SIGNALING PATHWAYS OF TACHYKININS IN THE REGULATION OF URINARY BLADDER SMOOTH MUSCLE CONTRACTION

PhD thesis

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1. Introduction

Overactive bladder (OAB) syndrome is defined as the symptoms of frequency (>8 micturition/24 h) and urgency, with/without incontinence. The prevalence of OAB syndrome is estimated 16–17%. OAB syndrome makes everyday activities burdensome, including social activities, traveling, sexual life, and even sleep. The currently used pharmacological treatments have frequent and inconvenient side effects, hence there is an unmet medical need for new, more specific therapies.

The mammalian tachykinins (TKs) represent one of the largest families of neuropeptides and released together from the nerve terminals with the classic cholinergic/adrenergic neurotransmitters regulating the function of the lower urinary tract. There are three classical members of the mammalian TKs: neurokinin A (NKA), neurokinin B (NKB) and substance P (SP). TKs can bind to three distinct receptor subtypes: NK1R, NK2R, and NK3R. All three TK receptors belong to the superfamily of G protein-coupled receptors.

NK2Rs are expressed in the mouse and human urinary bladder and have been shown to mediate the contractile effects of NKA and its synthetic analog in the detrusor muscle. NK2R was shown to be involved in the regulation of bladder voiding reflexes, and its inhibition reduced the amplitude of detrusor muscle contractions in experimental bladder obstruction and cystitis models. Furthermore, in capsaicin-induced hyperactivity model in rats, the NK2R antagonist SR 48965 ameliorated the hyperactive reflexes without interfering physiological micturition. This indicates the importance of NK2R in bladder overactivity.

2. Objectives

In the urinary bladder, the exact mechanism of TK signal transduction is still unexplored. Data in the literature indicate that NK2Rs play an important role in the pathophysiology of OAB. Therefore, our main aim was to characterize the signaling of TKs, specifically NK2Rs, in the urinary bladder smooth muscle (UBSM), providing a theoretical basis for the development of future medications for bladder disorders, including OAB.

3. Methods

Experiments were carried out on urinary bladders of adult male wild-type (C57BL/6) and genetically modified mice (mouse lines with smooth muscle-specific inducible deletion of the $G_{q/11}$ signaling pathway (*Gna11*, $G\alpha_{q/11}$-KO). Tamoxifen-treated served as controls and are referred to as $G\alpha_{q/11}$-CTRL. Similarly, smooth muscle-specific $G_{12/13}$-KO mice were generated with the floxed alleles of $G\alpha_{13}$ (*Gna13*) on the background of null alleles of Gα12 (*Gna12*).

After dissection the urinary bladders were placed into Krebs solution at room temperature. We cut from the dome of the bladder towards the urethral opening ensuring that the majority of a strip consists of the detrusor muscle.

Detrusor muscle contraction force was registered by myographs under isometric conditions. TK-induced tension changes were normalized to the reference contraction induced by 124 mM K^+-containing Krebs solution.

To measure intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), urinary bladders were slightly stretched and fixed on plastic rings with needles. Bladders were incubated with fluo-4-AM fluorescent dye. Ca^{2+} signals evoked by NK2R specific agonist [β-Ala^8]-NKA(4–10) were normalized to the amplitude of $[Ca^{2+}]_i$ induced by ionomycin.

Activated RhoA protein was quantified with a pull-down ELISA kit and activated RhoA-GTP values were normalized to the total RhoA content.

All data are presented as means ± SD.

4. Results

First, we evaluated the role of NK2R in mediating the effects of TKs. NKA, NKB, and SP induced strong contractions *(Figure 1./A–C)* in which a phasic and a tonic component could be discriminated. The phasic contractions, characterized by the maximal elevation of the tension, were markedly inhibited by the NK2R-specific antagonist MEN10376 in the case of all three TKs *(Figure 1./A–C)*. In contrast, the amplitude of the tonic contractions, determined at 5 mins, remained unaltered in the presence of MEN10376 in the case of NKA and NKB, whereas it was abolished in the case of SP. Since all TKs increased UBSM tension in a MEN10376-reversible manner, it was concluded that the NK2R is likely to play an important role in the control of urinary bladder functions.

