoked by CCh were markedly decreased in smooth muscle-specific Gα$_{q/11}$-knockout (KO) mice and were diminished in the presence of the ROCK inhibitor Y-27632 (10 μM) in the Gα$_{q/11}$-deficient animals. B: CCh (3×10^{-5} M)-induced RhoA activation was unaffected by the lack of Gα$_{q/11}$ in UBSM. A: Gα$_{q/11}$-CTRL: n = 15, Gα$_{q/11}$-KO: n = 22, Gα$_{q/11}$-KO + Y-27632: n = 8, B: Gα$_{q/11}$-CTRL: n = 5, Gα$_{q/11}$-KO: n = 5. Gα$_{q/11}$-CTRL vs Gα$_{q/11}$-KO: ****P < 0.0001, Gα$_{q/11}$-KO vs Gα$_{q/11}$-KO + Y-27632: $^{\#\#\#\#}P$ < 0.0001. B: Mann-Whitney test, P < 0.05 was considered as significant difference.

5. Discussion

OAB is a common clinical condition with the symptoms of frequency, nocturia and urgency with or without incontinence. It significantly impairs the overall quality of life, the performance of daily activities, work, sexual function, sleep and mental health (165-167). About 11-16 % of the population is affected by the symptoms of OAB, although the literature is somewhat controversial on this matter (168-171). In addition, the prevalence of OAB shows a steep increase after the age of 40 years; furthermore, it is prognosticated that over 400 million people will suffer from OAB symptoms in the world's eight major markets by 2030 (172) as a consequence of the aging population (166, 173).

Previously, OAB was suggested to be a nonspecific storage symptom complex without any characteristics of urinary tract infection (174). Even though OAB is considered to be an idiopathic condition, recently several studies proposed its multifaceted pathogenesis. Multiple studies have proposed low-count bacteriuria and inflammation along with the symptoms of OAB (175-178). Moreover, the implication of several pathophysiological factors has been proposed with the occurrence of OAB-like metabolic syndrome (179, 180) and vitamin D deficiency (181); furthermore, detrusor overactivity has been implicated in ovariectomized rats (139, 182).

As muscarinic receptors and the Rho–ROCK pathway play a pivotal role in the regulation of micturition, several studies examined their role and expression levels in animal models of voiding disorders. Tong *et al.* (2007) have demonstrated elevated levels of M_2 and M_3 receptors in the UB of animals suffering from metabolic syndrome (183). Additionally, the expression level of muscarinic receptors has been elevated in obstructed, hypertrophic rat bladders (184). This study has also confirmed the increased contribution of M_2 receptors to detrusor contraction in hypertrophic bladders. Moreover, observations suggested that the ROCK pathway is more privileged in studies of denervated than in normal UBs as inhibition of the ROCK has a greater effect on the concentration-response curve of CCh after bilateral denervation in the rat UB (132). In addition, enhanced enzyme activity has been proposed with aging in human detrusor muscle based on the evidence that the ROCK plays a greater role in the contraction of the detrusor muscle induced by CCh in elderly patients (136). The importance of the

ROCK under pathophysiological conditions is further demonstrated by the fact that per os administration of its specific inhibitor fasudil, relieved the symptoms of detrusor hyperactivity *in vivo* in rats (135).

Given the crucial role of ACh, antimuscarinic drugs are considered to be the mainstay for the treatment of OAB. However, despite the development of bladder selective agents, adherence to the therapy remains a challenge due to the extensive side-effect profile (e.g. dry mouth, obstipation) worsening patients' tolerability (185, 186). Recently, the β_3-AR agonist mirabegron offered an alternative treatment option with an improved side effect profile; however, it seems that it is less effective as compared to antimuscarinics (185, 187).

Earlier observations indicate that muscarinic receptors and the Rho–ROCK pathway are pivotal in detrusor muscle contraction and are associated with the etiology of OAB. Nevertheless, our knowledge of the exact signaling pathways involved in muscarinic receptor-mediated detrusor contractions still remains incomplete. Hence, these examinations incite us to characterize further the signaling pathway of muscarinic receptor activation and detrusor muscle contraction focusing on the role of the RhoA-ROCK pathway for the purpose of providing novel, more specific therapeutic targets for the treatment of bladder dysfunctions.

