Targeted optogenetic stimulation of parasympathetic cholinergic nerve fibers reveals cholinergic-purinergic cotransmission in the bladder detrusor muscle

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1 Introduction

1.1 Overactive Bladder

Overactive bladder (OAB) is urgency, with or without urge incontinence, with increased frequency of voiding and nocturia. Symptoms of an OAB are highly prevalent in the general population, which has a considerable impact on patients health-related quality of life (Milson et al. 2001). The prevalence of OAB from population-based studies ranges from 7% to 27% in men and 9% to 43% in women (Corcos and Schick 2004).

Like other chronic conditions, OAB requires long-term therapy. Behavioral modification and training of pelvic floor muscle are safe and recommended treatment options for all patients (Burgio et al. 1998). However, only 20 % of patients in clinical studies achieve complete remission of their symptoms. For this case, pharmacotherapy is an important adjunct. Antimuscarinic agents are commonly used first-line pharmacological therapy (Guidelines of the European Association of Urology 2015). They block muscarinic receptors in the bladder wall, reducing detrusor contractility and sensation. However, only 10-25% of patients continue their treatment after 12 months (Sexton et al. 2011). The main reason is the adverse effects of antimuscarinic drugs such as dry mouth, dizziness, constipation, and ocular hypertension. On the other hand, it seems as if the effect of therapy does not satisfy the patients (Chapple 2000).

OAB is preferentially associated with the following conditions: aging, diabetes mellitus, bladder outlet obstruction, interstitial cystitis, spinal cord and brain injury, stroke, Parkinson's disease, and multiple sclerosis. Still, the pathophysiology of OAB is not well understood. The International Continence Society (ICS) assumes as underlying pathological processes in the formation of OAB an increased afferent nerve activity and mechanisms involved in the abnormal handling of afferent signals (Abrams et al. 2003). The increased afferent activity can be explained by changes in urothelial receptor function and neurotransmitter release, as well as by changes in the sensitivity and coupling of the suburothelial myofibroblasts. Therefore, this state amplifies the sensation of bladder fullness, leading to the urgency and predisposes the activation of the micturition reflex. This is the urothelium based hypothesis. Another hypothesis is the myogenic hypothesis. In the myogenic hypothesis, the involuntary contraction of the detrusor muscle is

provoked by changes in myocyte excitability and coupling of myocytes with other myocytes or myofibroblasts (Brading 1997, Chapple 2014).

The mechanisms involved in the abnormal handling of afferent signals originate from the damage of central inhibitory pathways (de Groat 1997). The neural circuitry that participates in this process is complex. Several recent findings support a key role for purinergic transmission in overactive bladder. O'Reilly et al. reported that purinergic P2X2 receptors were significantly increased in bladder tissue specimens obtained from women with idiopathic OAB (O'Reilly et al. 2002). Additionally, substantially increased nerve-mediated contraction due to ATP release were observed in these bladder samples. The issue of the study was that there is an abnormal purinergic transmission in the bladder of patients with idiopathic OAB, which can lead to the above-mentioned symptoms.

There is also increasing evidence that ATP mediated detrusor contraction increases with age and plays an important role in bladder disorders such as OAB. Recent data also show an age-related reduction in cholinergic transmission. However, the importance of these changes in the pathophysiology of OAB is not clear. The future research of OAB should be directed towards an in-depth understanding of the sensory, myogenic and motor mechanisms and how they cooperate.

1.2 Anatomy of the lower urinary tract

The lower urinary tract (LUT) is composed of the bladder and urethra. Extensive differences between males and females LUT concern the structure of bladder neck and urethra. The characteristic features of the male urethra are the prostate gland in the proximal portion of the urethra, and the longer distal portion due to its extension to the urethral plate of the penis (Elbadawi et al. 1966).

The empty bladder has a tetrahedral form and a capacity of nearly 500 ml. Superiorly, the bladder is covered by peritoneum. The anterior abdominal wall borders to the anterior surface of the bladder. Posteriorly, the peritoneum extends to the seminal vesicles and connects with the peritoneal sheet of the rectum to form the rectovesical space. In the female, the bladder superiorly and posteriorly borders to the uterus and vagina, forming the vesicouterine pouch. Inferiorly and laterally, the bladder is surrounded by the pelvic sidewall.

The bladder can be divided into a body, located above the ureter orifices, and a base consisting of the trigone and bladder neck (Tanagho et al. 1982). Donker and colleagues showed in their histologic examination that the myofibrils of the bladder body are arranged into randomly directed fascicles and muscle layers are not separable (Donker et al. 1976). This network of the detrusor muscle is ideally constructed for emptying the bladder.

The trigonal part of the bladder base consists of three distinct muscle layers: a superficial layer, derived from the longitudinal muscle of the ureter; a deep layer, which extends from the fibromuscular (Waldeyer) sheath, and the detrusor muscle formed by longitudinal and circular smooth muscle. As the ureter approaches the urinary bladder, the mural smooth muscle fibers run longitudinal and obliquely pierce the bladder wall. In the intercrossing of intramural ureter and detrusor, the ureter is compressed and forms a physiological stricture. This intercrossing acts as a flap valve and provides the passive closure of the ureter during bladder filling (Thompson et al. 1994).

Adjacent to the bladder neck, the detrusor muscle is separable into the above-mentioned three layers, and it persists of many finer fibers. The middle layer shows structural differences in male and female gender. In men, this is expressed in a competent and circular ring-like smooth muscle, the internal urethral sphincter (Gosling et al. 1981). The internal urethral sphincter is efficient to maintain continence in men, even if the striated (external) urethral sphincter is damaged (Waterhouse et al. 1973). In contrast, in 50% of continent women, urine enters the proximal urethra during coughing due to the absence of circular smooth muscle (Versi et al. 1986). In addition, the closure of the bladder neck in men promotes anterograde ejaculation. Sympathetic nerve fibers richly innervate the bladder neck. Their activation leads to contraction of the bladder neck during ejaculation. De Groat and Booth described in women a lower density of sympathetic nerves than in men (De Groat and Booth 1993).

The urethra begins at the bladder outlet and expands to the external meatus. There are four segments in the male urethra: preprostatic urethra, prostatic urethra, membranous urethra, spongy urethra (bulbar and penile part). Circular smooth muscle from the bladder neck extends to the preprostatic part of the urethra (Gosling et al. 1999). The length of the preprostatic urethra varies from 0.5-1.5 cm.

The prostatic part extends through the length of the gland. The urethral crest builds a verumontanum on the posterior wall. On the apex of the verumontanum, there is a

Müllerian duct residue, the prostatic utricle. Ejaculatory ducts open from either side of the utriculum.

The membranous urethra courses from the apex of the prostate to the perineal membrane. The average length of the membranous part varies from 2-2.5 cm (1.2-5 cm) (Myers et al. 1991). A striated external urethral sphincter is located in the membranous urethra. This is an important structure to maintain continence. The external urethral sphincter has a broad base and a narrowing at the apex of the prostate in the urogenital hiatus of the levator ani.

In contrast to males, the female urethra is short and approximately 4 cm in length. Several authors deny the existence of an internal urethral sphincter (Gosling et al. 1981, Lassmann et al. 1984). However, the external urethral sphincter builds up two-thirds of the distal urethra (Oelrich et al. 1983). The sphincter mechanism of females is divided into two components. In the proximal part, it forms a complete ring around the urethra and distally the fibers do not meet posteriorly, forming two arch-shaped straps of muscle (De Groat and Yoshimura 2015). Contraction of the distal part leads to compression of the urethra against the fixed anterior vaginal wall.

1.3 Bladder compartments

1.3.1 Urothelium

The urothelium is a multilayer epithelium that consists, depending on species, of up to seven cell layers (Fawcett et al. 1984). There are three important layers (Fig.1.1): basal, intermediate and apical (umbrella cells). Umbrella cells are the largest epithelial cells covering the luminal surface of the urothelium (Lewis et al. 2000). The name “umbrella” is given due to its shape. Each umbrella cell can cover several intermediate cells and can reach the basal membrane with a stem-line process. Besides the primary urine-plasma barrier function, umbrella cells can stretch during bladder filling. One of the explanations of this phenomenon is a large number of discoid vesicles under the apical membrane. While stretching the amount of vesicle decreases rapidly through exocytosis and leads to an increase of the cell surface (Porter et al. 1967, Staehelin et al 1972, Sarikas and Chlapowsky 1986).

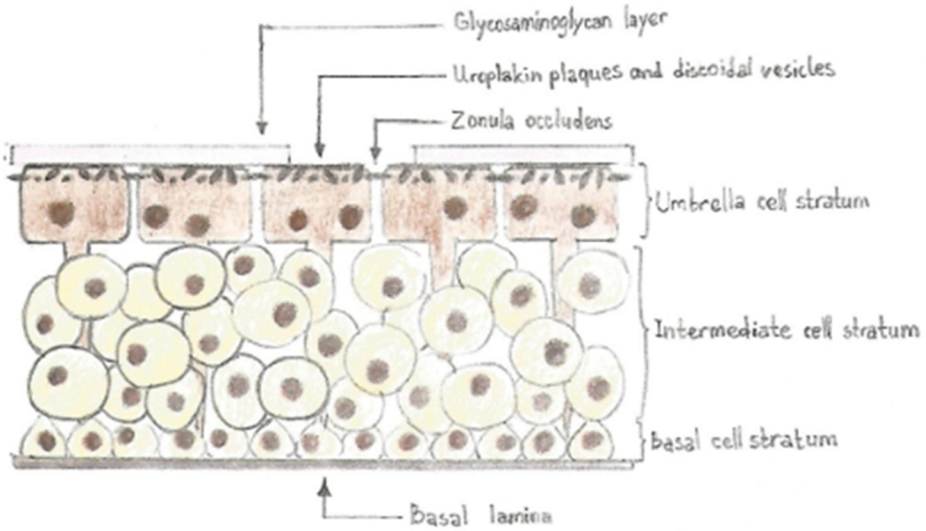


Figure 1.1 Diagrammatic representation of the bladder urothelium. The lumen of the bladder is on top and the lamina propria is below.

One essential and highly discussed function of the urothelium is its barrier function. In the seventies, Parsons and Mulholland assumed that the glucosaminoglycan (GAG) layer plays an essential role in the barrier function of the urothelium. GAG is a polysaccharide, which covers the luminal surface of umbrella cells. Their theory was based on increased bacterial adherence in rabbit urothelium after topical acid instillation and recovery of normal barrier function within 24 hours after injury, supposing that the GAG layer is a secretory factor produced by the urothelium to recover itself (Parsons et al. 1975, Muhlholland et al. 1976). Further studies showed that the secretory factor was, in fact, the mucin glycoprotein (MUC-1) rather than a glucosaminoglycan (Buckley et al. 1996, Higuchi et al. 2000). Later, Lavelle and colleagues demonstrated that GAG and MUC-1 have no effect on the transcellular permeability of water and urea in the urothelium (Lavelle et al. 1997). Overall, there were controversial results and no definite evidence that the GAG layer operates as a primary barrier between urine and plasma in the urothelium.

On the other hand, animal models have demonstrated that umbrella cells play an important role in the permeability of water and urea. Specifically, Lavelle and coworkers convincingly showed that the destruction of the apical layer leads to increased

permeability, apparently through leakage of urine solutes into the lamina propria (Lavelle et al. 1998, Lavelle et al. 2000).

There is now increasing evidence that the urothelium has additional signal transduction properties. This statement is based on the presence of many receptors found in recent years in the urothelium (receptors for bradykinin, neurotrophins, purines, norepinephrine, acetylcholine (ACh), transient receptor potential (TRP) channels and others). Activation of these receptors in a response to chemical or mechanical stimuli leads to release of mediators such as NO, ATP, ACh and substance P (SP) (Ferguson et al. 1997, Birder et al. 1998, Burnstock et al. 2001a). These mediators may act directly on afferent nerves or indirectly through suburothelial interstitial cells – myofibroblasts. Myofibroblasts are broadly connected by gap junctions and can respond to mediators that in turn modulate afferent nerves (Fowler et al. 2008). Therefore, it is believed that urothelial cells and myofibroblasts can cooperate in sensory mechanisms in the urinary tract by chemical coupling to the bordering sensory nerves.

1.3.2 Lamina propria

The lamina propria (LP) is located between the basal membrane of the urothelium and the detrusor muscle (Fig. 1.2). It includes an extracellular matrix, interstitial cells (IC), afferent and efferent nerve endings, and a rich vascular network (Dixon and Gosling 1983, Paner et al. 2007, Paner et al. 2009, Aitken and Bägli 2009). Interestingly, many authors deny the presence of muscularis mucosae in the LP of the human urinary bladder, unlike in the intestine, so that in some articles the LP is referred to as a true “submucosa” (Dixon and Gosling 1983, Paner et al. 2009). On the other hand, Ro and co-workers claimed that the muscularis mucosae consist of irregularly arranged muscle bundles that form a discontinuous layer (Ro et al. 1987).

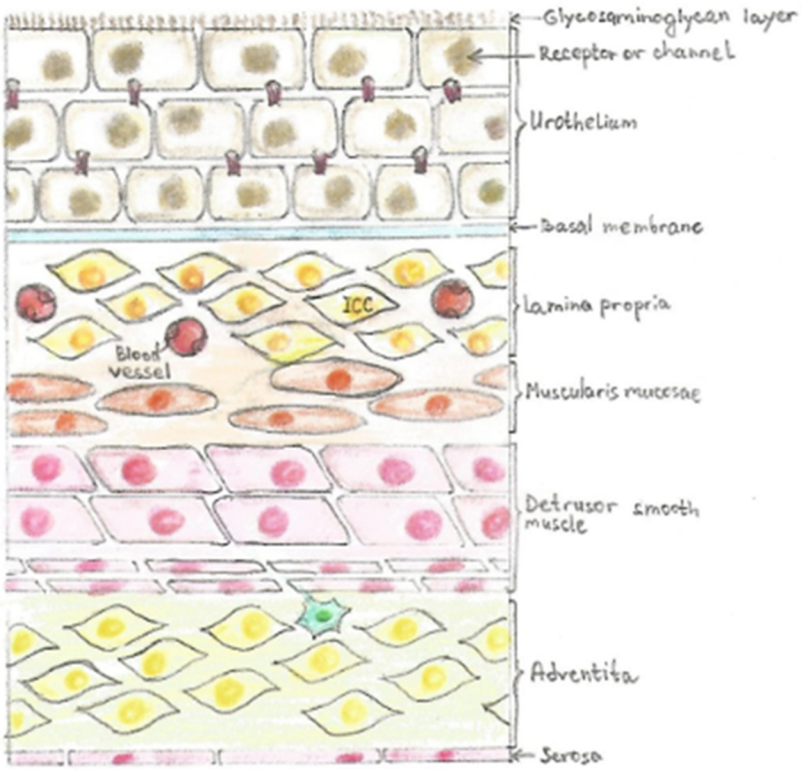


Figure 1.2 Anatomical components of the urinary bladder wall.

Interstitial cells (IC) of Cajal were first identified as pacemakers (driving the peristaltic activity of smooth muscle and transmit the signals from nerves to smooth muscle) in the gastrointestinal tract by Sanders in 1996 (Sanders 1996). In analogy, the presence of IC was presumed in the urinary bladder (Smet et al. 1996, Davidson and McCloskey 2005). Over the last decade, increasing evidence argues for the role of IC in the modulation of bladder smooth muscle activity and sensory processing. To identify IC in the bladder, different antibodies were used, such as those against vimentin, tyrosine receptor KIT, and platelet-derived growth factor receptor alpha (PDGFRα) (Koh et al. 2012, Monaghan et al. 2012). Unfortunately, these antibodies did not label exactly the same cellular population. Moreover, it was observed that different subtypes of IC in the urinary bladder could co-express two or more of these markers (Andersson et al. 2014). Up to this day, we distinguish four different subtypes of IC in the bladder wall. IC of the lamina propria (IC-LP) forms a cellular network interconnected by gap junctions consisting of connexin 43 (Davidson and McCloskey 2005). Detrusor-IC (IC-IM), unlike IC-LP, do not build up

a network and are arranged on the border of smooth muscle bundles (er et al. 2013). Interbundle IC (IC-IB) as a name tells us that they are located between detrusor bundles. The last known subtype is perivascular IC described by Cunningham (Cunningham et al. 2011). All IC according to their morphology, regardless of their subtype, are located close to nerves (Davidson and McCloskey 2005, Sui et al. 2002, Johnston et al. 2010).

Mukerji and coworkers reported the expression of the muscarinic M2 and M3 receptors in IC-LP. This finding was confirmed later also by Grol et al. so that they suggested that these cells respond to muscarinic stimuli (Mukerji et al. 2006, Grol et al. 2009). However, Sui and coworkers reported that the suburothelial IC did not respond to the application of the muscarinic agonist, carbachol, but are sensitive to ATP application. ATP induces Ca^{2+}-activated chloride currents (Sui et al. 2004, Sui et al. 2008). This could be explained by the expression of purinergic receptors (P2X3, P2Y2, P2Y4, P2Y6) (Sui et al. 2006). Ost reported also an expression of transient receptor potential cation channel subfamily V member 1 (TRPV1) on IC-LP (Ost et al. 2002). Different studies have concluded that IC-LP may be associated with many other Slosksignal systems (Nile et al. 2010, Rahnama' I et al. 2010, Nile and Gillespie 2012). However, the functions of bladder ICs are not completely understood yet.

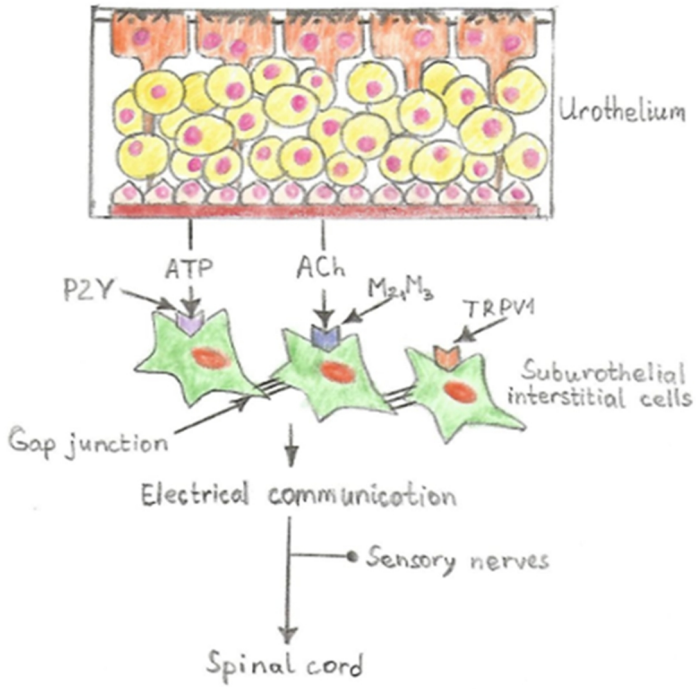


Figure 1.3 Schematic representation of suburothelial interstitial cells. Substances released from the basolateral surface during stretches, such as adenosine triphosphate (ATP) and

acetylcholine (ACh), activate afferents in the suburothelial layer. Interstitial cells express purinergic P2Y receptors, muscarinic M2, and M3- receptors, or capsaicin receptors (TRPV1) receptors, and are connected to one another by gap junctions.

1.3.3 Smooth muscle

The architecture of the bladder detrusor differs from smooth muscle layers in the ureter or gastrointestinal tract. One particular feature is a distinctive organization in fascicles. They settle in a random direction (Donker et al. 1982). Each muscle cell in a bundle is connected with others and they form a functional syncytium.

Postganglionic parasympathetic nerve fibers innervate the detrusor muscle. Not each smooth muscle cell receives direct synaptic contact. Due to the presence of gap junctions, the excitation spreads through the smooth muscle syncytium (Gillespie et al. 2006). Neuronally induced detrusor contraction is mainly mediated through ACh and ATP. When muscarinic and purinergic receptors are activated by their agonists, these receptors initiate the excitation-contraction coupling of the smooth muscle in the urinary bladder (Andersson and Arner 2004, Nausch et al. 2010, Bayliss et al. 1999).