Since the main goal of the present study was to investigate the intracellular signaling pathways mediating the detrusor muscle contraction in response to NK2R activation, we next tested the effects of NK2R-specific agonist [β-Ala^8]-NKA(4–10). Both phasic and tonic components of the contraction induced by the submaximal concentration (10^{-5} M) of [β-Ala^8]-NKA(4–10) were diminished by MEN10376 *(Figure 1./D)*. Therefore, in further experiments, 10^{-5} M [β-Ala^8]-NKA(4–10) was used to activate specifically NK2R-mediated signaling in the UBSM.

Next, we aimed to identify the G-proteins mediating the effect of NK2Rs in UBSM. To examine the contribution of the G_q/G_{11} signaling pathway, the consequences of $G\alpha_{q/11}$ deletion on NK2R-mediated smooth muscle contraction and Ca^{2+} signaling were analyzed. In $G\alpha_{q/11}$-KO animals, both phases of the contraction response induced by [β-Ala^8]-NKA(4–10) were eliminated (*Figure 2/A*). [β-Ala^8]-NKA(4–10) not only elicited contraction but also triggered $[Ca^{2+}]_i$ increase, which also

disappeared in $G\alpha_{q/11}$-KO bladders (*Figure 2/B*). Taken together, these results indicate that $G_{q/11}$ proteins have an exclusive role in mediating the intracellular signaling of NK2Rs in the UBSM.

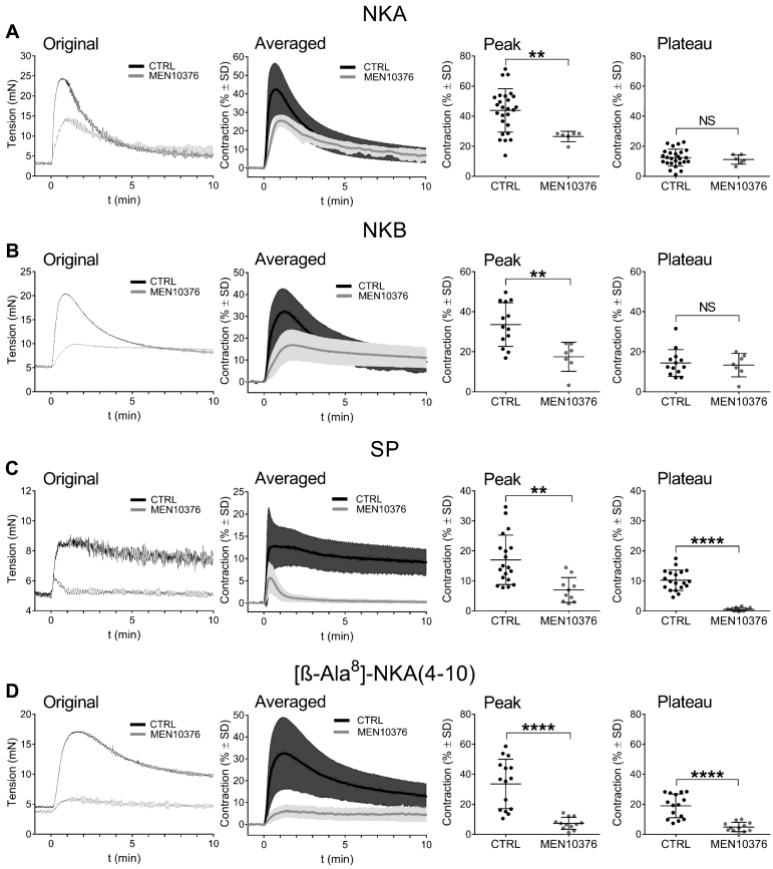

Figure 1. **Role of NK2Rs in mediating TK-induced detrusor muscle contraction.**
NKA: neurokinin A; NKB: neurokinin B; SP: substance P. NS, not significant. ***P* < 0.01; *****P* < 0.0001