The CCh-induced UBSM contractions were markedly suppressed in the presence of the ROCK inhibitor Y-27632. These results indicate that the ROCK enzyme has a crucial role in detrusor muscle contractions induced by CCh either in murine and human UB in accordance with previous reports (11, 107).

Furthermore, our goal was to identify the muscarinic receptors that contribute to detrusor muscle contraction and the activation of the RhoA–ROCK pathway in the murine UB. As previous results show that mostly the M_2 and M_3 receptors are present in the mouse UBSM, we focused on the role of these two muscarinic receptor subtypes in the detrusor contractions. Results gained from M_2-, M_3- and M_2/M_3-KO mice show that, in line with the hypothesis, the M_2 and the M_3 muscarinic receptors mediate CCh-induced UBSM contractions simultaneously in the mouse UB indicated by the loss of detrusor muscle contraction in mice lack of either M_2 and M_3 muscarinic receptors. These observations are in accordance with the claims of other groups (65, 188). Therefore, we aimed to examine the role of these two muscarinic receptor subtypes in

the RhoA–ROCK activation in mouse UB. Our findings suggest that RhoA activation is mediated by both the M_2 and the M_3 receptors but no other subtypes in the murine UB based on data gained from M_2-, M_3- and M_2/M_3-KO animals. In addition, there was no significant difference in the expression levels of the five muscarinic receptor subtypes in the UB strips from M_2-, M_3- and M_2/M_3-KO mice. Moreover, the expression level of the $ROCK_1$ was not affected by the gene deletion of M_2 and M_3 receptors; however, the $ROCK_2$ enzyme manifestation was slightly elevated in double-knockout animals suggesting a compensatory upregulation in the absence of M_2 and M_3 receptors. Previous studies proposed the role of the M_2 receptors in gastrointestinal smooth muscle (188) and UBSM contractions (132); nonetheless, to the best of our knowledge, this is the first study that proves the direct relationship between the M_2 receptor and the RhoA-ROCK pathway in the mouse UB.

It has been suggested that the M_2 and the M_3 receptors are coupled preferentially to $G\alpha_i$ and $G\alpha_{q/11}$ proteins, respectively (56, 58). Therefore, we examined if these G proteins contribute to the activation of RhoA induced by CCh in the mouse detrusor muscle. The concentration-response curve of CCh was shifted to the right after the inhibition of the $G\alpha_i$ by PTX suggesting the involvement of $G\alpha_i$ signaling upon muscarinic receptor activation. Furthermore, the amount of the activated RhoA protein was significantly decreased in PTX-treated animals indicating the direct link between the $G\alpha_i$ and RhoA proteins in the mouse detrusor. The activation of the RhoA protein by $G\alpha_i$ has been investigated previously by other research groups in human airway smooth muscle and HEK-TsA201 cells (189, 190) but this is the first study suggesting the $G\alpha_i$-dependent RhoA activation in the UBSM.

Finally, we investigated the involvement of the $G\alpha_{q/11}$ protein in CCh-induced contractions and RhoA activation in the UB. CCh-induced detrusor muscle contractions were significantly reduced in the UBSM from mice deficient in $G\alpha_{q/11}$ protein and abolished in the presence of the ROCK inhibitor. These results suggest that the $G\alpha_{q/11}$ protein plays a decisive role in the detrusor contractions induced by CCh. However, contrary to our earlier findings with other mediators (158, 191) and the hypothesized association between the $G\alpha_{q/11}$ and the RhoA–ROCK pathway, the lack of the $G\alpha_{q/11}$ protein did not affect the magnitude of the CCh-induced RhoA activation in the UBSM. Therefore, it appears to be mediated by the $G\alpha_i$ proteins in the murine UB. Based on our