1.4 Peripheral neural control of the lower urinary tract

The innervation of LUT is accomplished by three sets of efferent peripheral nerves: sacral parasympathetic (running in the pelvic nerves), thoracolumbar sympathetic (in hypogastric nerves) and sacral somatic pathway (in pudendal nerves). Each of these nerves contains afferent in addition to these efferent (motor) axons. The afferent axons in the pelvic, hypogastric, pudendal and levator ani nerves convey information from the LUT and pelvic floor to the dorsal horn in the lumbosacral spinal cord (de Groat 1986, Yoshimura and de Groat 1997).

1.4.1 Parasympathetic pathway

The parasympathetic preganglionic neurons are located in the lateral part of the sacral gray matter (Nadelhaft et al. 1980, Morgan et al. 1981, de Groat et al. 1993). They are carrying information from the spinal cord to the bladder and urethra. Parasympathetic

postganglionic neurons are effectors of excitatory transmission in the bladder (de Groat and Booth 1993). They located in the detrusor wall as well as in the pelvic plexus (De Groat et al. 1993, De Groat et al. 1996). This location is crucial to know in patients with lesion of the spina equina or the pelvic plexus because afferent and efferent neuron interconnection is possible at the level of the intramural ganglia.

The activation of the postganglionic parasympathetic nerve leads to the release of ACh and ATP in the bladder (Andersson 1993, Andersson and Arner 2004). However, it is unclear whether the purinergic ligands are released together with ACh from the same nerve fibers as a cotransmitter or from a separate fiber population. The concept of cotransmission is now well accepted as an integral feature of neurotransmission (Burnstock 1976, Burnstock 1990a). Unfortunately, there is little information about cholinergic/ATP-cotransmission within the parasympathetic nervous system (Burnstock et al. 2001b). The paucity of data addressing parasympathetic cotransmission is probably due to the fact that it is harder to surgically or chemically denervate postganglionic parasympathetic nerves than postganglionic sympathetic nerves (Burnstock et al. 2001b). Several studies present indirect evidence for cholinergic/purinergic cotransmission. In the seventies, Hoyes et al. proposed that ACh and purinergic transmitters are colocalized in small agranular and large opaque vesicles in nerve terminals (Hoyes et al. 1975). Further, MacKenzie and coworkers showed a significant reduction of the excitatory response of both cholinergic and purinergic component to nerve stimulation by botulinum neurotoxin type A in the guinea-pig bladder (MacKenzie et al. 1982). Other indirect evidence for purinergic cotransmission was described by Parija and coworkers. Using adenosine in the rat bladder, they achieved prejunctional inhibition of both cholinergic and purinergic components of the nerve-mediated responses (Parija et al. 1991). It appears to be likely from pharmacological studies that a range of nerves exists, containing diverse proportions of ATP and ACh, from predominantly ATP in cat and guinea-pig through to roughly 50:50 in rat and dog to predominantly ACh in the human bladder (Burnstock et al. 2001a).

ACh acts upon postjunctional muscarinic M2 and M3 receptors. M2 receptors inhibit adenylate cyclase. This causes bladder contraction due to suppression of the stimulation of adenylate cyclase through β-adrenergic receptors (Andersson and Arner 2004). However, investigation on knockout mice revealed that the M3 is the dominant receptor involved in the transmission (Andersson et al. 1993, Andersson and Arner 2004, Matsui et al. 2000, Matsui et al 2002). Activation of M3 receptors causes an increase in the

intracellular Ca^{2+}-concentration. There are two known mechanisms leading to this increase in intracellular Ca^{2+}-concentration: 1) extracellular entry through L-type voltage-dependent calcium channel (VDCC) (Anderson and Arner 2004, Anderson and Wein 2004, Schneider et al. 2004a, b, Nausch et al. 2010), 2) release of Ca^{2+} from intracellular Ca^{2+} stores in the sarcoplasmic reticulum (SR) (Iacovou et al. 1990, Eglen et al. 1994, Fry et al. 2002, Braverman et al. 2006a). The activation of muscarinic M3 receptors leads to activation of a Gq-protein α-subunit and subsequent activation of the membrane-bound enzyme, phospholipase C (PLC). The PLC cleaves a membrane phospholipid, phosphatidylinositol 4, 5-biphosphate (PIP2), and forms cytosolic inositol triphosphate (IP3). IP3 binds to its receptor on the SR, thereby provoking Ca^{2+}-release.

The role of the intracellular Ca^{2+}-increase in the contraction of the smooth muscle is currently under debate. Nausch et al. described that during electrical field stimulation (EFS) – a stimulus that excites nerve endings to release transmitters – smooth muscle contraction is reduced after application of a muscarinic inhibitor (atropine) and a VDCC blocker (diltiazem) (Nausch et al. 2010), but not by blocking of the IP3-receptor or inhibition of the PLC. It should be mentioned that in this study the purinergic components were blocked through α,β-methylene ATP.

The excitation of bladder smooth muscle by ATP is mediated through P2X1 receptors. The P2X1 receptor is known as the main subtype of P2X receptors in the rat and human bladder (Ralevic et al. 1998, Burnstock et al. 2001b). It is disputed that purinergic transmission has an important role in excitation of animal bladders but not in the healthy human bladder. Bayliss et al. forwarded the theory that ACh mediates contraction of the bladder in the normal healthy state (Bayliss et al. 1999). In patients with LUT disorders, neurotransmission works mainly through ATP. His study of human bladders from patients with LUT obstruction and OAB revealed that the atropine-resistant contraction was blocked by α,β-methylene ATP, a blocker of P2X receptors. Yoshida et al. demonstrated an increasing dependence of the purinergic signaling with the aging bladder (Yoshida et al. 2001). Recent studies in animal models revealed that activation of both purinergic and muscarinic receptors leads to bladder contraction (Heppner et al. 2009).

1.4.2 Thoracolumbar sympathetic pathways

Sympathetic preganglionic pathways originate from the T11 to L2 level, pass through the synaptic chain ganglia to the inferior mesenteric ganglia and then follow the hypogastric nerves to the pelvic plexus. Sympathetic postganglionic nerve fibers release norepinephrine, leading to the activation of the smooth muscle of the bladder base and urethra as well as inhibition of the smooth muscle of the bladder body. Anderson et al. described the prevalence of α1-adrenergic receptors in the bladder base and proximal urethra, whereas β3-adrenergic receptors are predominantly expressed in the bladder body (Andersson and Arner 2004).

It is assumed that ATP is contained together with noradrenaline (NA) in sympathetic nerve terminals. The first evidence for sympathetic cotransmission involving ATP together with NA came from Su and coworkers. They demonstrated the release of ATP during the stimulation of periarterial sympathetic nerves and blocking of both ATP and NA release by guanethidine (Su et al. 1971). The most extensive evidence for sympathetic cotransmission came from the investigation on the vas deference by Westfall and colleagues. Their study showed that ATP is both a potent contractile agent and is present in the adrenergic storage complex in the vas deferens (Westfall et al. 1978). Further, Theobald mentioned in 1983 that ATP is also released from the hypogastric nerve. In his experiments, after the stimulation of the hypogastric nerve and contraction of the bladder of the cat, he used arylazido aminopropionyl ATP (ANAPP3) to reduce the contraction, implying that ATP was released. 6-Hydroxydopamine, which destroys noradrenergic sympathetic nerve fibers, prevents this contractile response, indicating that ATP was released from sympathetic nerve terminals (Theobald 1983). Due to this strong evidence that ATP acts as a cotransmitter with noradrenaline in the sympathetic nervous system, the concept of purinergic-noradrenergic cotransmission is now well accepted.

1.4.3 Sacral somatic pathway

The efferent pathway to the external urethral sphincter (EUS) arises from Onuf´s nucleus at the lateral border of the ventral horn in the third and fourth sacral segment (Thor et al. 1989). The fibers travel in the pudendal nerve. As the pudendal nerve passes through Alcock's canal, it branches into the inferior rectal nerve (which innervates the anal rhabdosphincter), the perineal nerve (which innervates the striated urethral sphincter, the

bulbospongiosus muscle, the ischiocavernosus muscle, superficial perineal muscle and labial skin), and the dorsal nerve of the clitoris or penis, respectively (de Groat and Yoshimura 2015).

1.4.4 Afferent Pathways

Afferent axons in the pelvic, hypogastric and pudendal nerves transmit information from the LUT to the spinal cord. The cell bodies of these neurons are located in lumbosacral dorsal root ganglia (DRG). Afferent nerve fibers that innervate the bladder and the urethra are divided into two major populations: myelinated (Aδ) and unmyelinated C-fibers.

Aδ-fibers are mechanoreceptors that respond to passive distension as well as the active contraction of the bladder. They are silent when the bladder is empty, but increase firing frequency during slow filling of the bladder, indicating that they are tension receptors (Häbler et al. 1993, Bruns et al. 2011). Electrophysiological studies in rats have revealed that the normal micturition reflex is managed by myelinated Aδ-fiber afferents (Mallory et al. 1989).

In contrast to the Aδ bladder afferents, the C bladder afferents are not mechano-sensitive (Häbler et al. 1990). These afferents are nociceptive and respond to cold or chemical stimuli (Häbler et al. 1990). The exposure of the bladder to chemical stimuli such as high potassium, low pH, and high osmolarity, leads C-fibers to acquire mechanoreceptive properties. It is believed that C-fiber afferents are not essential for normal voiding (de Groat et al. 1990). However, the bladder overactivity induced by noxious stimuli can be reduced by the application of capsaicin, indicating that C-fiber afferents play an important role in LUT dysfunction in pathological conditions (Cheng et al. 1995, de Groat and Yoshimura 2009, de Groat and Yoshimura 2015).

Immunohistochemical studies demonstrated that afferent neurons contain numerous neuropeptides, such as SP, calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal peptide (VIP), and putative excitatory amino acid transmitters (Keast and de Groat 1992, Maggi 1993, Keast and Stephensen 2000, Brumovsky et al. 2013).

Tachykinins (SP, neurokinin A, neurokinin B) and CGRP act on neurokinin (NK) 1, NK2, NK3 or CGRP receptors, respectively (Andersson 2002). The sensory functions of the

tachykinins include regulation of micturition threshold, activation of cardiovascular reflexes and perception of pain from the urinary bladder (Maggie et al. 1995, Andersson 2002). Expressions of tachykinins in the LUT have been reported in animal models of bladder inflammation and in the clinical syndrome of interstitial cystitis and painful bladder syndrome. It has been demonstrated that intrathecal injection of SP antagonists reduces cyclophosphamide-induced bladder hyperreflexia (Lecci et al. 1994).

1.5 Muscarinic acetylcholine receptors in the bladder smooth muscle

Muscarinic receptors are essential for the contraction of the detrusor smooth muscle. They consist of five subtypes (M1-M5) encoded by five distinct genes. In the human urinary bladder, all five subtypes of muscarinic receptors are demonstrated (Sigala et al. 2002, Bschleipfer et al. 2007). M2 and M3 receptors are dominant in the human bladder (Sigala et al. 2002, Yamaguchi et al. 1996). Muscarinic receptors are coupled to G-proteins. The signal transduction of M3 and M2 receptors are different. After many years of the research, the calcium influx via L-type VDCC and increased sensitivity of the actinomyosin cross-bridge cycling to calcium through activation of Rho-kinase is considered as the main pathway for the contraction of the human detrusor through the M3 receptor. This leads to the inhibition of the myosin light chain phosphatase (MLCP) (Scheider et al. 2004a, Andersson et al. 2004a), thus, tipping the balance toward the contraction of the smooth muscle. The M2 receptor inhibits the adenylyl cyclase activity, accordingly reducing the efficacy of β-adrenoreceptor mediated responses (Hegde et al. 1997).

In the detrusor smooth muscle of the human bladder, 10% of the muscarinic receptors are of the M1 subtype, 20% of the M3 subtype and 70% of the M2 subtype (Mansfield et al. 2005). The ratio between M2 and M3 can vary from 9:1 to 3:1, depending on the species (Wang et al. 1995, Yamanishi et al. 2000). Interesting results were derived from the cystometric examination of mutant mice. For example, prolonged voiding intervals, larger micturition volume and larger bladder capacity than the wild-type mice were observed in M3-knockout mice (Igawa et al. 2004). The residual volume was the same in both groups, which indicates normal bladder emptying. Probably the lack of M3 receptors is compensated by non-cholinergic receptors (purinergic) or by another subtype of

muscarinic receptors. Cystometric measurements in M2-knockout mice did not show a significant increase in the micturition frequency and volume (Igawa et al. 2004). This result again suggests that the M2 receptor mediates contraction indirectly through inhibiting adrenergic relaxation.

1.6 Purinergic receptors in the bladder smooth muscle

Since the participation of ATP in bladder contraction is known, many efforts were undertaken to recognize ATP receptors involved in this activity. In 1994, Abbracchio and Burnstock described two types of ATP receptors: ionotropic P2X receptors and metabotropic P2Y receptors (Abbracchio and Burnstock 1994). Up to date, there are 7 known subtypes of P2X ion channel receptors and 8 subtypes of P2Y G-protein coupled receptors (Burnstock 2007, Ralevic and Burnstock 1998).

While it is well established that ATP contracts the urinary bladder, it is assumed that ATP can also relax the detrusor muscle (Burnstock 2014). This is most likely due to the expression of multiple purinergic receptors in the bladder.

1.6.1 P2X receptors

It seems that the P2X1 receptor is the dominant P2X receptor subtype in the smooth muscle cell membrane of the urinary bladder, as it has been shown in immunohistochemical studies (Lee et al. 2000). Other immunohistochemical studies reported that P2X1 receptors spread not only in the smooth muscle of the bladder but also in parasympathetic nerve varicosities (Hansen et al. 1998, Dutton et al. 1999).

Other molecular genetic investigations, such as Northern blotting and in situ hybridization, described the presence of P2X1 mRNA in the urinary bladder (Valera et al. 1995). Further Northern blot analyses detected also an expression of the P2X4 receptor gene in the human bladder (Dhulipala et al. 1998). However, despite the detection other receptor subtypes in the bladder, all authors agree that the P2X1 subtype is the functional receptor subtype mediating contraction (Burnstock et al. 1978, Valera et al. 1994, Evans et al. 1995, Hansen et al. 1998).

1.6.2 P2Y receptors

In contrast to P2X receptors, the function of P2Y receptors in the bladder is under debate. It is discussed that these receptors are involved in the inhibition of bladder contraction (Theobald and de Groat 1989). There are two theories on the mechanisms underlying the inhibitory function of P2Y receptors: 1) direct initiation of detrusor relaxation via postjunctional P2Y receptors on the smooth muscle, 2) prejunctional inhibition of the release of excitatory neurotransmitters (Theobald et al. 1992).

In the early 90´s, Boland et al. and Bolega et al. reported that the stimulation of purinergic receptors with ATP induces a biphasic force response in bladder muscle strips (Boland et al. 1993, Bolego et al. 1995). The biphasic response was characterized by an initial fast contraction and a slower long lasting relaxation. This effect had been described already in the respiratory, vascular, digestive and genital tract (Brown and Burnstock 1981, Manzini et al. 1985, Ralevic and Burnstock 1991, Boland et al. 1992). Evidence advanced that the long-lasting relaxation is mediated through a P2Y receptor in the bladder smooth muscle. Especially, Boland et al. in their experiment precisely examined the effect of ATP on the smooth muscle. The aim of their experiment was to induce tachyphylaxis, which was specific for P2X receptors, applying ATP twice. After the second stimulation, the contractile response to ATP was decreased, this effect is called tachyphylaxis. Interestingly, however, the relaxation caused by ATP was not decreased. These results lead to the conclusion that the two opposing effects are mediated through two different purinergic receptors (Boland et al. 1993).

Further studies, using molecular analysis such as RT-PCR (reverse transcription-polymerase chain reaction), Northern blotting and in situ hybridization demonstrated the expression of P2Y1, P2Y2 and P2Y4 receptors in the smooth muscle of the urinary bladder (Obara et al. 1998).

The physiological function of P2Y receptors in the detrusor muscle is not completely known. They could play an essential role in relaxing the bladder muscle during the storage phase. On the other hand, recent data describe the presence of P2Y receptors on the bladder neck (Tong et al. 1995). Thereby, a role in micturition through relaxing the bladder neck needs also to be considered.

1.7 Neuromodulation of detrusor contraction

Nerves innervating the detrusor mostly express acetylcholinesterase and the vesicular acetylcholine transporter (VAChT) and are considered to be parasympathetic (Ek et al. 1977, Dixon et al. 1983). Such parasympathetic postganglionic fibers release ACh, resulting in detrusor contraction. In addition, early studies demonstrated that also ATP plays an important role in detrusor contraction (Burnstock et al. 1978, Valera et al. 1994, Evans et al. 1995). EFS studies have been used to investigate the neurotransmitter released from muscle strips. These studies revealed that EFS responses are blocked by muscarinic receptor antagonists combined with purinergic antagonists (Lawrence et al. 2010) so that both ACh and ATP seem to provide most of the excitatory input (Fig. 1.4). Beside ACh and ATP, there are additional substances present in parasympathetic efferents (nitric oxide synthase (NOS), vasoactive intestinal polypeptide (VIP), β- NAD, etc.), which raise the question whether extra transmitters (other than ACh and ATP) have a part in regulating normal micturition or in disease pathophysiology (Lundberg 1981, Callsen-Cencic and Mense 1997, Breen et al. 2006).

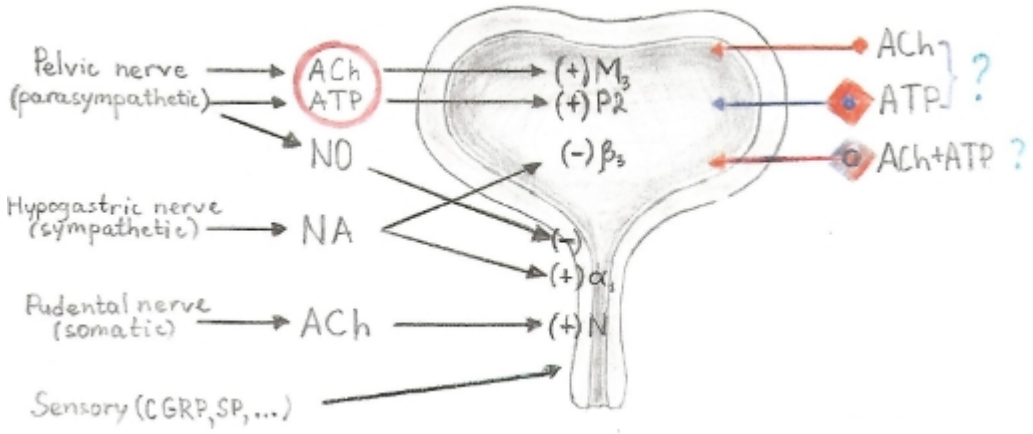


Figure 1.4 The innervation of the bladder, urethra and external urethral sphincter. The parasympathetic fiber of the pelvic nerve stimulates the bladder detrusor muscle, mediated by muscarinic and purinergic receptors and relaxes the urethral smooth muscle, mediated by nitric oxide. The sympathetic hypogastric nerve stimulates the urethral smooth muscle, mediated by α1-adrenergic receptors and inhibits bladder detrusor contraction, mediated by β3-adrenergic receptors. The somatic pudendal nerve stimulates striated muscle of the external urethral sphincter, mediated by nicotinic receptors. Sensory nerves are located in the whole urothelium mediated by tachykinins, CGRP or neurokinin receptors. Plus and minus signs describes neural stimulation and inhibition,

respectively. ACh: acetylcholine; ATP: adenosine triphosphate; NO: nitric oxide; NA: noradrenaline; CGRP: calcitonin gene-related peptide; SP: substance P.