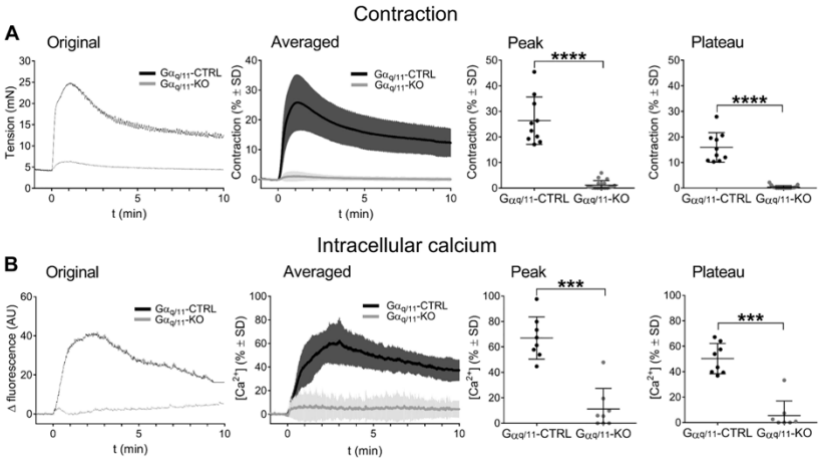

Figure 2. **$G_{q/11}$ proteins mediate intracellular signaling of NK2R in the UBSM muscle.** ****P* < 0.001; *****P* < 0.0001

To exclude the potential overlapping function of multiple G proteins in eliciting contraction, we investigated the effects of genetic $G_{12/13}$ deletion on [β-Ala^8]-NKA(4–10) induced contraction. We generated $G\alpha_{12/13}$-KO mice with a similar approach as we applied in $G\alpha_{q/11}$-KO. Bladders of $G_{12/13}$-KO mice showed no significant difference in the phasic or tonic phase of contraction compared to $G_{12/13}$-CTRL mice (*phasic*: $G\alpha_{12/13}$-CTRL: 32.0±2.7% vs. $G\alpha_{12/13}$-KO: 29.8±2.9%, P = 0.578; *tonic*: $G\alpha_{12/13}$-CTRL: 20.2±2.0% vs. $G\alpha_{12/13}$-KO: 15.7±1.5%, P = 0.094; n=12 bladder strips, three biological

replicates each group; Student's *t*-test). It appears that $G_{q/11}$ solely mediates NK2R signaling in urinary bladder smooth muscle *ex vivo*.

Although NK2R is coupled exclusively to $G_{q/11}$ *ex vivo* in detrusor muscle, in different systems $G_{12/13}$ protein and the Ca^{2+} independent pathway can also elicit smooth muscle contraction. Prostaglandins can induce contraction under physiological conditions; and their TP receptor has been shown to exert its effects via $G_{12/13}$ proteins in platelets. We hypothesized prostaglandins mediate contraction via the $G\alpha_{12/13}$-Rho-ROCK pathway in the urinary bladder smooth muscle. The contractile responses evoked by prostaglandins (PGE_2 and $PGF_{2\alpha}$, 10^{-5} M) were reduced in $G\alpha_{12/13}$-KO animals and in the presence of ROCK inhibitor Y-27632 to a similar magnitude suggesting the conventional activation of Ca^{2+} independent pathway (*Figure 3.*). Therefore, the predominance of the $G_{q/11}$ pathway, as observed in the case of NK2R, is not a general phenomenon for all GPCR-mediated UBSM contractions.