findings, M_3 receptors but not $G\alpha_{q/11}$ protein are involved in RhoA activation. These results contradict the claims about the conventional wisdom of the exclusive coupling of M_3 receptors to $G\alpha_{q/11}$ protein. However, Offermanns *et al.* (1994) suggested that the M_3 receptors can also couple to $G\alpha_{i1,3}$ protein which can explain our results gained from murine UB detrusor strips deficient in $G\alpha_{q/11}$-protein (58). In summary, Figure 10 shows our proposed model of muscarinic receptor signaling. The inconsistency between the inhibitory effect of PTX on CCh-induced contractions and RhoA activation can also be explained according to this paradigm. In the presence of PTX, the G_{i1} and G_{i3} proteins are inactivated but the fortified M_3-$G\alpha_{q/11}$ coupling could compensate for the loss of the Ca^{2+} sensitizing mechanism in detrusor contraction.

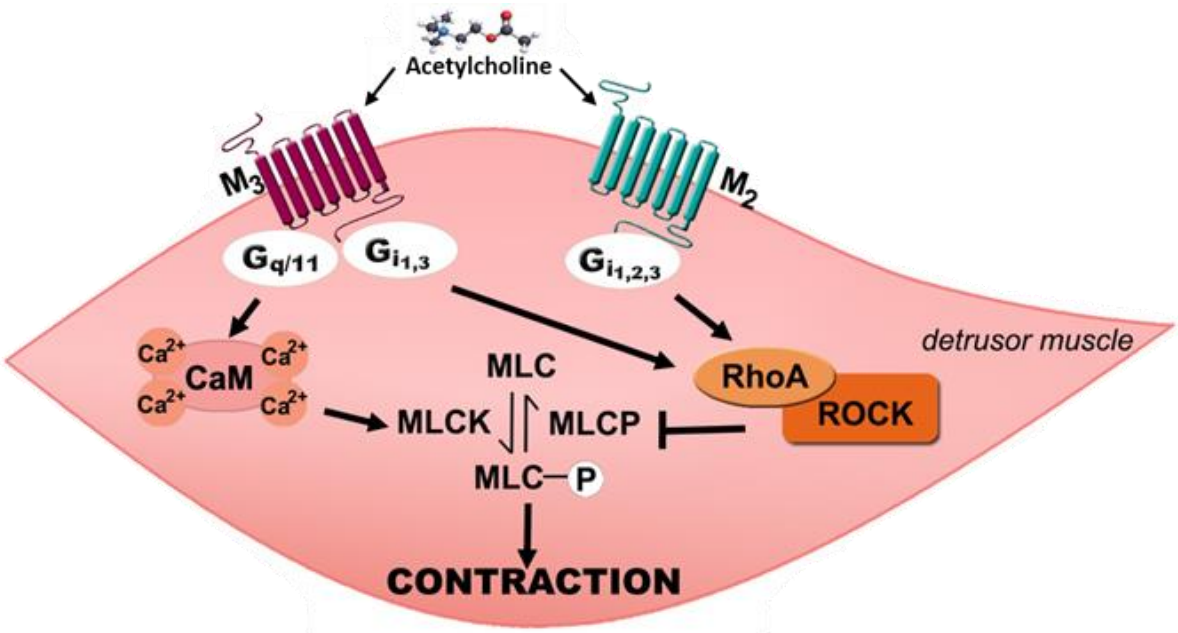

Figure 10 | Proposed model of muscarinic receptor signaling in the murine urinary bladder smooth muscle based on our findings and Offermanns *et al.* (1994) (58, 150). CaM, calmodulin; MLC, myosin light chain; MLCK, myosin light-chain kinase; MLCP, myosin light-chain phosphatase; ROCK, Rho-associated coiled-coil-containing protein kinase.