Additionally, it is still not clarified if ATP is released from separate nerve fibers or together with ACh as a cotransmitter from postganglionic parasympathetic nerve fibers in the urinary bladder. By the early seventies, evidence that nerves could in fact release more than one substance as a neurotransmitter was accumulating (Su et al. 1971, Dowdall et al. 1974, Lagercrantz and Stajärne 1974). Numerous techniques, such as myography, electrophysiology and biochemical measurement of neurotransmitter release, have been used to show that ATP has cotransmitter roles in peripheral nerves. Still, all these techniques affect more than a single fiber population. Electrophysiological stimulation, for example, excites all nerve fibers and, thus, has a lack of specificity for the cell type. Pharmacological manipulation is specific for receptors, but these may be expressed by several functionally distinct nerve fiber populations. Further, this technique has a low temporal resolution. To overcome these problems, we used a new technique with temporal precision and high cell specificity. This technique is called optogenetic stimulation.

1.8 Optogenetics

In 1979, Francis Crick proposed the need for a method, which activates all neurons of one type, leaving the other unaltered (Crick 1979). Many different manipulations were developed to fulfill the criterion set by Crick, but only optogenetics succeeded.

“Optogenetics is the combination of genetic and optical methods to achieve gain or loss of function of the well-defined event in the specific cells of living tissue.” (Deisseroth 2010). Initially, optogenetics was developed to enhance our knowledge of fundamental neuroscience. To this day, optogenetics expands our knowledge beyond the nervous system. The reason is that optogenetics promotes the control of defined events in a defined cell type and at a defined time (Deisseroth 2011). In other words, this technique allows turning defined groups of neurons on and off safely in freely moving animals, avoiding the development of compensatory effects.

The basic principle of optogenetics is to express photosensitive proteins in a defined cell group. These proteins are ion channels, so that illumination with light of certain wavelengths allows the inflow of cations or anions in the cell and, hence, causes

depolarization (excitation) or hyperpolarization (inhibition) of this cell. In nature, there are several natural photosensitive proteins: bacteriorhodopsin (BR), described by Stockenius and Oesterhelt in 1971, halorhodopsin (NpHR), described by Matsuno-Yagi and Mukohata in 1979, and channelrhodopsin (ChR), described in 2002 by Nagel (Oesterhelt et al. 1971, Matsuno-Yagi et al. 1997, Nagel et al. 2002).

Miesenböck reported a pioneering work of a cell-specific activation of neurons. He and his coworkers described the interaction of invertebrate rhodopsin with other proteins through activation of ligand-gated ion channels during the stimulation of a synthetic photo-caged precursor (Zemelman et al. 2002). Around this time, Nagel and colleagues showed that rhodopsins are directly light-gated ion channels (Nagel et al. 2002). In their experiments, they expressed channelrhodopsin-1 (ChR-1), from the green algae *Chlamydomonas reinhardtii*, in mammalian cells. A year later Nagel et al. described that mammalian cells expressing channelrhodopsin-2 (ChR-2) can be depolarized by a light stimulus (Nagel et al. 2003). In 2006, Deisseroth and his group successfully delivered the gene expressing ChR2 to rat hippocampal neurons using a viral delivery method (Deisseroth et al. 2006).

All opsins have structural similarities with each other. However, in their working principle, ChRs are completely different from NpHR and BRs (Archaerhodopsin (Arch) and ArchaerhodopsinT (ArchT)) (Fig. 1.5). ChRs are non-selective cation channels that open when exposed to 470 nm blue light. The passive influx of Na^+ and less Ca^{2+} ions leads to depolarization (Nagel et al. 2003). In contrast, the stimulation of NpHR with 589 nm yellow light results in an influx of chloride ions into the cell and causes a hyperpolarization with consecutive silencing of the targeted cell. Light of a wavelength of 566 nm leads to the activation of Arch and ArchT and induces hyperpolarization of the cell through efflux of H^+ (Chow et al. 2010, Han et al. 2011).

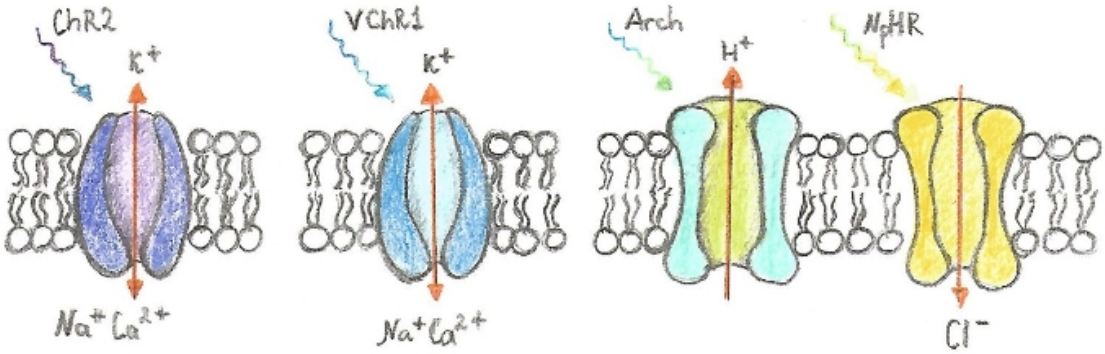


Figure 1.5 The optogenetic principle. Activating tools: ChR2 from *Chlamydomonas reinhardtii* and ChR1 from *Volvox carteri* (VChR1); silencing tools: archaerhodopsin-3 (Arch) from *Halorubrum sodomense* and Halorhodopsin (NpHR) from *Natronomonas pharaonic*.

In order to function, opsin requires a chromophore retinal. Trans-Retinal is an aldehyde derivate of vitamin A that serves for absorption of a photon. During the illumination, all-trans-retinal changes its conformation to 13-cis retinal (Nagel et al. 2003). This leads to a channel opening or a pump activation and inhibition or activation of the neuronal activity. Within milliseconds retinal returns to its trans-retinal state. The retinal is sufficiently present in the mammalian tissue so that optogenetic tools could be used without exogenous retinal supplementation. Nevertheless, invertebrate models need the retinal supplementation in order for optogenetic tools to function.

There are two known methods of targeted delivery of the optogenetic tools. The first one is viral gene transfer in vivo. This method has been effectively applied to mice, rats, and non-human primates. Various viral vectors such as adeno-associated viruses (AAV), lentiviruses and, rarely, herpes simplex virus (HSV) have been used for targeting (Adamantidis et al. 2007; Carter et al. 2010; Covington et al. 2010). The advantages of viral targeting are a short interval from virus production to the opsin expression and broad applicability. However, the length of the genetic information a virus can carry is limited. The second option of targeting is a generation of a transgenic animal. Using a strong promoter in combination with the Cre-*loxP* system achieves a cell type-specific expression. In other words, the promoter drives the expression of ChR2 or NpHR. An advantage of this method is the possibility to express the rhodopsin in a specific cell type

throughout the body. However, it takes more effort and time to generate a stable transgenic line.

1.9 Channelrhodopsin

Channelrhodopsins (ChR) are the only known light-gated ion channels and are derived from *Chlamydomonas reinhardtii*. These channels are seven transmembrane helix proteins with covalently linked retinal that provide green algae with photophobic and phototaxis responses (Nagel et al. 2003). The photoreceptor is located in the pigmented eyespot region. After only a few years of the first introduction the transformation of algal channelrhodopsin into neurons, it became one of the important tools in neuroscience (Boyden et al. 2005). It also has been applied to activate cardiac myocytes, skeletal muscle or used to control the behavior of invertebrates (Aponte et al. 2010, Pianca et al. 2017).

ChRs are inserted into the plasma membrane and mediate changes in the membrane potential in response to blue light (Boyden et al. 2005). This membrane potential change should be ideally instantaneous and predictable. However, properties of the plasma membrane such as resistance and capacitance can limit the rate and level of the obtainable depolarization. In addition, the unique properties of each ChR have a significant impact during the experiment to control the depolarization with light. The following seven properties of ChRs can influence the precision of managing the membrane potential during the experiment (Lin et al. 2010).

1. Channel conductance directly decides the capability of the light-induced depolarization. The single-channel conductance of ChR2 is less than the conductance of a common membrane channel and is less than 1 pS (picosiemens) (Nagel et al. 2003).

2. Ion selectivity. All ChRs have broad cation conductance and are non-selective towards H^+, Na^+, K^+ and Ca^{2+} (Tsunoda and Hegemann 2009, Gunaydin et al. 2010).

3. Kinetics. Opening and closure rates of ChRs are basic factors accomplishing transient, exact control of the membrane potential. The ideal kinetics of ChR should be as fast as it is physically possible. However, very rapid kinetics leads to decreasing light sensitivity (Lin et al. 2009a).

4. Desensitization and recovery from the desensitized state. During the strong, continuous or pulsed stimulation, the response of ChR2 extinct to 80% from a peak response (Nagel et al. 2003). All known ChRs desensitize, and some variants do not recover (Schoenenberger et al. 2009).

5. Light sensitivity. The ChRs have different light sensitivity. As mentioned before, each mutation that leads to an increase in sensitivity has a negative effect on the kinetic properties of the channel (Lin et al. 2009a).

6. Spectral response. The maximal and steady-state components of the photocurrents can have different spectral excitation peaks. For example, using the second light pulse with a different wavelength on ChR could speed up the recovery time after the desensitization (Lin et al. 2009 a, b).

7. Membrane trafficking and expression. Low expression of ChRs results in low effectiveness on membrane depolarization (Lin et al. 2009b). Overexpression of an exogenous membrane protein can adversely affect the membrane properties and possibly lead to cell death.

Several wild-types and engineered variations of ChR already have been reported. The first wild-type ChR that was characterized ex vivo was named ChR1 (Nagel et al. 2002). ChR1 has a low conductance at pH 7 and this limits its use as a scientific tool. In 2005, Boyden et al. introduced another wild-type ChR, ChR2. This group was able to excite neurons expressing ChR2 with light. ChR2 has a rapid on-rate and a moderate channel closing time. The conductance of ChR2 is between 50-250 femtosiemens (fS). The main disadvantage of ChR2 is the high level of desensitization. The recovery time after the desensitization is estimated to be 25 s in the dark (Lin et al. 2009a).

ChR-2 with H134R mutation shows a slight decrease in desensitization, a slight increase in light sensitivity and a slower channel closing (Lin et al. 2009a, b). These changes lead to stagnation in the kinetics and less temporal precision of ChR2/H134R than ChR2. Each published variation of ChR2 has its own advantages, and these benefits could be usefully applied in experiments. For example, ChR2/C128X is useful for inducing a prolonged depolarization when temporal precision is not critical. CheTA (E123T mutation in ChR2) is useful for high-frequency stimulations. Chimera EF with I170V mutation is the best ChR2 for experiments with high frequency, repetitive stimulation or continuous

illumination (Lin et al. 2010). Despite many ChR modifications have been developed, many properties still need to be optimized to generate better ChRs.

1.10 Aim of the study

Micturition is initiated and controlled by the autonomic nervous system. The two major neurotransmitters leading to detrusor contraction are ACh and ATP. Currently, it is still unclear if both neurotransmitters are co-released together from single nerve fibers or separately from different sets of autonomic neurons which then could be differently affected in disease but also selectively targeted in therapeutic approaches. A widely used technique to investigate for potential co-occurrence of transmitters in neurons is multiple-labeling immunohistochemistry that allows for precise localization even at the subcellular level. In case of small molecular weight transmitters against which antibodies can be hardly generated, the respective transmitter synthesizing enzyme is often taken as a useful surrogate. This allows for reliable identification of cholinergic neurons by demonstration of the ACh synthesizing enzyme, choline acetyltransferase (ChAT). ATP, however, is synthesized by all cells as it generally serves as an energy source for multiple cellular reactions. Thus, a pure demonstration of its presence within a cell does not provide clues as to its use as a transmitter, and a functional readout is needed. Organ bath experiments recording detrusor contraction represent such a suitable readout. To decide whether ATP is indeed released from cholinergic nerve fibers, a method that allows for selective stimulation of only cholinergic neurons is required. While general depolarization of all axons within the preparation by electrical field stimulation does not fulfill the selectivity criterion, optogenetics present as a suitable tool. Thus, we first generated mouse strains expressing ChR2 selectively in cholinergic neurons, validated this model, and then analyzed transmitters inducing detrusor contraction which are released from cholinergic nerve fibers by pharmacological profiling.

2 Materials and Methodology

2.1 Materials

2.1.1 Experimental animals

All studies involving animals described in this work were carried out conforming to the German animal welfare act and were registered by the Regierungspräsidium Giessen (Animal euthanasia - Tiertötung Meldung Nr: 572_M and Nr: 555_M).

Using the Cre/loxP recombinase system, B6N.129S6(B6)-Chat$^{tm2(cre)Lowl}$/J (006410, Jackson Lab, Sacramento, USA) and B6;129-Gt(ROSA)26Sor$^{tm1(CAG-COP4*E123T*H134R,-tdTomato)Gfng}$/J (012567, Jackson Lab, Sacramento, USA) mouse lines were crossed, resulting in a transgenic mouse strain with channelrhodopsin-tomato protein expression in cholinergic neurons (ChAT-ChR2-tdTomato) (Fig. 2.1). As a control group, we used C57BL/6 strain (000664, Jackson Lab, Sacramento, USA) and ChAT^{BAC}-eGFP (007902, Jackson Lab, Sacramento, USA) transgenic mice line with fluorescent visualization of cholinergic nerve fibers. Colonies were maintained in the central animal facility of the Justus-Liebig-University Giessen. For euthanasia, we applied 0.5 ml liquid 5% isoflurane (Baxter GmbH, Unterschleissheim, Germany) to absorbent material in the open-drop system. During the euthanasia, the mouse was separated from the absorbent material by a physical barrier in a closed 3 l glass container. The isoflurane was scavenged through a ventilated hood.

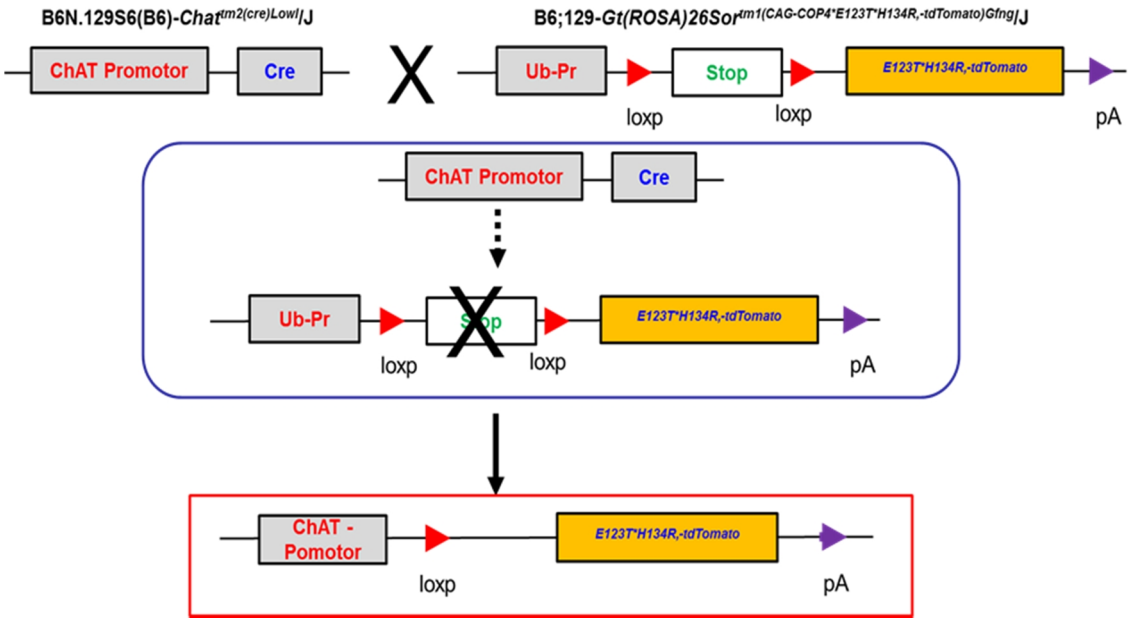


Figure 2.1 Generation of a mouse strain expressing ChR2 in cholinergic neurons by cross-breeding of B6N.129S6(B6)-Chat$^{tm2(cre)Lowl}$/J (express Cre recombinase under the control of the choline acetyltransferase promoter) and B6;129-Gt(ROSA)26Sor$^{tm1(CAG-COP4*E123T*H134R,-tdTomato)Gfng}$/J (express E123T*H134R gene and tdTomato fluorescence protein). ChAT: choline acetyltransferase; Ub-Pr: ubiquitin promoter; E123T*H134, tdTomato: gen expressing ChR-2 and tomato protein.

2.1.2 Pharmacological substances

All pharmacological substances used during the experiment are listed in Table 2.1.

Substance	Function	Target	Concentration	Solvent	Source
Muscarine chloride	Agonist	Muscarinic receptors	10^{-5}–10^{-8} M	Ampuwa®	Sigma Aldrich, Steinheim, Germany
Atropine sulfate	Antagonist	Muscarinic receptors	2 µM	Ampuwa®	Sigma Aldrich, Steinheim, Germany
Mecamyl-amine	Antagonist	Nicotinic receptors	20 µM	Ampuwa®	Sigma, Missouri, USA
Suramin sodium salt	Non-selective antagonist	Purinergic receptors	300 µM	Ampuwa®	Sigma Aldrich, Steinheim, Germany
Pyridoxal-phosphate-6-azophenyl-2', 4'-disulfonic acid (PPADS) tetrasodium salt	Non-selective antagonist	Purinergic receptors	100 µM	Ampuwa®	Sigma Aldrich, Steinheim, Germany

Table 2.1 Agonist and antagonists used in organ bath experiments.

2.1.3 Antibodies

The characteristics of primary and secondary antibodies used in immunohistochemistry are listed in Table 2.2 and Table 2.3, respectively.

Antigen	Host	Dilution	Source
ChAT	Goat, Polyclonal	1:250	Millipore, Temecula, California
VAChT	Goat, Polyclonal	1:100	Biotrend, Cologne, Germany
Tyrosine Hydroxylase	Sheep, Polyclonal	1:800	Millipore, Temecula, California
Substance P	Rat, Monoclonal	1:400	Santa-Cruz, Heidelberg, Germany

Table 2.2 Characteristics of primary antibodies. ChAT: choline acetyltransferase; VAChT: vesicular acetylcholine transporter.

Antigen	Host	Conjugate	Dilution	Source
Rat-Ig	Donkey	FITC	1:400	Dianova, Hamburg
Goat-Ig	Donkey	CF 488A	1:2000	Dianova, Hamburg

Table 2.3 Characteristics of secondary antibodies. Ig: immunoglobulin; FITC: fluorescein isothiocyanate.

2.1.4 Solutions and Buffers

Aqua ad injectabila (Ampuwa® (B. Braun, Melsungen, Germany)

Water to aliquot the pharmacological substances.

1% Penicillin-streptomycin in minimum essential medium (MEM)

50 U Penicillin and 500 g streptomycin in 500 ml MEM solution without glutamine and phenol red (ThermoFischer Scientific, Dreieich, Germany).

Paraformaldehyde (PFA) 4% in 0.1 M phosphate buffer pH 7.2-7.4

40 g Paraformaldehyde powder solved in 500 ml distilled water was heated to approximately 70 °C in a ventilated hood. Afterward, 2 M NaOH was added dropwise with a pipette until the solution clears. Once the paraformaldehyde was dissolved, the solution was cooled and filtered. Lastly, we added 500 ml 0.2 M phosphate buffer and adjusted the pH at 7.4. All reagents were obtained from Merck (Darmstadt, Germany).

Phosphate buffered saline (PBS)

NaCl 137 mM, KCl 2.7 mM, phosphate 12 mM (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.42 g, KH_2PO_4 0.27 g in one liter distilled water); all reagents from Sigma Aldrich (Steinheim, Germany).