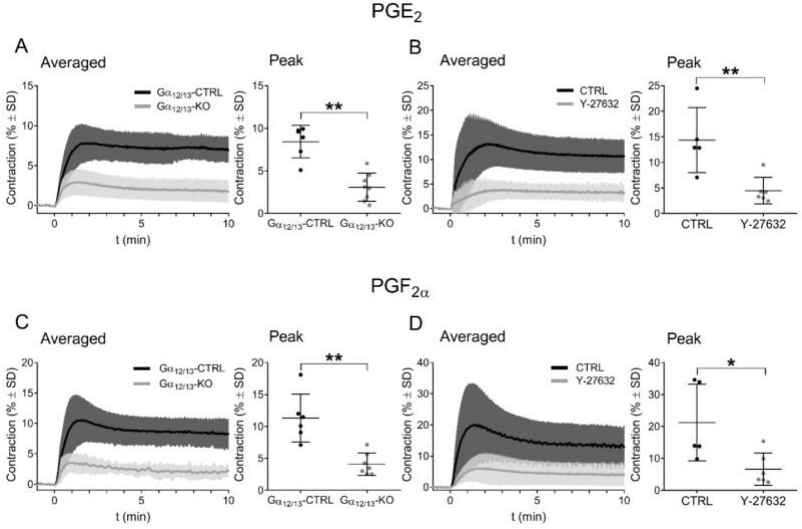

Figure 3. **Prostaglandin E_2 and $F_{2\alpha}$ induce urinary bladder smooth muscle contraction primarily via $G\alpha_{12/13}$-Rho-ROCK pathway.** * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Our next aim was to identify the downstream signaling partner(s) of $G_{q/11}$ proteins in mediating NK2R-induced UBSM contraction. $G_{q/11}$ proteins are linked primarily to PLC-β, which can evoke intracellular Ca^{2+} release by producing IP_3. However, two different types of PLC-β inhibitors (U-73122 and edelfosine) both failed to have any effect on contraction force, either in the acute (*Figure 4./A*) or during the tonic (*Figure 4./B*) phase of contraction. Therefore, PLC-β does not appear to be involved in the $G_{q/11}$-mediated signal transduction pathway of the UBSM.

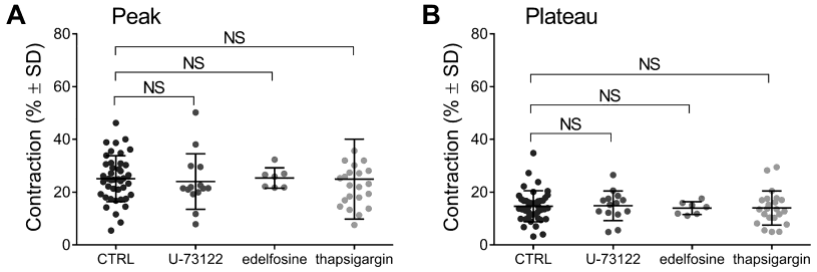

Figure 4. **Ineffectiveness of PLC-β inhibition or intracellular Ca^{2+} store depletion on NK2R-mediated detrusor muscle contraction.** NS, not significant

Next, we aimed to clarify whether the source of the Ca^{2+} signal is intracellular or extracellular. Intracellular Ca^{2+} store depletion by thapsigargin, which inhibits SERCA, did not alter the contractile effect of [β-Ala^8]-NKA(4–10) (*Figure 4./A and B*). In contrast, the removal of Ca^{2+} from Krebs solution resulted in a marked reduction of the contraction force in both the phasic and tonic phase of the response (*Figure 5.*), indicating the

predominant role of Ca^{2+} influx from the extracellular space in mediating UBSM contraction upon activation of NK2Rs.

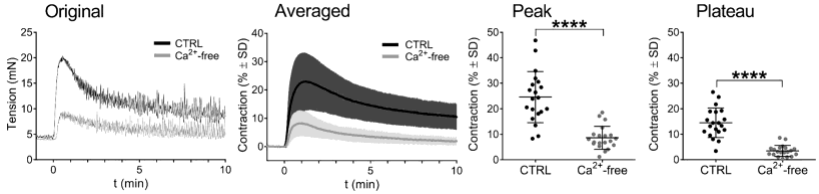

***Figure 5*. Role of extracellular Ca^{2+} in mediating [β-Ala^8]-NKA(4–10)-induced urinary bladder contraction.**
****P < 0.0001 (Student's *t*-test)

Different Ca^{2+} channels have been implicated in regulating the tone of the UBSM, of which VDCCs appear to have the most widespread biological function. Therefore, we aimed to evaluate their role in NK2R-mediated UBSM contraction. The VDCC blocker diltiazem induced similar inhibition of the contractile effect of [β-Ala^8]-NKA(4–10) as was observed in the absence of extracellular Ca^{2+} (*Figure 6.*). Taken together, these results indicate that Ca^{2+} influx through VDCCs mediates the contractile effect of NK2R stimulation.