There may be some limitations in this study that could be addressed in future research. First of all, neuronal control cannot be investigated *in vitro*, though its importance is unquestionable in the micturition process under physiological as well as pathological conditions. In this context, the role of M_2 receptors in bladder function can

be particularly interesting, as it can modulate sympathetically-mediated relaxant effects by reversing β-adrenoreceptor-mediated detrusor muscle relaxation (192, 193). Furthermore, Igawa *et al.* (2004) have confirmed that the M_3 receptor has a major role in UB contraction in *in vivo* experiments and that noncholinergic mechanisms can compensate for a chronic loss of M_3 receptors (194). Thus, further studies on M_3 receptor-mediated signaling in the bladder under *in vivo* conditions could also contribute significantly to a better understanding of bladder function, as this pathway is still incompletely understood. Besides, as ROCK appears to play an undoubted role in micturition under physiological conditions, addressing the proper dosage of the ROCK inhibitors can be an important concern about their therapeutic usage. However, it has to be mentioned that ROCK inhibitors have been clinically approved to treat glaucoma in oral formulation in Japan without severe adverse effects but hyperaemia (195).

In conclusion, our study contributes to a deeper understanding of the intracellular signaling pathways of muscarinic receptors and the pivotal role of the RhoA–ROCK pathway in detrusor contraction in human and murine UB as well. These data may define a therapeutic potential in the treatment of bladder dysfunctions, including OAB.

6. Conclusion

Muscarinic acetylcholine receptors are of utmost importance in the physiological regulation of micturition and also in the development of voiding disorders. Our results gained from the *ex vivo* experiments performed on murine and human UB detrusor strips are the following:

- We demonstrate that the RhoA–ROCK pathway plays a crucial role in contractions induced by cholinergic stimulation in the murine and human detrusor muscle.
- Both M_2 and M_3 receptors mediate the CCh-induced detrusor contractions and the activation of RhoA.
- The muscarinic receptor-mediated RhoA activation is regulated by G_i but not $G_{q/11}$ proteins.

The G_i–Rho–ROCK pathway may provide a novel therapeutic target for overactive voiding disorders.

7. Summary

The mAChRs play an unquestionable role in the regulation of micturition and antimuscarinic drugs are mainstays for the treatment of the worldwide symptom complex, OAB. Nevertheless, the exact signaling pathway of mAChRs is obscure even though a more detailed understanding of the signal transduction pathway may help to find more specific drug targets with fewer adverse effects for OAB.

Therefore, in the present study, we aimed to analyze the role of the M_2 and M_3 muscarinic receptors and their corresponding heterotrimeric G proteins (G_i and $G_{q/11}$, respectively) in the regulation of detrusor function, concentrating on the interaction with the Rho–ROCK pathway. We demonstrated that the ROCK plays a pivotal role in the detrusor contraction induced by the muscarinic receptor agonist, CCh. Moreover, the CCh-induced contractions and RhoA activation are mediated by both the M_2 and the M_3 receptors according to the results gained from M_2-, M_3- and M_2/M_3-KO animals. In addition, the expression levels of other muscarinic receptors were not altered by the genetic deletion of M_2 and M_3 receptors indicating that no other muscarinic receptors contribute to the UBSM contraction induced by CCh. Furthermore, pharmacological inhibition of the ROCK enzyme had an additional inhibitory effect on the concentration-response curve of CCh in M_2- and M_3-KO mice. Interestingly, there was no change in the expression of the $ROCK_1$ enzyme in detrusor muscle from mice deficient in M_2 or M_3 receptors; however, the $ROCK_2$ enzyme expression was elevated in M_2/M_3-KO mice suggesting a compensatory upregulation of ROCK in the absence of both receptors. PTX treatment of WT mice shifted the concentration-response curve of CCh to the right parallel with a steep decrease in RhoA activation. The CCh-induced contractions were diminished in $G_{q/11}$-KO animals; however, the RhoA activation did not change in mice deficient in $G_{q/11}$ compared to that of WT. These results indicate that muscarinic receptor-mediated RhoA activation is not mediated by $G_{q/11}$ but rather by G_i.

These observations can support the development of novel, more specific therapeutic targets that can help to improve patient adherence along with better-tailored therapeutic strategies of OAB to maximize patients' quality of life and cost-effective care.

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