Histoblock solution

10% Horse serum (HS), 0.5% Tween 20, 0.1% bovine serum albumin (BSA) in PBS. All reagents were obtained from Merck (Darmstadt, Germany).

Zamboni fixative

Zamboni fixative contains 800 ml distilled water, 4.3 g NaOH, 20 g PFA, 18.8 g NaH_2PO_4 and 150 ml saturated picric acid. All reagents were obtained from Merck (Darmstadt, Germany).

Carbonate-buffered glycerol

Glycerol and buffer (50 ml 0.5 M sodium hydrogen carbonate solution and 0.5 M sodium carbonate at pH 8.6; all reagents from Sigma Aldrich, Steinheim, Germany) are mixed in a ratio of 1:1.

2.2 Methods

2.2.1 Genotyping of mice

Genotyping was performed by Dr. Amir Rafiq, Institute for Anatomy and Cell Biology, Justus-Liebig-University Giessen. Mouse-tail deoxyribonucleic acid (DNA) for genotyping was extracted following instructions provided with KAPA mouse genotyping kit (VWR International GmbH, Darmstadt, Germany). 2x KAPA2G Fast Genotyping Mix with Dye was obtained from VWR International GmbH, Darmstadt, Germany. This master mix contains all necessary ingredients for polymerase chain reaction (PCR) such

as DNA polymerase, buffer salt solution and deoxynucleotides (dNTPs) and gel loading dye. ChR2-tdTomato genotyping was performed by using forward primer "caacgtgctggttattgtgc" and reverse primer "gccgaattcgatctagcttg". The composition of the PCR mixture is given in Table 2.4.

Component	Per 30 µl reaction	Final concentration
PCR-grade water	12.37 µl	N/A
2x KAPA2G Fast Genotyping Mix with Dye	15 µl	1x
Forward and reverse primer	0.63 µl	500 nM
Template DNA	1.0 µl	10 ng/µl

Table 2.4 Components for preparing PCR mixture.

The PCR was conducted using a thermal cycler (Master Cycler®, Eppendorf, Hamburg, Germany) using the conditions specified in Table 2.5.

Step	Temperature	Time duration	Cycles
Initial denaturation	95 ˚C	4 min	1
Denaturation	95 ˚C	20 s	35 cycles
Annealing	60 ˚C	20 s	
Extension	72 ˚C	30 s	
Final extension	72 ˚C	5 min	1

Table 2.5 Steps and temperature cycles of PCR.

The PCR products were analyzed by 2% Tris-acetate-EDTA (ethylenediaminetetra-acetic acid) agarose gel electrophoresis.

2.2.2 Preparation of the tissue

2.2.2.1 Dissection of the urinary bladder

After euthanasia with an overdose of isoflurane, we settled a mouse on a supine position. The extremities were fixed. We performed a midline transperitoneal incision that extended from just over the pubis to well above the umbilicus. As a next step, we opened the peritoneum in the midline, retracted the intestine and dissected the aorta abdominalis to bleed the mouse. Then, the urinary bladder was freed from perivesical fat and removed.

After cystectomy, the urinary bladder was placed in a 10 ml centrifuge tube filled with 1% penicillin-streptomycin in a modified MEM solution without glutamine and phenol red. The conveyance lasted approximately 10 min.

The urinary bladder was fixed with needles on the bottom of a petri dish covered with sylgard. The bladder was divided along the longitudinal axis into two equal parts. We trapped each part between two clamps at six and twelve o´clock position. One of the clamps had a silk suture so that the tissue could be fixed and strained on a tensiometer (PowerLab 8/30 ADInstruments, Bella Vista, Australia) in the organ bath.

2.2.2.2 Extraction of control tissue

To characterize the expression of the ChR2-tdTomato fusion protein in ChAT-ChR2-tdTomato mice, control tissues with known cholinergic innervation were taken from 10 animals and processed for cryosectioning and immunohistochemistry as described above for urinary bladders. These controls were larynx, trachea, lung, esophagus, and duodenum.

2.2.3 Organ bath experiments

For organ bath experiments, we used a 4-chamber tissue bath system produced by Radnoti (Radnoti LLC, Monrovia, USA). Each tissue bath contained 15 ml of buffer solution. As a buffer solution, we used 1% penicillin-streptomycin in a modified MEM solution. The oxygen supply was maintained via direct saturation of the buffer solution in a chamber with a carbogen (95% oxygen and 5 % carbon dioxide). The flow pressure was preinstalled at 1 psi, furthermore, the aeration stream was controlled individually for each

tissue bath with a needle valve at the bottom of the chamber. This setup is schematically shown in Figure 2.2. A stable temperature of 37°C was maintained through a Thermos Bath Heater (Radnoti LLC, Monrovia, USA).

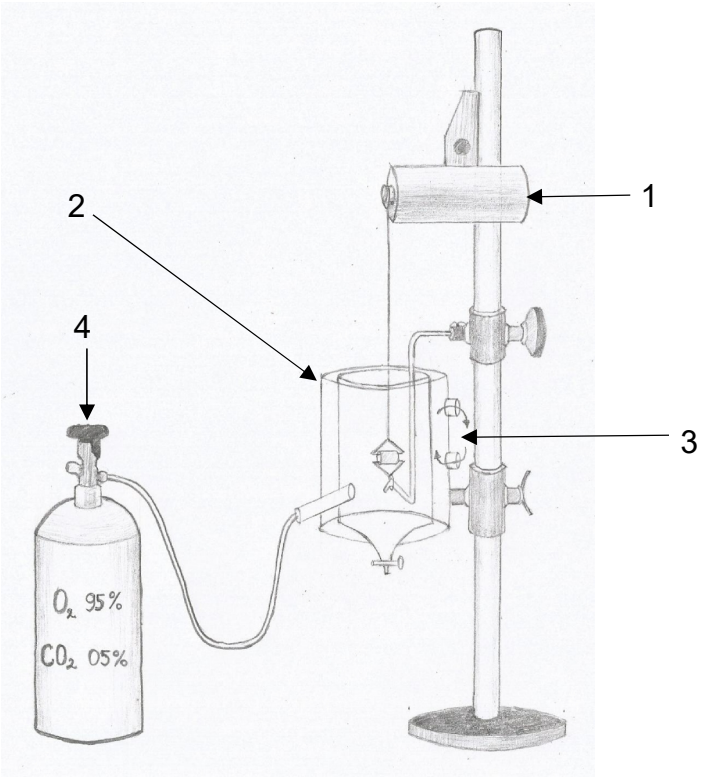


Figure 2.2 Schematic representation of the organ bath. 1: Force transducer; 2: Organ bath chamber; 3: Connection to the thermal circulation pump; 4: Bottle of the gas mixture: carbogen (95% oxygen and 5 % carbon dioxide)

The urinary bladder was placed in the bath. To measure tension, one end of the urinary bladder was attached to a fixed hook with paddles for electrical impulse and the other end strained to a force transducer. The force transducer converted the force generated by the tissue, measured in grams (g), after stimulation into an electrical signal. These signals were collected on a LabChart7.0 recorder (ADInstruments, Bella Vista, Australia) (Fig. 2.3).

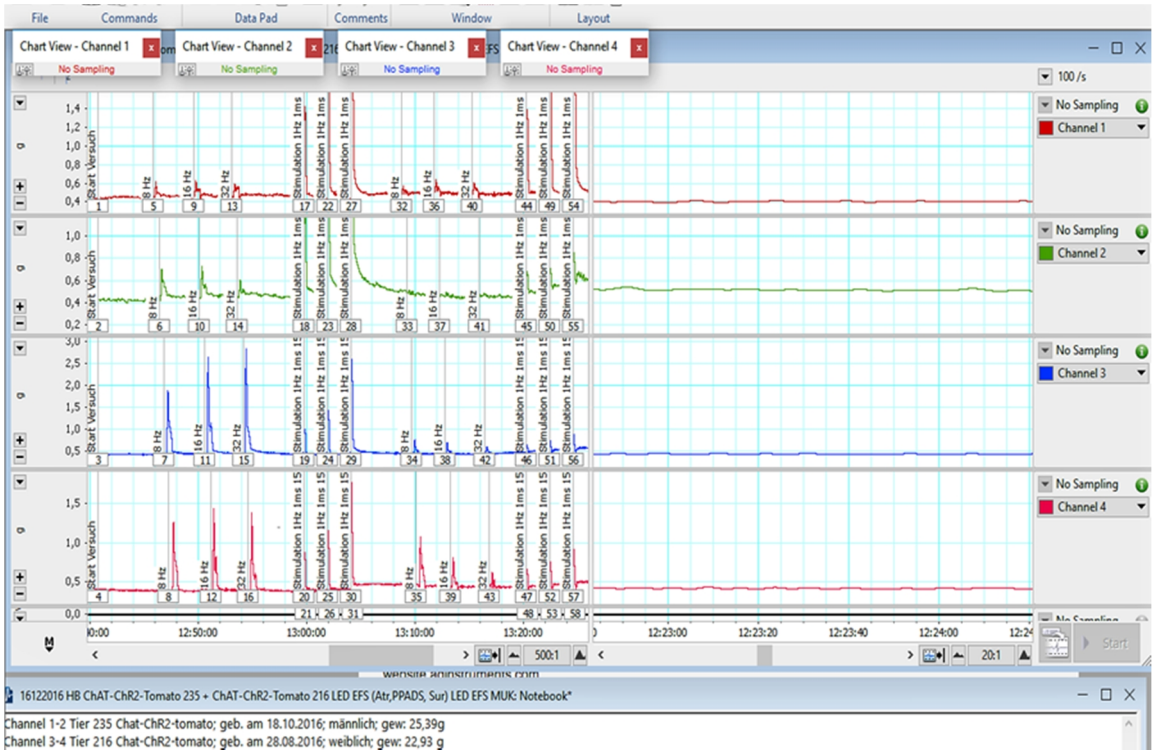


Figure 2.3 Lab Chart Data screenshot. Data received from four channels during the stimulation of bladders from ChAT-ChR2 mice. Here, the light stimulation (8, 16 and 32 Hz) alternates with EFS (8, 16 and 32 Hz)

The stimulation of the tissue was provoked by EFS via electrodes in the organ bath, or by direct illumination of the tissue with LED, or by adding pharmacological agents in the buffer solution.

As a light source, we used a Prizmatix Optogenetics-LED-Blue (Givat-Shmuel, Israel). This is a fiber-coupled LED application. The module yields high power blue light (~460 nm) that triggers the opening of the channel protein ChR2. Through the pulser module, we generated trains of pulses for optogenetic activation directly from the computer. The setup is shown in Figure 2.4.

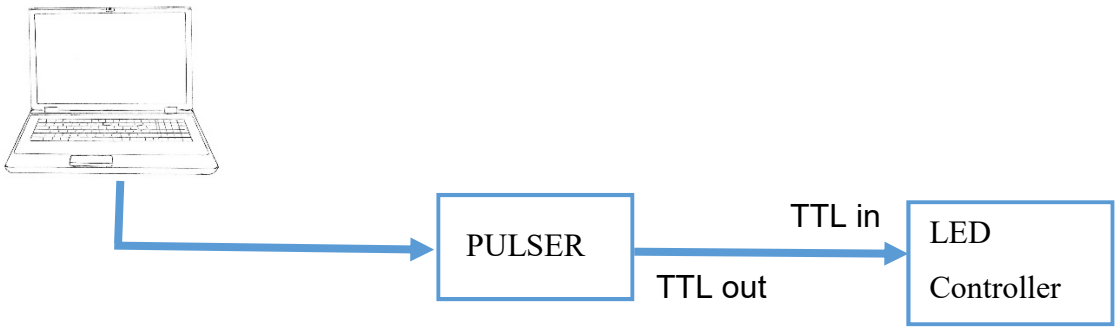


Figure 2.4 PC control with a direct connection to LED. TTL: Transistor-transistor logic

To enable the visual design of the various pulse conditions, a Graphic User Interface (GUI) was used. Figure 2.5 demonstrates the setting of GUI for an example stimulus.

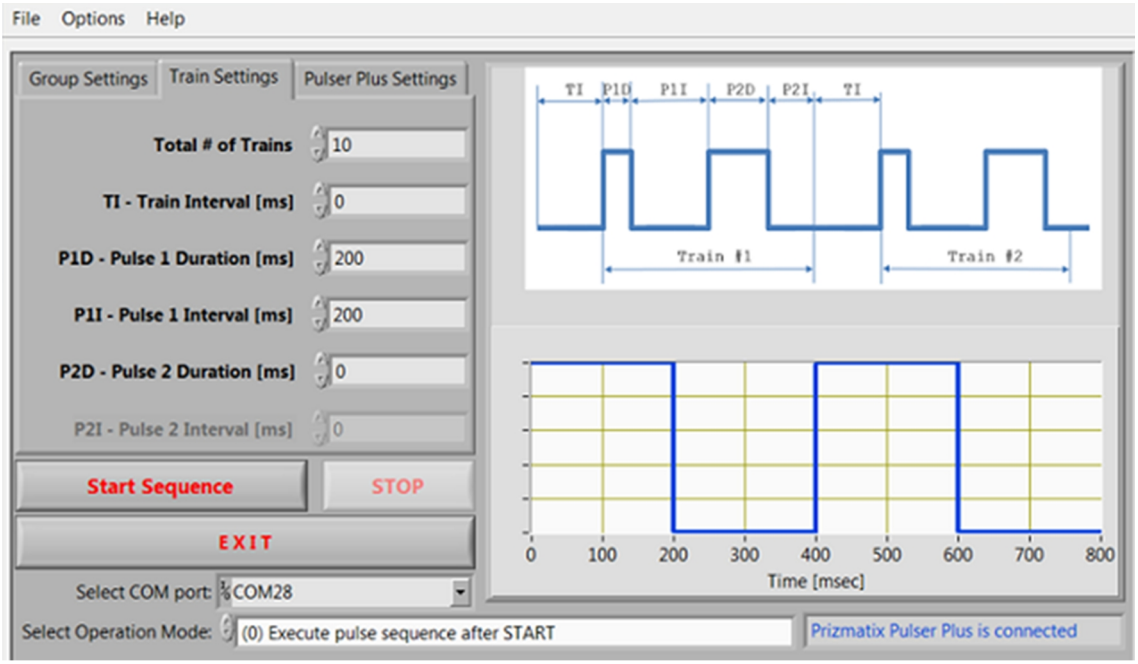


Figure 2.5 Train setting Tab, screenshot from Prizmatix Pulser Software Version 2.3.1 (Givat-Shmuel, Israel). On the upper right side of the GUI exposed the legend of user definable parameters. The bottom plane demonstrates the waveform that was programmed with the user settings on the left side

The urinary bladder was pre-stretched between 0.5-0.7 g for 30 min. After this calibration period, we started the experiment.

Two different settings were used in our experiment. In a first step, we recorded the response curve of bladders from ChAT-ChR2tdTomato mice (n=6) to illumination (460 nm; 1 Hz; 2 Hz; 4 Hz; 8 Hz; 16 Hz; 32 Hz; pulse duration respectively: 500 ms; 250 ms; 125 ms; 62.5 ms; 31.25 ms; 15.63 ms; pulse train 25 s) and then to EFS (10 V; 150 mA; 1 Hz; 2 Hz; 4 Hz; 8 Hz; 16 Hz; 32 Hz; pulse duration: 2 ms and pulse train: 10 s). Afterward, the contractility and viability of the urinary bladder were controlled by adding increasing concentrations of muscarine (10^{-5} M) (Fig. 2.6). The same procedure was carried out for control bladders taken from ChAT-eGFP (n=6) and C57BL/6J (n=6) mice.

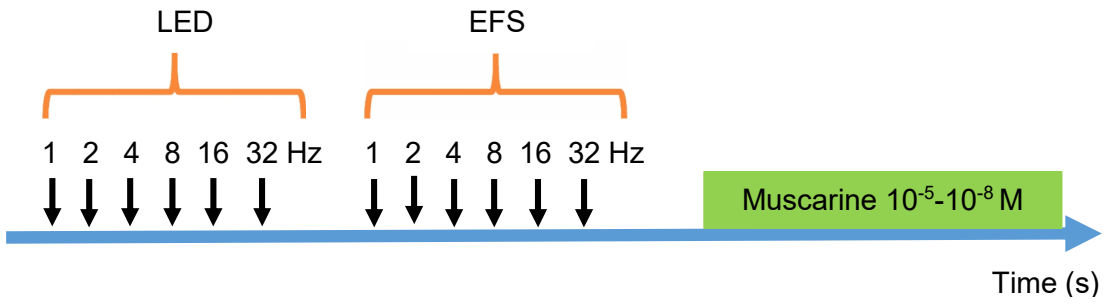
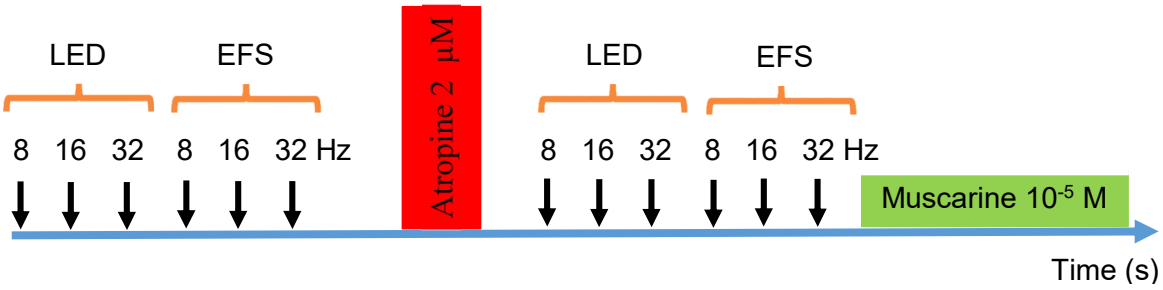


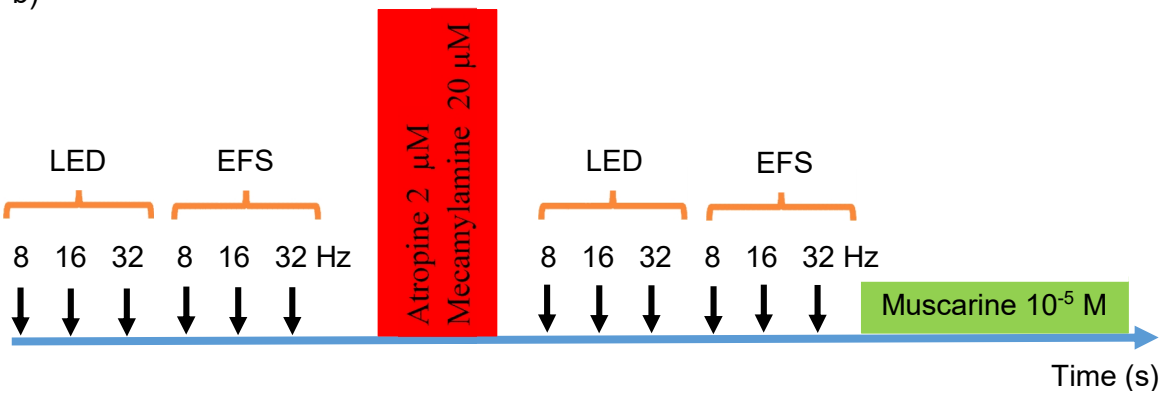
Figure 2.6 Protocol for obtaining stimulus-response curves. LED is light exposure with 1-32 Hz (460 nm; 2-500 ms; 25 s). EFS is electrical field stimulation at 1-32 Hz (10 V; 150 mA; 2 ms; 10 s). Muscarine was added at an increasing concentration (10^{-5}-10^{-8} M)

In a further step, we stimulated urinary bladders of Chat-ChR2-dtTomato mice with LED (8, 16, 32 Hz) and EFS (8, 16, 32 Hz). Then, we added 2 µM of the muscarinic inhibitor atropine sulfate and repeated the LED and EFS excitation (Fig. 2.7). Afterward, the EFS and LED stimulation of detrusor were performed in the presence and absence of the muscarinic inhibitor atropine (2 µM) and nicotinic inhibitor mecamylamine (20 µM). The same protocol was run using purinergic inhibitors, i.e. 300 µM suramin sodium salt and 100 µM PPADS tetrasodium salt. Lastly, we used all three antagonists to inhibit the response of urinary bladders (atropine sulfate 2 µM, suramin sodium salt 300 µM, PPADS 100 µM). In all protocols, muscarine 10^{-5} M was added at the end of the experiment to validate the effectiveness of atropine addition in protocol №1, №2 and №4 and viability of the preparation in protocol №3. For protocol №1, №3, №4 were six organ bath experiments conducted. Five organ bath experiments were carried out using protocol №2.