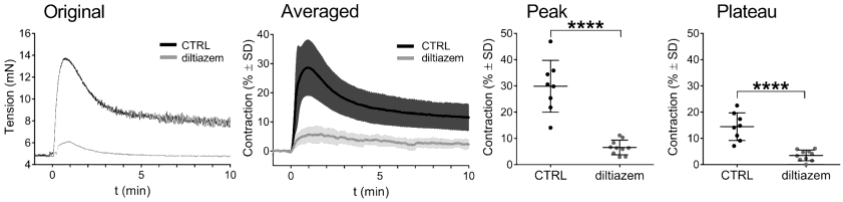

***Figure 6.* Involvement of VDCCs in NK2R-mediated detrusor muscle contraction.** ****P < 0.0001

Interestingly, neither removal of extracellular Ca^{2+} nor administration of diltiazem was able to diminish the effect of [β-Ala^8]-NKA(4–10) as completely as the deletion of $G\alpha_{q/11}$ proteins did, indicating the presence of an alternative intracellular signaling mechanism. We hypothesized that this might be the RhoA-ROCK signaling pathway since a previous study indicated its importance in the regulation of UBSM tone. Indeed, administration of [β-Ala^8]-NKA(4–10) increased RhoA activity in the UBSM of CTRL mice, whereas this effect was absent in the urinary bladders of $G\alpha_{q/11}$-KO animals (*Figure 7./A*). In addition, the ROCK inhibitor Y-27632 reduced the amplitude of [β-Ala^8]-NKA(4–10)-induced contraction during both the phasic and tonic phases (*Figure 7./B*). The combination of Y-27632 with the removal of extracellular Ca^{2+} or administration of diltiazem abolished completely the contractile effect of [β-Ala^8]-NKA(4–10) (*Figure 7./B*). Therefore, our results indicate that NK2Rs simultaneously activate VDCC-mediated Ca^{2+} influx and the RhoA-ROCK signaling pathway, both of which contribute significantly to UBSM contraction.