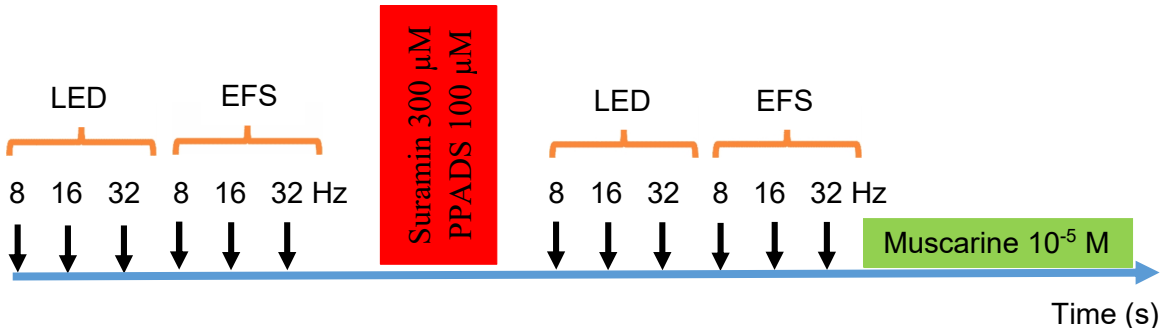
a)


LED
EFS
8 16 32 8 16 32 Hz
Atropine 2 µM
LED
EFS
8 16 32 8 16 32 Hz
Muscarine 10-5 M
Time (s)

b)


LED
EFS
8 16 32 8 16 32 Hz
Atropine 2 µM
Mecamylamine 20 µM
LED
EFS
8 16 32 8 16 32 Hz
Muscarine 10-5 M
Time (s)

c)


LED
EFS
8 16 32 8 16 32 Hz
Suramin 300 µM
PPADS 100 µM
LED
EFS
8 16 32 8 16 32 Hz
Muscarine 10-5 M
Time (s)

d)

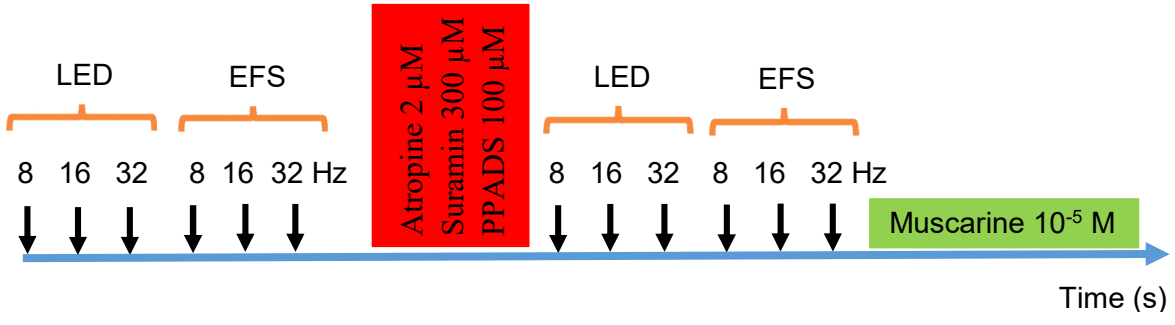

LED
EFS
8 16 32 8 16 32 Hz
Atropine 2 µM
Suramin 300 µM
PPADS 100 µM
LED
EFS
8 16 32 8 16 32 Hz
Muscarine 10-5 M
Time (s)

Figure 2.7 Protocol for determining the signal pathway of the light-induced contraction of the detrusor muscle in ChAT-ChR2 mice. a: Protocol №1, muscarinic inhibitor (atropine sulfate 2 µM); b: Protocol №2, muscarinic and nicotinic inhibitors (atropine sulfate 2 µM and mecamylamine 20 µM); c: Protocol №3, purinergic inhibitors (suramin sodium salt 300 µM and PPADS tetrasodium salt 100 µM); d: Protocol №4 cocktail of muscarinic and purinergic antagonists (atropine sulfate 2 µM, suramin sodium salt 300 µM, PPADS tetrasodium salt 100 µM)

2.2.4 Immunohistochemistry

After the experiments in an organ bath, the bladders were shortly rinsed in distilled water and then fixed by immersion for at least four hours in Zamboni fixative. Afterward, the fixed tissue was rinsed several times in PBS for 15 min until the yellow color was completely washed out. For cryoprotection, it was rinsed in PBS with 18% sucrose until it sank. The tissue was then embedded with Tissue Tek® O.C.T™ (Sakura, Zoeterwoude, Netherland) in cryomolds. Lastly, the tissue was rapidly frozen in melting isopentane (Fluka, Sigma, Taufkirchen, Germany) cooled with liquid nitrogen, and kept at -80°C until further processing.

Frozen sections from urinary bladder and control tissues were prepared with a cryostat (Leica CM 1900, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) at -20°C. For this purpose, we fastened the bladder tissue using Tissue-Tek on a sample holder inside the cryostat. We cut the sample to 10 µm sections. The sections were mounted on microscope slides (SuperFrost Plus, Menze-Glaeser, Braunschweig, Germany) and air-dried. The sections were then covered with 100 µl Histoblock solution for one hour and washed in PBS for 20 min. During incubation, the slides were stored in moist chambers to prevent evaporation of the applied solution. This step was followed by the application of primary antibody overnight (Tab. 2.2). After washing the slides in PBS solution, they were coated with a secondary antibody for one hour (Tab. 2.3). The samples were washed 20 min in PBS solution, fixed 10 min in 4 % PFA, washed again in PBS, and coverslipped with carbonate-buffered glycerol.

Slides were evaluated with an epifluorescence microscope (Axioplan 2, Carl Zeiss, Jena, Germany). Fluorescent dyes (tdTomato, fluorescein isothiocyanate (FITC), CF488A and Alexa Fluor® 488) and appropriate excitation and barrier filters are given in Table 2.6.

Fluorescence dye	Color	Excitation filter (nm)	Barrier filter (nm)
tdTomato	red-orange	530-560	572-647
FITC	Green	460-500	512-542
CF488A	Green	460-500	512-542
Alexa Fluor® 488	cyan-green	460-500	512-542

Table 2.6 Fluorophores and filter combinations used in fluorescence microscopy.

The documentation was performed using the AxioCam HR camera (Carl Zeiss, Jena, Germany) and the Axiovision 4.7.1 software (Carl Zeiss, Jena, Germany).

2.2.5 Statistical analysis

Statistics were calculated using IBM SPSS Statistics (Armonk, USA) version 23 for Windows 10. The values in tables and graphs are expressed as mean ± SE. Data were checked for normality of distribution using the Shapiro-Wilk normality test. The data were normally distributed. Comparison of delta values before and after application of antagonist for the same stimuli within one ChR2 mice was performed using paired T-Test. Additionally, we used one-way ANOVA analysis for calculating the frequency dependency of detrusor contraction to EFS and LED-stimuli after application of muscarinic and nicotinic inhibitor cocktail. P values less than 0.05 were regarded as statistically significant. All graphs were created using the Microsoft Excel (Redmond, USA) software.

3 Results

3.1 Cellular expression of ChAT-ChR2-tdTomato

3.1.1 Native tdTomato-fluorescence in reference organs with cholinergic innervation

From the respiratory tract, a section of larynx, trachea, and lung were evaluated by fluorescence microscopy. Native tdTomato-fluorescence was seen without further tissue treatment in large axons, probably those of α-motoneurons, in the superior and recurrent laryngeal nerves. The same axons were immunoreactive for ChAT as demonstrated with a primary ChAT antibody visualized with a FITC-conjugated secondary antibody (Fig. 3.1). Consistent with ChR2 being a membrane protein and ChAT being a cytoplasmatic protein, the red fluorescence indicating the localization of the ChR2-tdTomato fusion protein presented as a ring around the green fluorescence representing ChAT-immunoreactivity (Fig. 3.1). Neurons of small parasympathetic ganglia located in the vicinity of the larynx and at the lung hilus also displayed membrane-bound tdTomato-fluorescence and homogeneous ChAT-immunoreactivity (Figs. 3.2 and 3.3). Through the entire length of the airways, their smooth muscle was densely innervated by nerve fibers with tdTomato-fluorescence whose ChAT-immunoreactivity was generally weak (Fig. 3.3). Colocalization with immunoreactivities for TH (noradrenaline synthesizing enzyme) and SP (neuropeptide) was not observed. Very rarely, tdTomato-fluorescence was observed in single skeletal muscle fibers of laryngeal muscles (Fig. 3.4) and in patches of epithelial cells in the alveolar region (Fig. 3.5).

From the gastrointestinal tract, sections of esophagus and duodenum were evaluated. Native tdTomato-fluorescence was seen in cell bodies of myenteric ganglia, numerous nerve fibers innervating the duodenal smooth muscle layer and terminals and, to a lesser extent, in the duodenal mucosa (Fig. 3.6). At the level of neuronal cell bodies, colocalization with ChAT-immunoreactivity was seen. At the level of muscular nerve terminals, there was also colocalization of tdTomato-fluorescence and ChAT-immunoreactivity, although the signals did not match 1:1 throughout (Fig. 3.7). It could not be resolved at the fluorescence microscope level whether this partial mismatch reflected labeling of different nerve fibers or occurred due to the differential subcellular localization – membrane versus axoplasm – of the underlying proteins. In the epithelial

layer, solitary triangular-shaped ChAT-immunoreactive cells, most probably cholinergic tuft (=brush) cells (Schütz et al. 2015), were observed. These cells did not exhibit native tdTomato-fluorescence (Fig. 3.7). In myenteric ganglia and in nerve fibers innervating the muscle layer, frequent colocalization of tdTomato-fluorescence with SP was visible (Fig. 3.8).

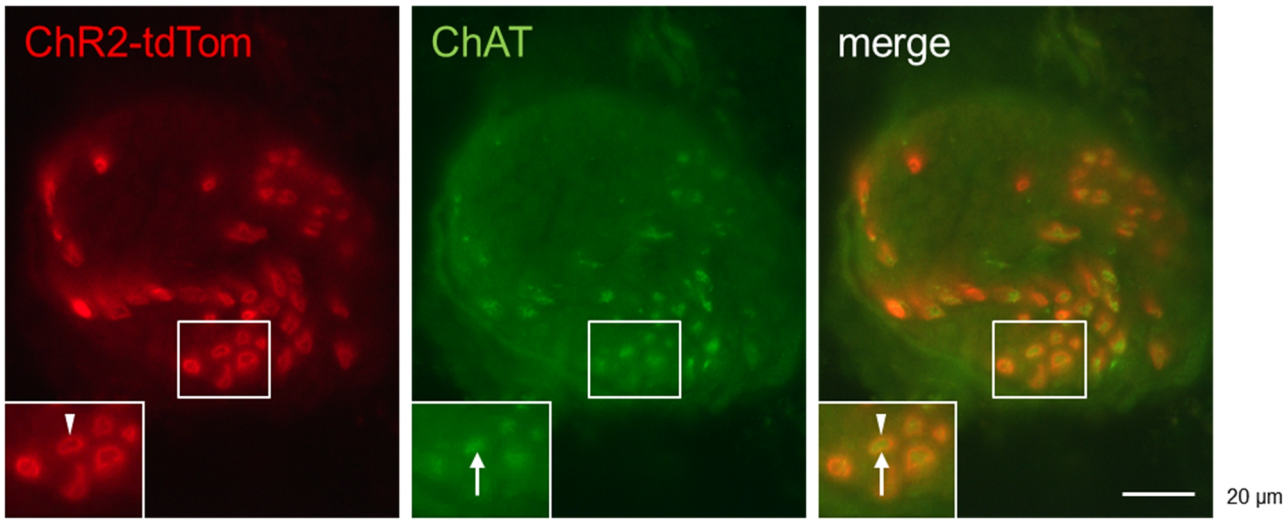


Figure 3.1 Native tdTomato-fluorescence (red) and ChAT-immunolabeling (green), recurrent laryngeal nerve. Red fluorescence indicates the localization of ChR2-tdTomato fusion protein. It is found in the membrane (arrowheads) of large diameter cholinergic axons with ChAT-immunoreactivity in the axoplasm (arrows). The boxed area is shown at higher magnification in the inset. Bar = 20 µm

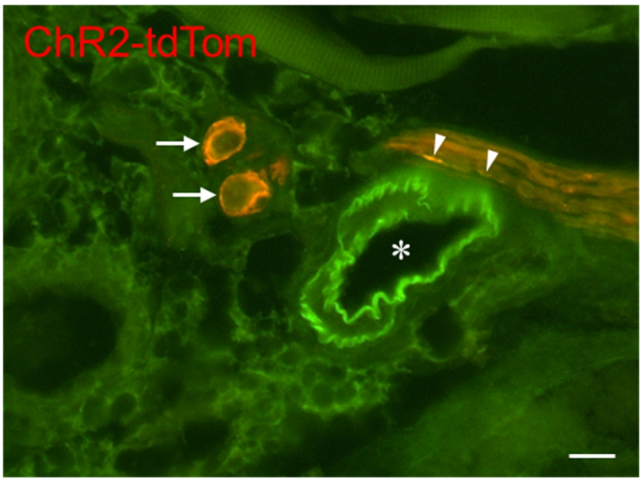


Figure 3.2 Larynx; red fluorescence indicates the localization of ChR2-tdTomato fusion protein, overall tissue morphology is demonstrated by green background fluorescence without antibody staining. Two neuronal cell bodies of a local parasympathetic ganglion display membrane-bound native tdTomato-fluorescence (arrows). The arrowhead points to positive large diameter fibers in a recurrent laryngeal nerve branch. * = small artery with autofluorescent internal and external elastic membrane. Bar = 20 µm

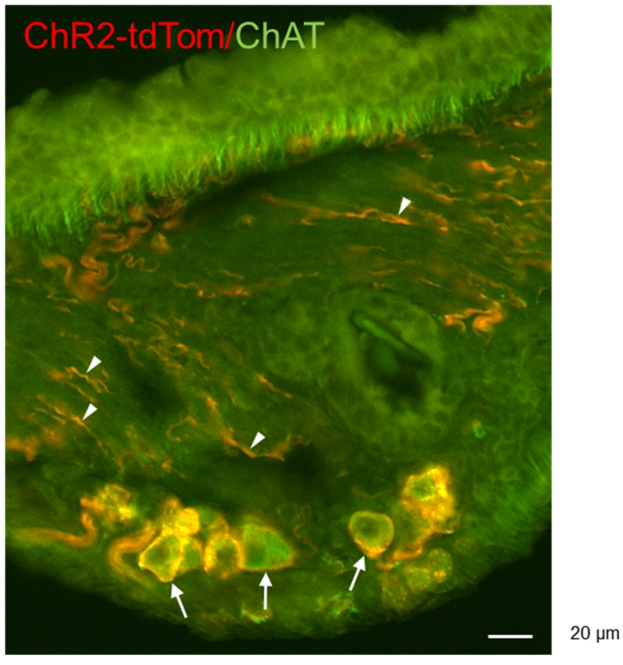


Figure 3.3 Lung hilus; native tdTomato-fluorescence (red) and ChAT-immunolabeling (green). Neuronal cell bodies of a local parasympathetic ganglion display membrane-bound native tdTomato-fluorescence (arrows) around ChAT-immunoreactive (green)

cytoplasm. The bronchial smooth muscle is densely innervated by tdTomato+/ChAT+ nerve fibers (arrowheads). Bar = 20 µm

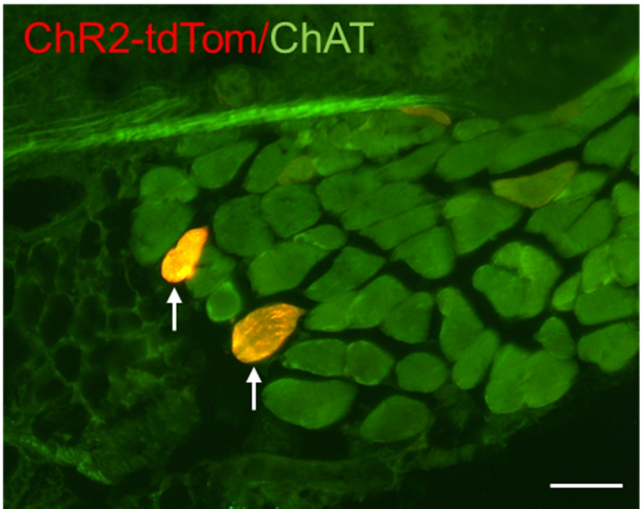


Figure 3.4 External laryngeal muscle; red fluorescence indicates the localization of ChR2-tdTomato fusion protein, overall tissue morphology is demonstrated by green background fluorescence. This section was processed for ChAT-immunolabeling, but ChAT-immunoreactive structures are not seen in the field of view. Two skeletal muscle fibers (arrows) strongly express native tdTomato-fluorescence without concomitant ChAT-immunoreactivity. Bar = 50 µm

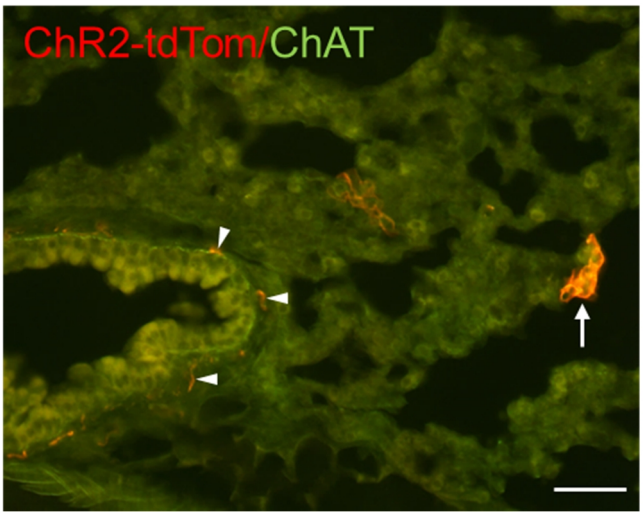


Figure 3.5 Lung; native tdTomato-fluorescence (red) and ChAT-immunolabeling (green). A group of epithelial cells in the wall of an alveolar duct (arrows) displays native

tdTomato-fluorescence without concomitant ChAT-immunoreactivity. Arrowheads point to the cholinergic innervation of bronchiolar smooth muscle. Bar = 50 µm

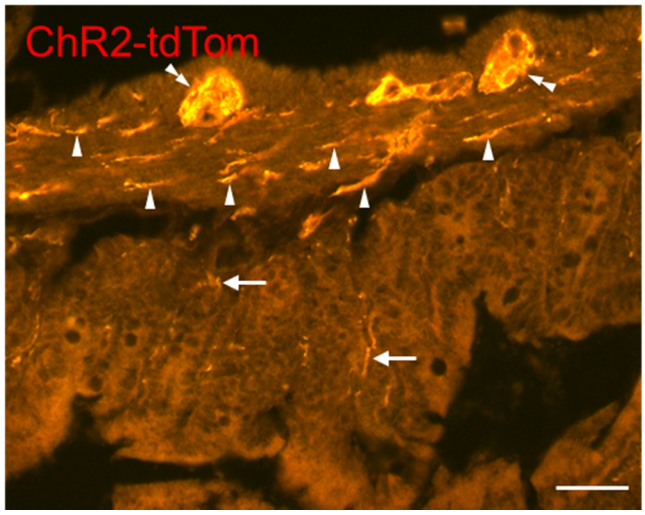


Figure 3.6 Duodenum; strong native tdTomato-fluorescence (red) in myenteric ganglia (double-headed arrowheads), numerous nerve fibers in the muscle layer (arrowheads) and fewer nerve fibers in the mucosa (arrows). Bar = 50 µm

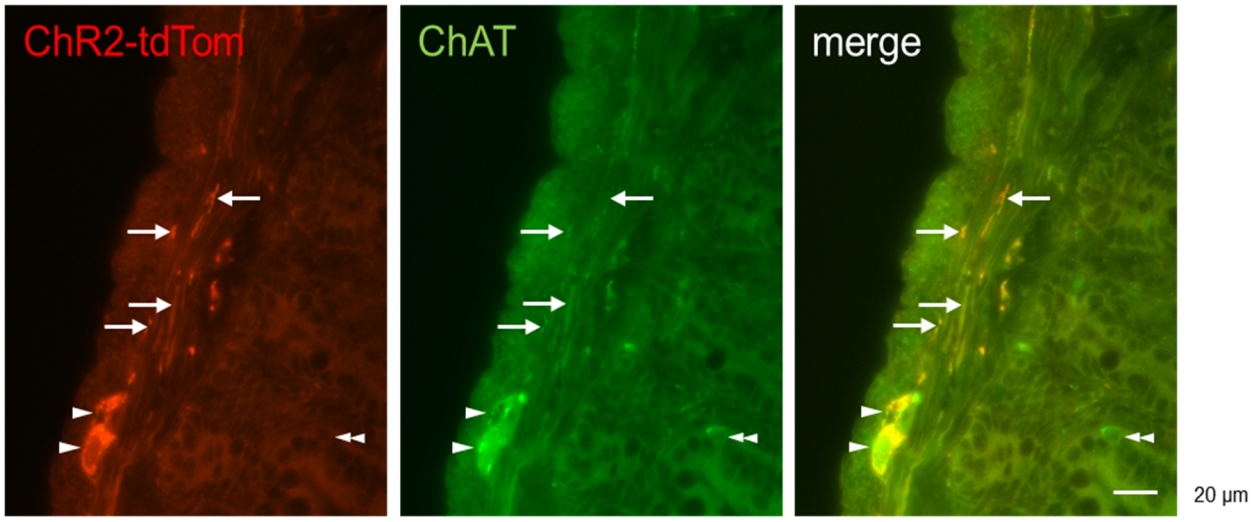


Figure 3.7 Duodenum; native tdTomato-fluorescence (red) and ChAT-immunolabeling (green) are colocalized in myenteric neurons (arrowheads) and nerve fibers (arrows), although the red fluorescence channel reveals more fibers than depicted by ChAT-immunofluorescence. Double-headed arrowhead depicts ChAT-positive epithelial cells that do not display tdTomato-fluorescence. Bar = 20 µm

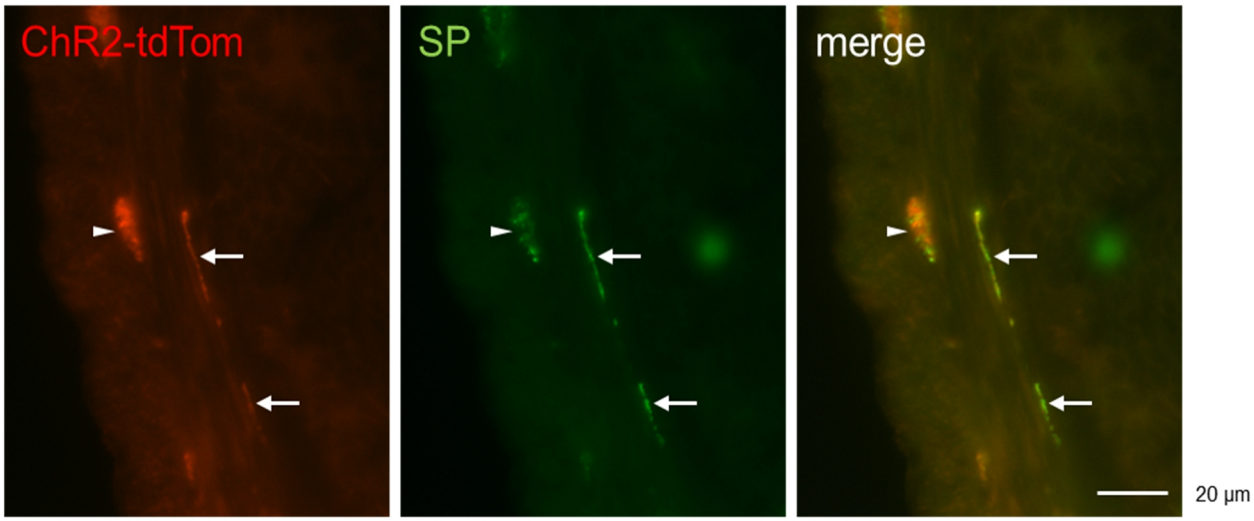


Figure 3.8 Duodenum; native tdTomato-fluorescence (red) and SP-immunolabeling (green) are colocalized in myenteric neurons (arrowheads) and nerve fibers (arrows). Bar = 20 µm

3.1.2 Urinary bladder

Neurons of intrinsic ganglia in the bladder wall and associated nerve fiber bundles displayed intense ChR2-tdTomato-fluorescence (Fig. 3.9a). A very dense innervation by nerve fibers with native tdTomato-fluorescence was observed in the detrusor muscle (Fig. 3.9b). Such nerve fibers also contained immunoreactivities for ChAT (Fig. 3.10) and VAChT (Fig. 3.11), but neither for TH (Fig. 3.12) nor for SP (Fig. 3.13). In some but not all bladders investigated (4 out of 12), single or small groups of detrusor smooth muscle cells also exhibited tdTomato-fluorescence (Fig. 3.14).

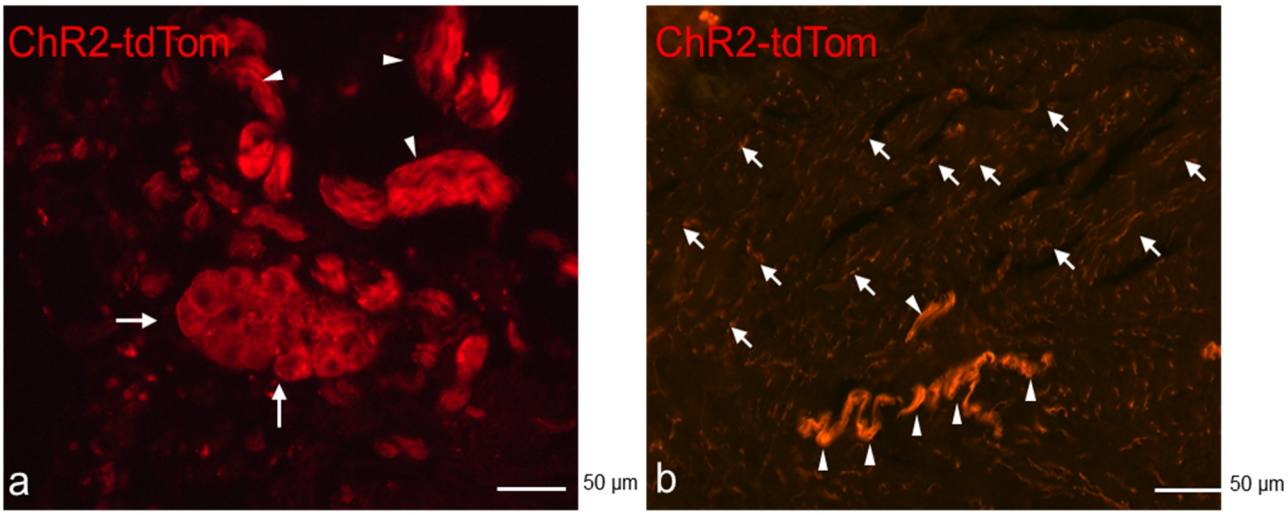


Figure 3.9 Urinary bladder; strong native tdTomato-fluorescence (red) a) in nerve trunks (arrowheads) and neurons in intrinsic ganglia (arrows), and b) in nerve fiber bundles (arrowheads) and ramifications (arrows) in the detrusor muscle. Bar = 50 µm

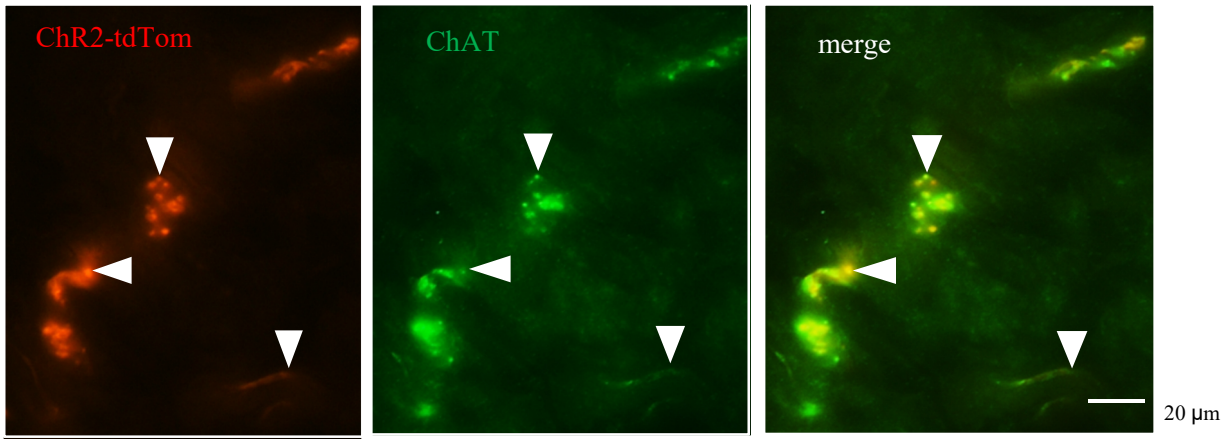


Figure 3.10 Urinary bladder, detrusor muscle; native tdTomato-fluorescence (red) and ChAT-immunolabeling (green) are colocalized in nerve fibers (arrowheads). Bar = 20 µm

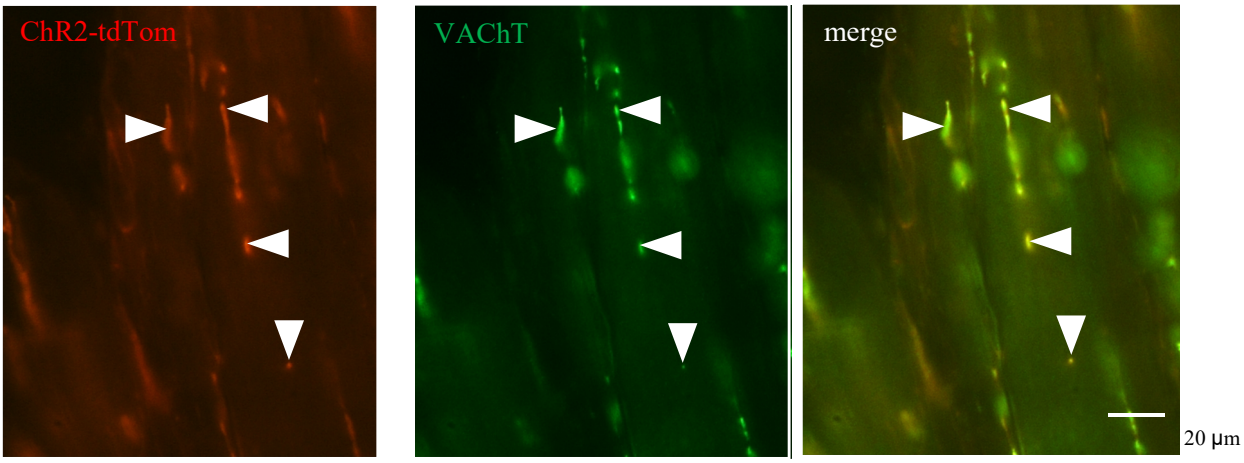


Figure 3.11 Urinary bladder, detrusor muscle; native tdTomato-fluorescence (red) and VAChT-immunolabeling (green) are colocalized in nerve fibers (arrowheads). Bar = 20 µm

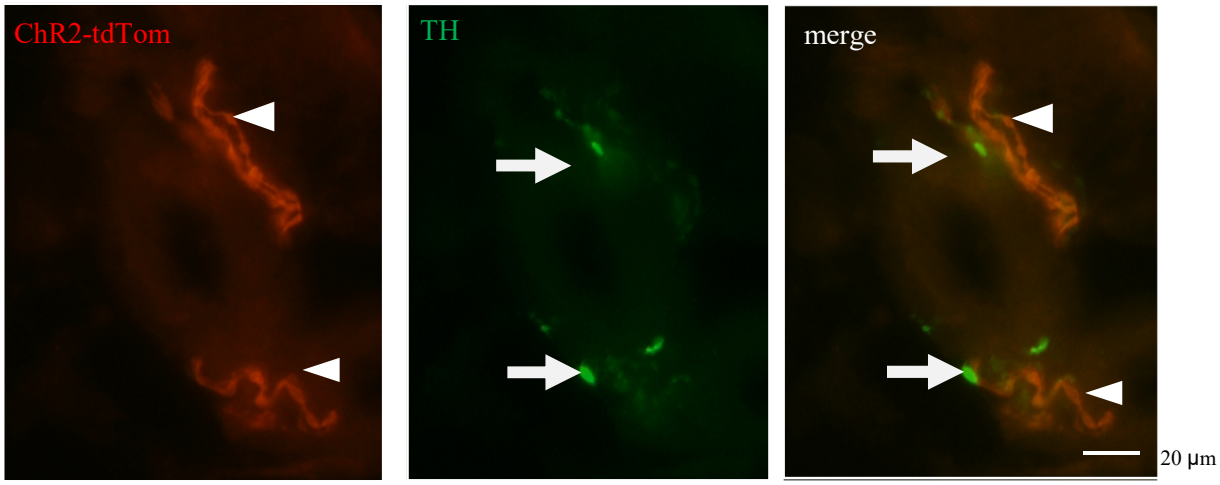


Figure 3.12 Urinary bladder, detrusor muscle; native tdTomato-fluorescence (red, arrowheads) and TH-immunolabeling (green, arrows) occur in separate populations of nerve fibers. Bar = 20 µm

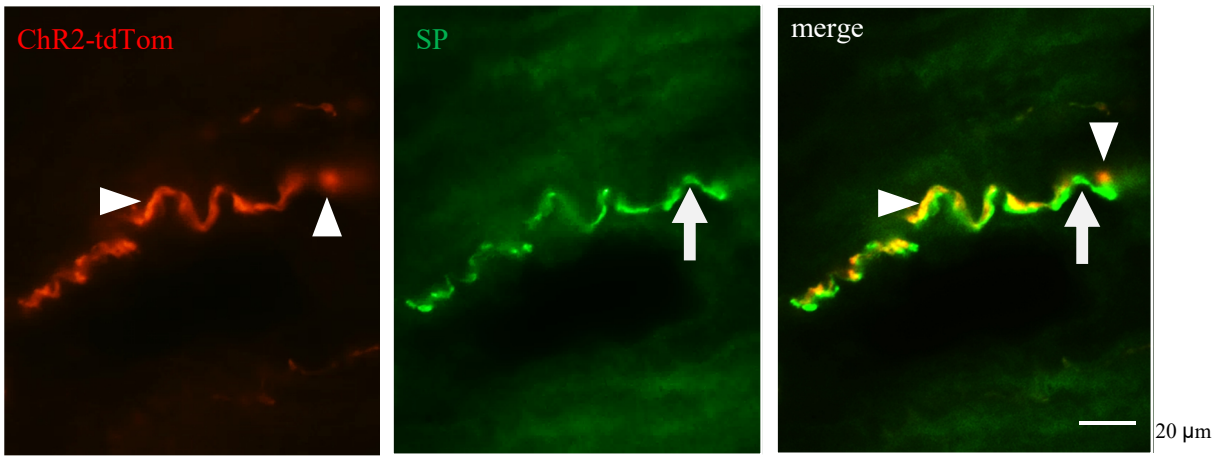


Figure 3.13 Urinary bladder, detrusor muscle; native tdTomato-fluorescence (red, arrowheads) and SP-immunolabeling (green, arrows) occur in separate populations of nerve fibers. Bar = 20 µm

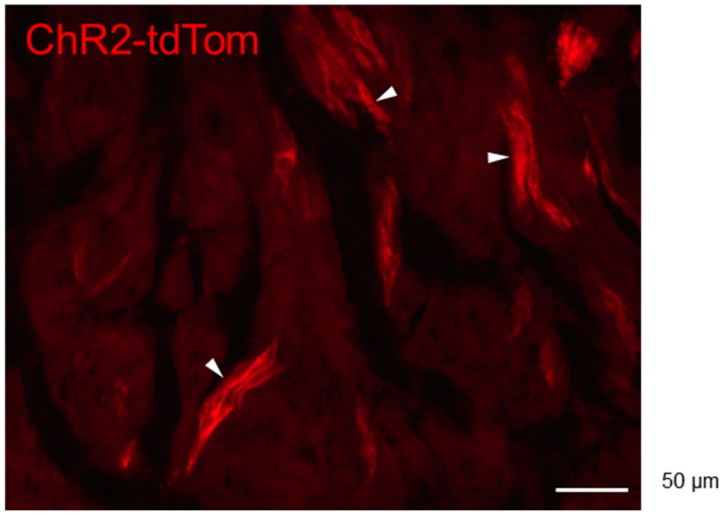


Figure 3.14 Urinary bladder; native tdTomato-fluorescence (red) is expressed by some smooth muscle fibers (arrowheads) of the detrusor muscle in this specimen. Bar = 50 µm

3.2 Organ bath experiments

3.2.1 Illumination of the detrusor muscle leads to a contraction in ChAT-ChR2-tdTomato, but not in ChAT-GFP mice

Urinary bladder strips from ChAT-ChR2-tdTomato and ChAT-GFP mice were subjected to LED and EFS stimulation and force was recorded in organ baths. EFS (8-32 Hz) induced frequency dependent detrusor contraction in both mouse strains with no significant differences between strains (Figs. 3.15 and 3.16, Tab. 3.3). LED, however, evoked detrusor contraction only in ChAT-ChR2-tdTomato mice but not in ChAT-GFP mice (Figs. 3.15 and 3.16). Constrictor responses were further characterized in ChR2-tdTomato mice. A graded increase in force development was seen in correlation with EFS frequency over a range of 1 to 32 Hz. At 32 Hz, contraction force almost reached that evoked by muscarine (10^{-5} M) (Fig. 3.17, Tab. 3.1). Such frequency dependence was not observed in LED stimulation with an increase of approximately 1 g in the range of 1 to 32 Hz (Fig. 3.18, Tab. 3.2). This increase was comparable to that evoked by EFS at 16 Hz, 2 ms, 10 V. It has to be noted, however, that pulse duration was kept constant in EFS (2 ms) whereas it was adapted to equal on-off durations in this protocol of LED stimulation (i.e. 500 ms at 1 Hz, 250 ms at 2 Hz, and so forth).