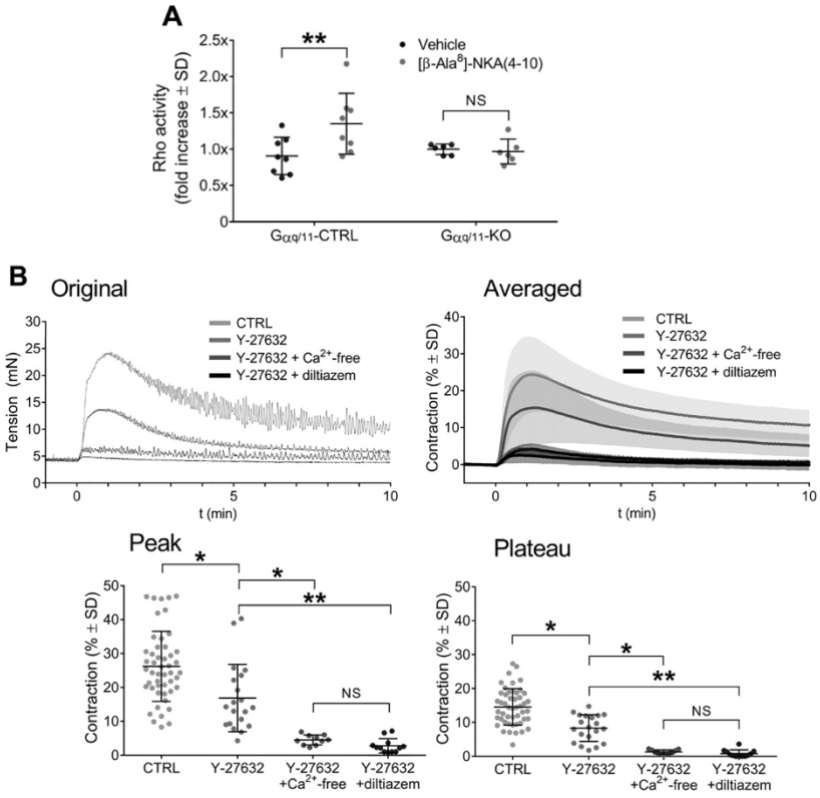

Figure 7. **Role of the RhoA-Rho kinase pathway in mediating detrusor contraction by [β-Ala^8]-NKA(4–10).**
**P* < 0.05; ***P* < 0.01

6. Conclusions

In our experiments, we aimed to investigate the intracellular signaling of TK-induced urinary bladder smooth muscle contraction under physiological conditions (*Figure 8.*).

Our results indicate that:

- All three TKs (NKA, NKB and SP) elicit detrusor contraction via NK2R which is coupled exclusively to $G_{q/11}$ protein
- PLC-β and intracellular Ca^{2+} release is not involved in the contractile response
- VDCC mediates contraction induced by NK2R activation
- Both the enzymatic assay readout and treatment with pharmacological inhibitor suggest that $G_{q/11}$ protein activates the Rho-ROCK pathway
- Prostaglandins mediate UBSM contraction via $G_{12/13}$-Rho-ROCK signaling

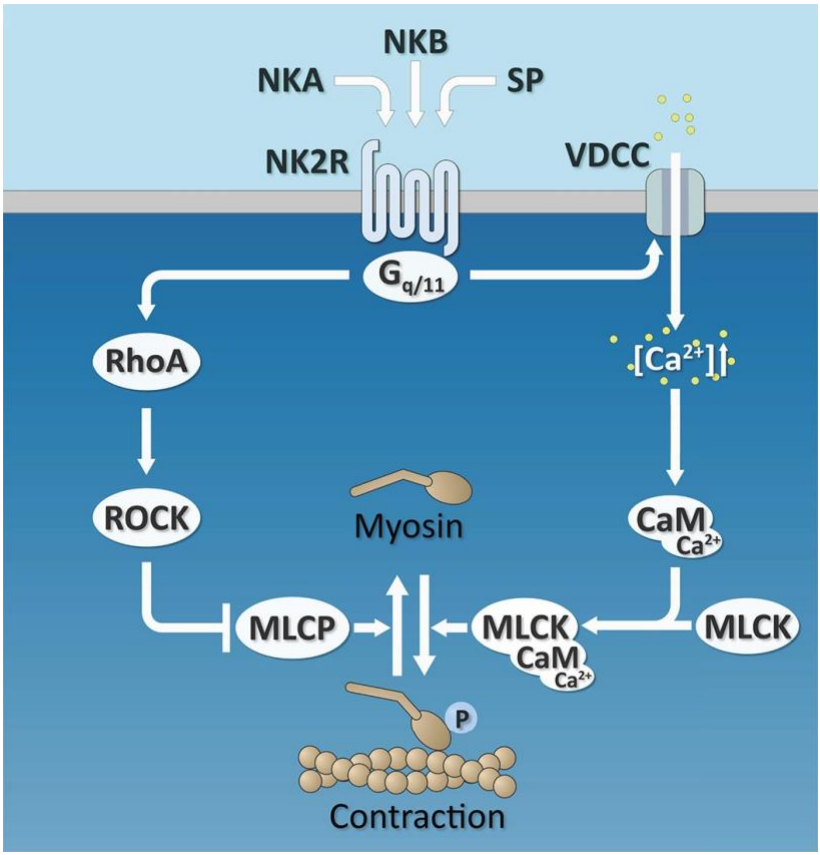

Figure 8. **Summary of NK2R signal transduction in the UBSM muscle.**

NKA: neurokinin A; NKB: neurokinin B; SP: substance P; ROCK: Rho kinase; MLCP: myosin light-chain phosphatase; MLCK: myosin light-chain kinase; CaM: calmodulin.

7. Bibliography of the candidate's publications

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