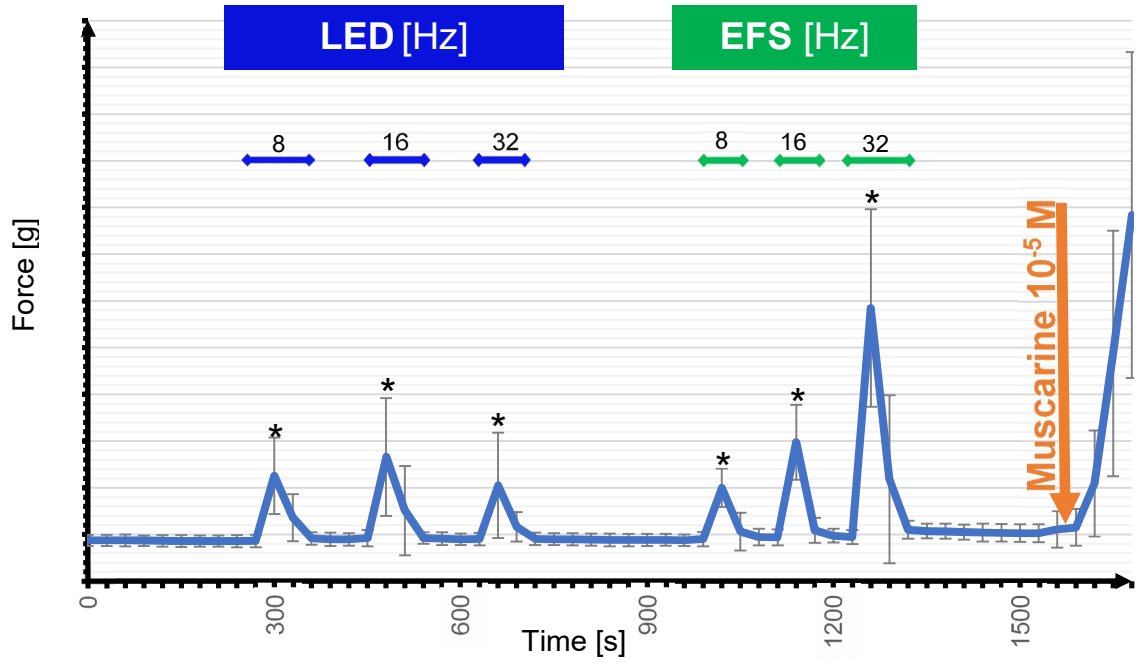


Figure 3.15 LED-, EFS-, and muscarine-induced contraction of the urinary bladder of ChAT-ChR2-td Tomato mice (n=21) in organ bath experiments. The responses of the urinary bladder to LED (8, 16, 32 Hz), EFS (8, 16, 32 Hz) and muscarine 10^{-5} M are shown in force (g). Mean ± S.E.M. Significant ($p \leq 0.05$, paired t-test) increases in tension, compared to baseline, are indicated by asterisks.

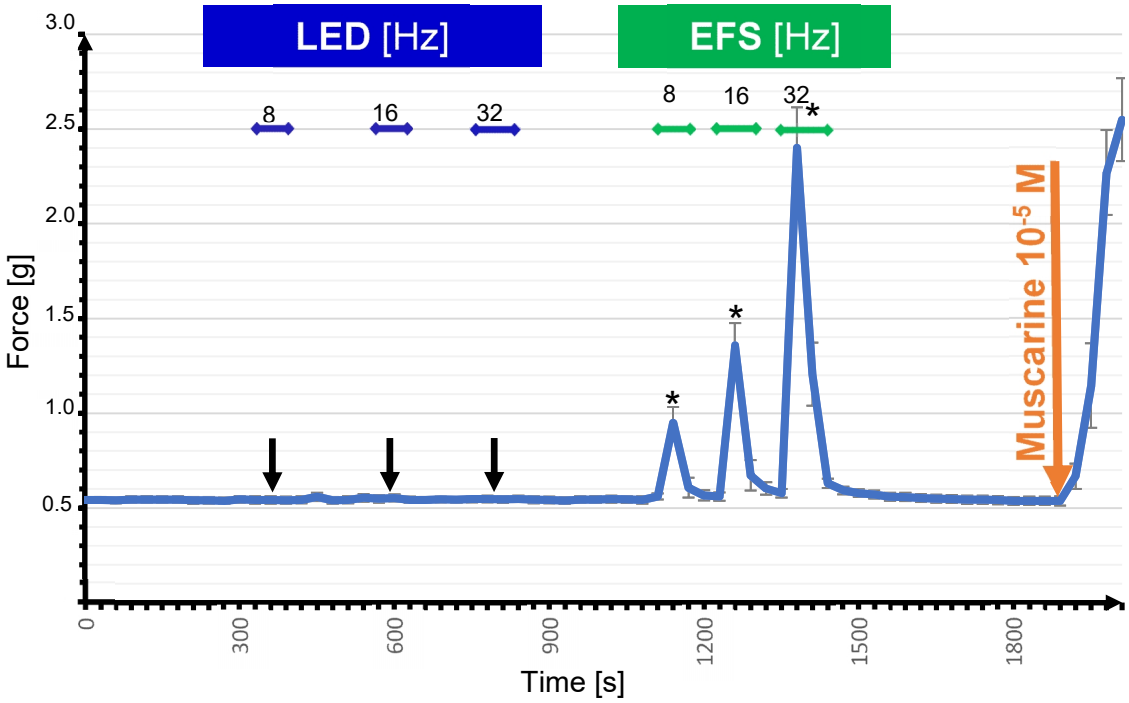


Figure 3.16 LED-, EFS-, and muscarine-induced contraction of the urinary bladder of ChAT-GFP mice (n=15) in organ bath experiments. The responses of the urinary bladder to LED (8, 16, 32 Hz), EFS (8, 16, 32 Hz) and muscarine 10^{-5} M are shown in force (g). Mean ± S.E.M. Significant ($p \leq 0.05$, paired t-test) increases in tension, compared to baseline, are indicated by asterisks.

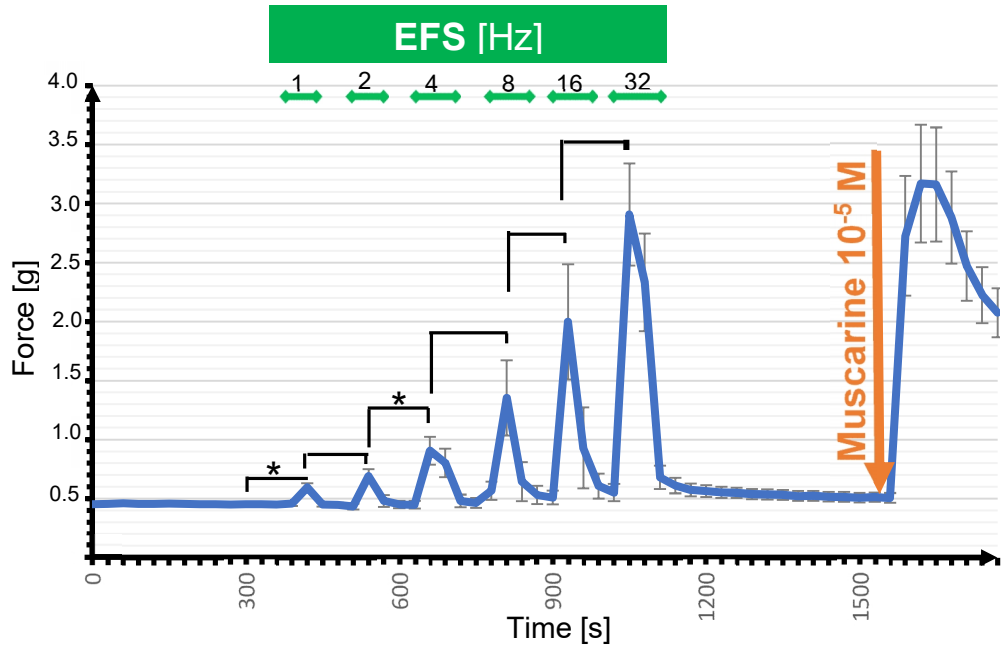


Figure 3.17 EFS-induced contraction of the urinary bladder of ChAT-ChR2 mice in an organ bath (n=5). Control of the viability of the tissue with muscarine 10^{-5} M. EFS stimuli: 1-32 Hz (10 V; 2 ms; 10 s). The response of the urinary bladder to stimuli is shown in force (g). Mean ± S.E.M. Significant ($p \leq 0.05$, paired t-test) increases in tension provoked by raising the frequency of the stimuli, compared to each other, are indicated by asterisks

Frequency	1 Hz	2 Hz	4 Hz	8 Hz	16 Hz	32 Hz
Increase in tension [g]	0.13±0.04	0.26±0.06	0.46±0.12	1.42±0.56	1.49±0.54	2.27±0.40

Table 3.1 EFS-induced contraction of the urinary bladder of ChAT-ChR2 mice in an organ bath (n=5). Mean ± S.E.M. EFS stimuli: 1-32 Hz (10 V; 2 ms; 10 s).

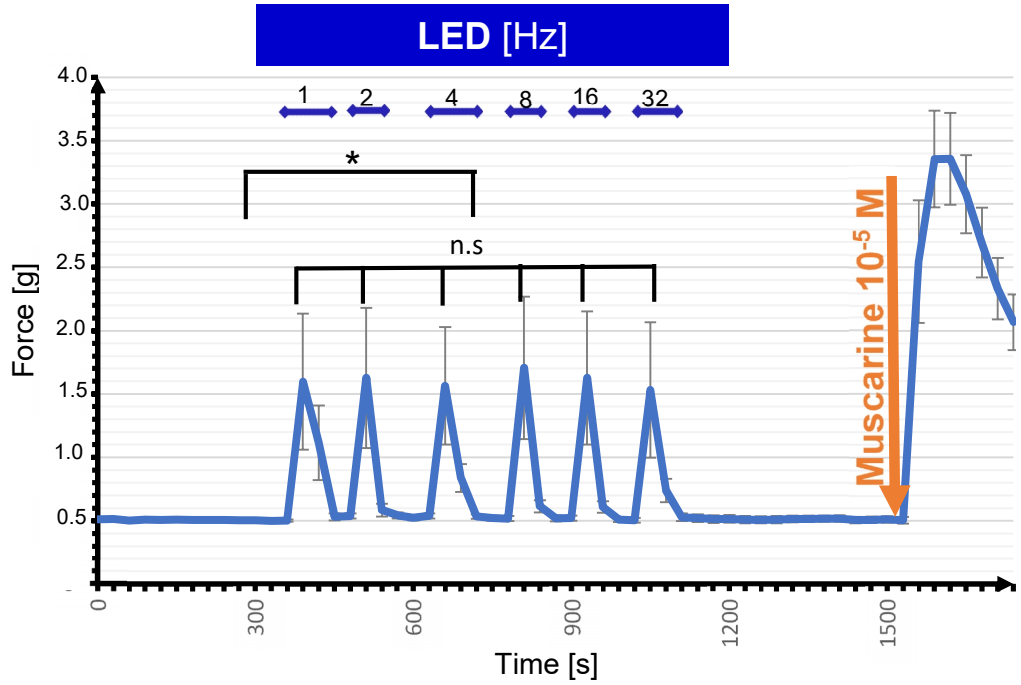


Figure 3.18 LED-induced contraction of the urinary bladder of ChAT-ChR2 mice in an organ bath (n=5). Control of the viability of the tissue with muscarine at 10^{-5} M. LED stimuli: 1-32 Hz (460 nm; 2-500 ms; 25 s). The response of the urinary bladder to stimuli is shown in force (g). Mean ± S.E.M. Significant ($p \leq 0.05$) is indicated by an asterisk, n.s-no significance (paired t-test) observed by comparing the tension provoked by raising the frequency of stimuli, among each other

Frequency	1 Hz	2 Hz	4 Hz	8 Hz	16 Hz	32 Hz
Increase in tension [g]	1.09±0.60	1.09±0.62	1.03±0.51	1.28±0.61	1.11±0.60	1.03±0.61

Table 3.2 LED-induced contraction of the urinary bladder of ChAT-ChR2 mice in an organ bath (n=5). Mean ± S.E.M. LED stimuli: 1-32 Hz (460 nm; 2-500 ms; 25 s).

Frequency [Hz]	Mouse strain	N	Increase in tension [g] Mean±S.E.M	p-value
8	ChAT-GFP	15	0.39±0.08	0.07
	ChAT-ChR2	21	0.55±0.04	
16	ChAT-GFP	15	0.80±0.11	0.11
	ChAT-ChR2	21	1.02±0.08	
32	ChAT-GFP	15	1.82±0.21	0.06
	ChAT-ChR2	21	2.45±0.22	

Table 3.3 EFS-induced increase in detrusor tension in ChAT-GFP and ChAT-ChR2 mouse strains. The response of the urinary bladder to stimuli is shown in force (g). Mean ± S.E.M. Significant ($p \leq 0.05$, independent t-test) increases in tension, among ChAT-ChR2 and ChAT-GFP mice. There is no significant difference between mouse strains.

3.2.2 The muscarinic inhibitor atropine partly reduces the detrusor contraction evoked by EFS and LED stimulation of cholinergic nerve fibers

Cholinergic detrusor contraction is entirely mediated by muscarinic receptors and can be fully antagonized by atropine (Andersson 1993, Andersson and Wein 2004). To estimate the cholinergic component, EFS- and LED-induced detrusor contraction was evoked first in the absence and then in the presence of atropine at 2 μM. The response to LED stimulation was reduced by 35-37% at all frequencies, while the response to EFS was frequency dependently reduced by 5 (at 8 Hz) to 21% (at 32 Hz) (Fig. 3.19, Tab. 3.4). Repetitive EFS and LED stimulation in the absence of atropine, however, did not show decreased responses (Fig. 3.20, Tab. 3.5).

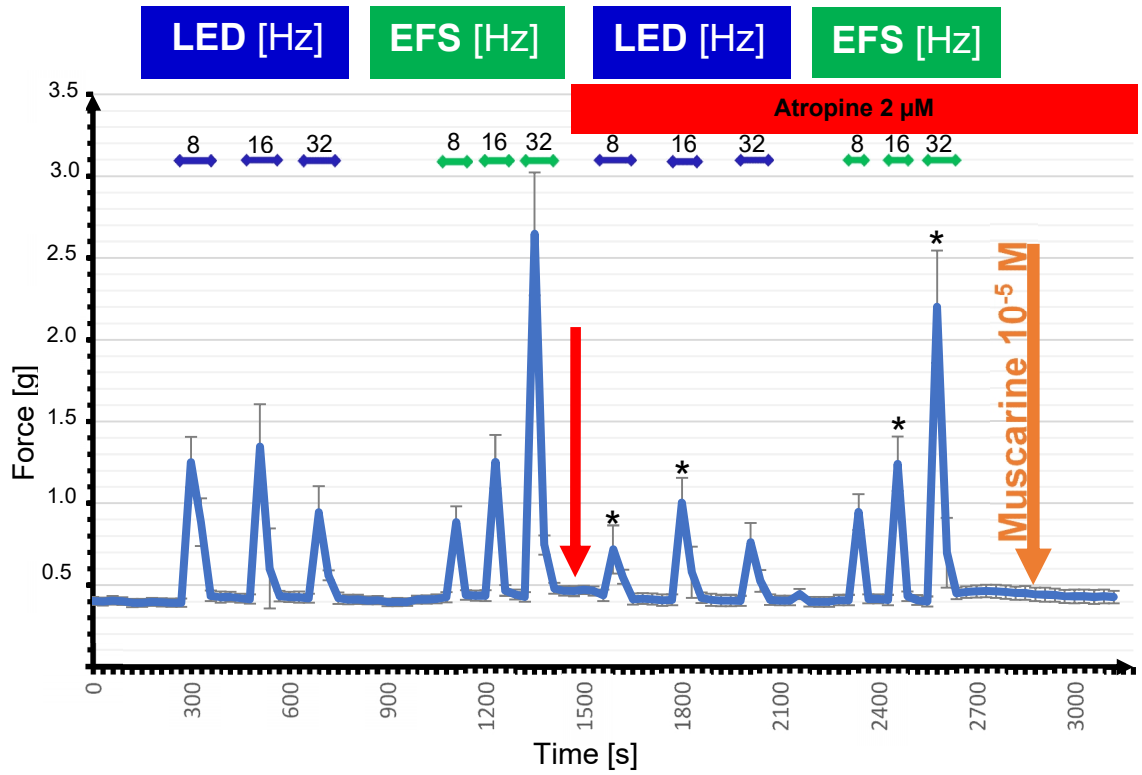


Figure 3.19 LED- and EFS-induced contraction of the urinary bladder of ChAT-ChR2 mice in organ bath in the presence and absence of the muscarinic inhibitor atropine (n=7). Efficacy of atropine treatment is demonstrated by loss of response to the muscarine. Significant ($p \leq 0.05$, paired t-test) differences in tension, compared to the same stimulus before atropine administration, are indicated by asterisks

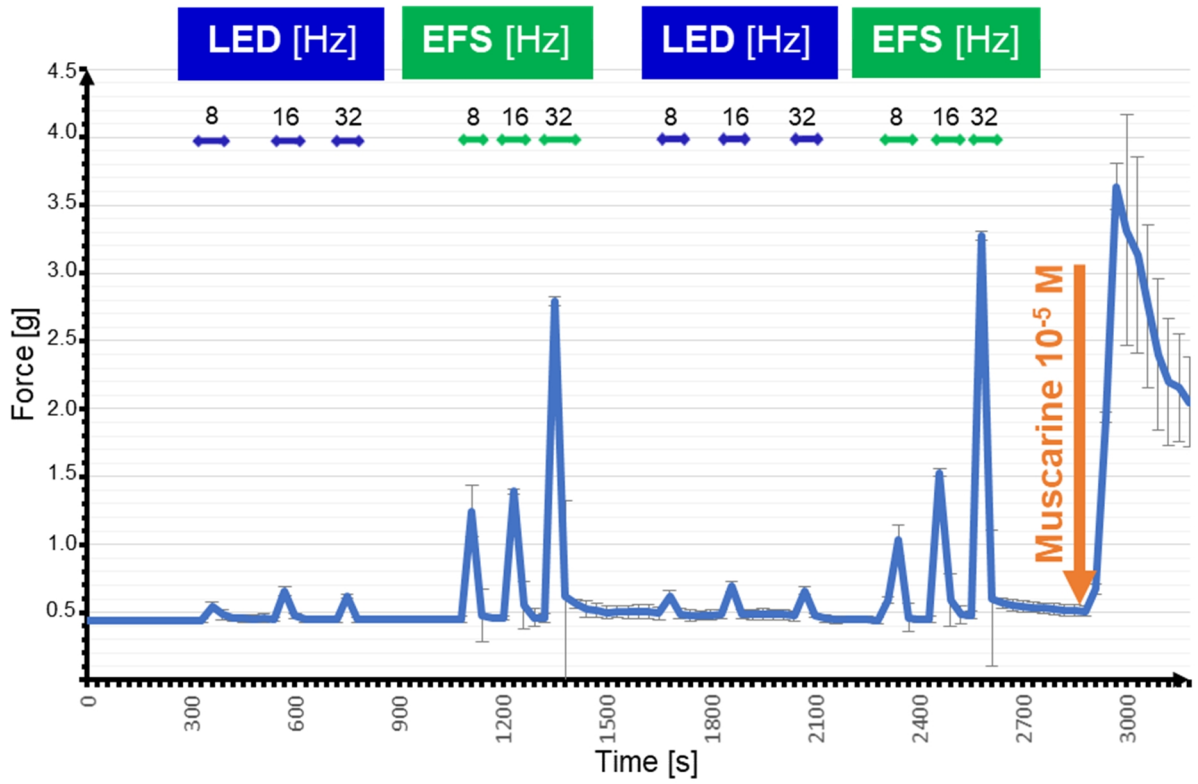


Figure 3.20 LED- and EFS-induced contraction of the urinary bladder of ChAT-ChR2 mice in an organ bath, repetitive stimulation without treatment of the tissue between the two series of stimulation (n=4). There were no significant differences in tension, compared to the same stimulus in the first series ($p>0.05$, paired t-test)

Stimuli	Increase in tension before atropine treatment Δ Force [g]	Increase in tension after atropine treatment Δ Force [g]	Diminution of increase in response to atropine treatment (ΔΔ Force)		p-value
	$\bar{X}$±*SEM*	$\bar{X}$±*SEM*	$\bar{X}$±*SEM* **[g]**	$\bar{X}$±*SEM* **[%]**	
LED 8 Hz	0.77±0.17	0.50±0.14	0.27±0.03	35.3±3.9	**0.038**
LED 16 Hz	0.98±0.28	0.63±0.15	0.35±0.13	36.1±13.3	**0.049**
LED 32 Hz	0.59±0.18	0.37±0.11	0.22±0.07	37.5±11.9	0.105
EFS 8 Hz	0.51±0.09	0.48±0.09	0.03±0.00	5.1±0.0	0.240
EFS 16 Hz	0.93±0.16	0.81±0.15	0.12±0.01	13.0±1.1	**0.031**
EFS 32 Hz	2.10±0.40	1.65±0.34	0.45±0.06	21.2±2.9	**0.042**

Table 3.4 Comparison of LED- and EFS-induced increases in detrusor tension in ChAT-ChR2 mice before and after atropine (2 μM) administration. P-values calculated by paired t-test (n=7).

Stimuli	Increase in tension by first stimuli Δ Force [g]	Increase in tension by second stimuli Δ Force [g]	Difference in the response to the repetitive stimuli (ΔΔ Force)		p-value
	$\bar{X}\pm SEM$	$\bar{X}\pm SEM$	$\bar{X}\pm SEM$ **[g]**	$\bar{X}\pm SEM$ **[%]**	
LED 8 Hz	0.11±0.03	0.12±0.02	-0.01±0.01	-9.1±9.1	0.622
LED 16 Hz	0.21±0.03	0.20±0.01	0.01±0.02	4.8±9.5	0.867
LED 32 Hz	0.17±0.03	0.19±0.03	-0.02±0.00	-11.8±0.0	0.616
EFS 8 Hz	0.80±0.24	0.44±0.15	0.36±0.09	45.0±11.3	0.325
EFS 16 Hz	0.93±0.19	1.08±0.20	-0.15±0.01	-16.2±1.1	0.074
EFS 32 Hz	2.33±0.78	2.79±0.56	-0.46±0.22	-19.7±9.4	0.155

Table 3.5 Comparison of LED- and EFS-induced changes in detrusor tension in ChAT-ChR2 mice by repetitive stimuli. P-values calculated by paired t-test (n=4).

3.2.3 Combined muscarinic and nicotinic inhibition only partly reduces the detrusor contraction evoked by EFS and LED stimulation of cholinergic nerve fibers

To assess a potential nicotinic component in the detrusor contraction upon stimulation of cholinergic nerve fibers, EFS- and LED-induced detrusor contraction was evoked first in the absence and then in presence of a blocker cocktail consisting of the muscarinic inhibitor atropine (2 µM) and the nicotinic inhibitor mecamylamine (20 µM). The response to LED stimulation was reduced by 8-18% at all frequencies, while the response to EFS was reduced between 24% at 8 Hz and 38% at 32 Hz (Fig. 3.21, Tab. 3.6). Despite this variation, there was no significant frequency dependency (ANOVA).

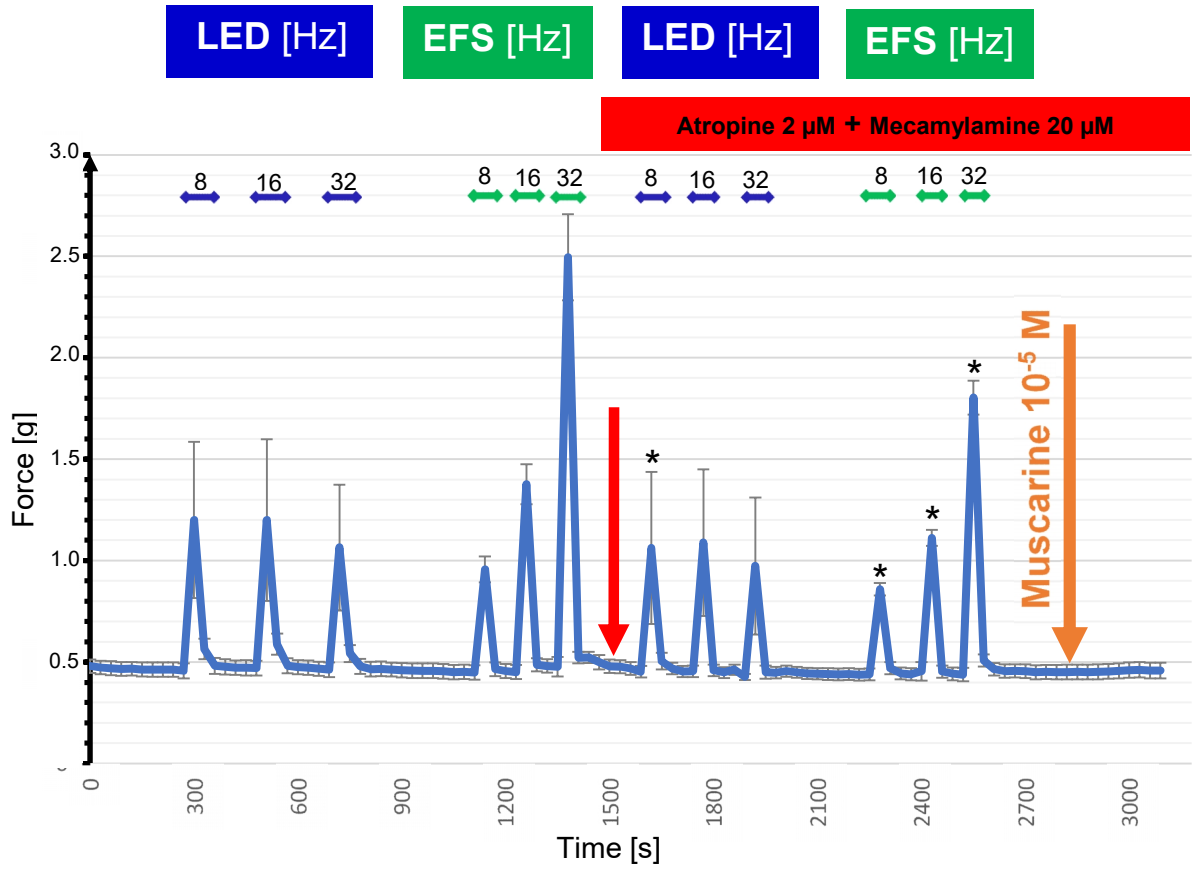


Figure 3.21 LED- and EFS-induced contraction of the urinary bladder of ChAT-ChR2 mice in organ bath in the presence and absence of a blocker cocktail consisting of the muscarinic inhibitor atropine (2 µM) and the nicotinic inhibitor mecamylamine (20 µM) (n=5). Efficacy of atropine treatment is demonstrated by loss of response to muscarine. Significant ($p \leq 0.05$, paired t-test) differences in tension, compared to the same stimulus before blocker administration, are indicated by asterisks

Stimuli	Increase in tension before a blocker cocktail (atropine and mecamylamine) Δ Force [g]	Increase in tension after a blocker cocktail (atropine and mecamylamine) Δ Force [g]	Diminution of increase in response to a blocker cocktail (atropine and mecamylamine) (ΔΔ Force)		p-value
	$\bar{X}\pm SEM$	$\bar{X}\pm SEM$	$\bar{X}\pm SEM$ **[g]**	$\bar{X}\pm SEM$ **[%]**	
LED 8 Hz	0.88±0.50	0.72±0.50	0.16±0.00	18.2±0.0	**0.010**
LED 16 Hz	0.87±0.51	0.77±0.47	0.10±0.04	11.5±4.6	0.169
LED 32 Hz	0.72±0.40	0.66±0.44	0.06±0.04	8.33±5.6	0.270
EFS 8 Hz	0.54±0.02	0.41±0.03	0.13±0.01	24.1±1.9	**0.033**
EFS 16 Hz	0.97±0.08	0.63±0.04	0.34±0.04	35.1±4.1	**0.042**
EFS 32 Hz	2.07±0.23	1.28±0.04	0.79±0.19	38.2±9.2	**0.034**

Table 3.6 Comparison of LED- and EFS-induced increases in detrusor tension in ChAT-ChR2 mice before and after administration of a blocker cocktail consisting of the muscarinic inhibitor atropine (2 µM) and the nicotinic inhibitor mecamylamine (20 µM). P-values calculated by paired t-test (n=5).

3.2.4 The purinergic inhibitors (PPADS and suramin) crucially reduce the detrusor contraction evoked by light-induced selective stimulation of cholinergic nerve fibers

Besides ACh, ATP plays also an important transmitter role in neutrally evoked detrusor contraction (Burnstock et al. 1978, Levin et al. 1990, Masuda et al. 1995). To assess a potential purinergic component in the detrusor contraction upon stimulation of cholinergic nerve fibers, EFS- and LED-induced detrusor contraction was evoked first in

the absence and then in presence of a blocker cocktail consisting of the P2 receptors PPADS (100 µM) and suramin (300 µM) (Fig. 3.22).

Responses to LED stimulation and EFS were significantly reduced by this blocker cocktail, but a residual contractile response remained. Contractions evoked by LED stimulation were reduced by 60-75 %, whereas those evoked by EFS were diminished by only 25-30% (Tab. 3.7). These differences between stimulation protocols were statistically significant ($p \leq 0.05$; paired t-test).

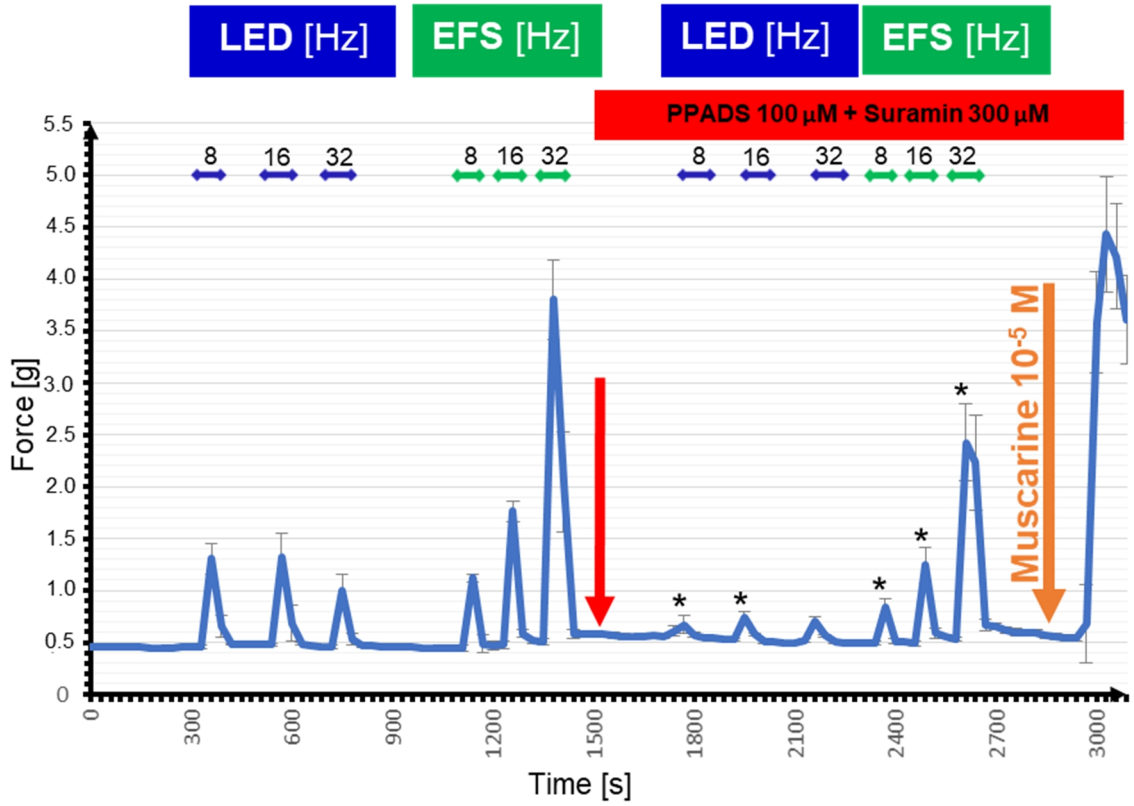


Figure 3.22 LED- and EFS-induced contraction of the urinary bladder of ChAT-ChR2 mice in organ bath in the presence and absence of a purinergic blocker cocktail consisting of PPADS (100 µM) and suramin (300 µM) (n=6). Control of the viability of the tissue with muscarine at 10^{-5} M. Significant ($p \leq 0.05$, paired t-test) differences in tension, compared to the same stimulus before blocker administration, are indicated by asterisks

Stimuli	Increase in tension before suramin and PPADS treatment Δ Force (g)	Increase in tension after suramin and PPADS treatment Δ Force (g)	Diminution of increase in response to suramin and PPADS treatment (ΔΔ Force)		p-value
	$\bar{X}$±*SEM*	$\bar{X}$±*SEM*	$\bar{X}$±*SEM* **[g]**	$\bar{X}$±*SEM* **[%]**	
LED 8 Hz	0.79±0.16	0.30±0.10	0.49±0.06	61.9±7.6	**0.027**
LED 16 Hz	0.94±0.24	0.25±0.07	0.69±0.17	73.9±18.1	**0.036**
LED 32 Hz	0.60±0.17	0.22±0.05	0.38±0.12	62.4±20.0	0.084
EFS 8 Hz	0.61±0.04	0.42±0.08	0.19±0.04	30.7±6.6	**0.019**
EFS 16 Hz	1.15±0.10	0.86±0.18	0.29±0.08	25.0±7.0	**0.036**
EFS 32 Hz	2.87±0.40	1.97±0.42	0.90±0.02	31.6±0.7	**0.001**

Table 3.7 Comparison of LED- and EFS-induced increases in detrusor tension in ChAT-ChR2 mice before and after PPADS (100 µM) and suramin (300 µM) administration. P-values calculated by paired t-test (n=6).

3.2.5 Residual detrusor contraction after using all three inhibitors (atropine, PPADS, suramin)

To further investigate if additional receptors or neurotransmitters participate in the detrusor contraction during the stimulation of cholinergic nerve fibers, we blocked purinergic P2X receptors through PPADS (100 µM) and suramin (300 µM), and muscarinic receptors through atropine (2 µM).

The contractile responses to LED stimulation and EFS were vastly reduced (about 80%) by a combination of these three inhibitors. Still, there was a residual contraction of about 20% evoked by both LED stimulation and EFS (Fig. 3.23; Table 3.8).

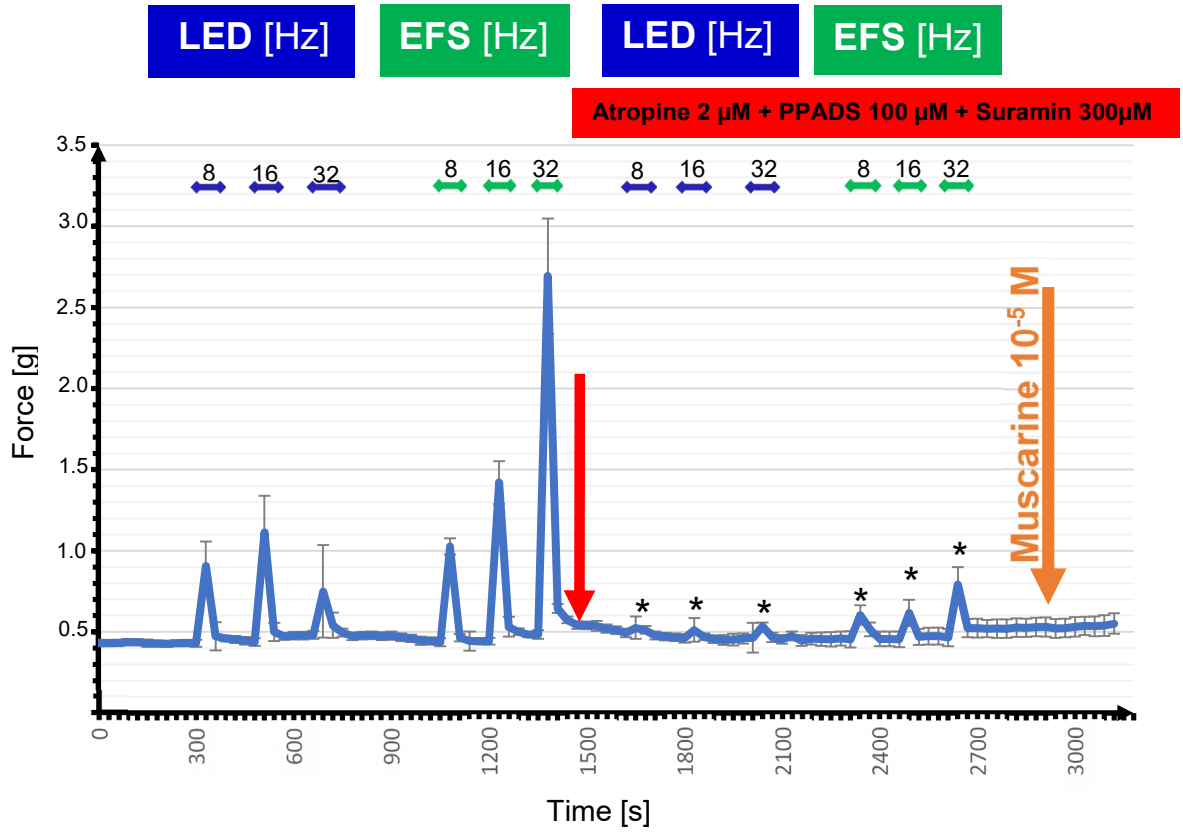


Figure 3.23 LED- and EFS-induced contraction of the urinary bladder of ChAT-ChR2 mice in organ bath in the presence and absence of a cholinergic and purinergic blocker cocktail consisting of atropine (2 µM), PPADS (100 µM) and suramin (300 µM) (n=7). Efficacy of atropine treatment is demonstrated by loss of response to the muscarine. Significant ($p \leq 0.05$, paired t-test) differences in tension, compared to the same stimulus before blocker administration, are indicated by asterisks

Stimuli	Increase in tension before atropine, suramin, and PPADS treatment Δ Force [g]	Increase in tension after atropine, suramin, and PPADS treatment Δ Force [g]	Diminution of increase in response to atropine, suramin, and PPADS treatment (ΔΔ Force)		p-value
	$\bar{X}\pm SEM$	$\bar{X}\pm SEM$	$\bar{X}\pm SEM$ **[g]**	$\bar{X}\pm SEM$ **[%]**	
LED 8 Hz	0.61±0.16	0.12±0.05	0.49±0.11	80.2±18.0	**0.012**
LED 16 Hz	0.81±0.25	0.13±0.05	0.68±0.20	83.5±24.7	**0.033**
LED 32 Hz	0.61±0.30	0.13±0.06	0.48±0.24	79.2±9.8	**0.016**
EFS 8 Hz	0.57±0.05	0.17±0.02	0.40±0.03	71.0±5.3	**0.001**
EFS 16 Hz	1.08±0.14	0.20±0.04	0.88±0.10	81.2±9.3	**0.001**
EFS 32 Hz	2.60±0.38	0.31±0.06	1.86±0.32	88.1±12.3	**0.001**

Table 3.8 Comparison of LED- and EFS-induced increases in detrusor tension in ChAT-ChR2 mice before and after administration of atropine (2 μM), PPADS (100 μM) and suramin (300 μM). P-values calculated by paired t-test (n=7).

4 Discussion

4.1 Validation of the optogenetic mouse model

The present study is based upon a mouse model with a cell-type specific expression of ChR2, a light-sensitive cation channel that is not encoded in the mammalian genome. Hence, cell type-specific gene transfer is required to obtain such a model. In the CNS, viral gene transfer is often used for this purpose (Carter et al. 2010, Adamantidis et al. 2007, Covington et al. 2010). This method is quick, broadly applicable and restricted to the area of viral injection. While this spatial limitation to the area of injection may be of advantage in several experimental settings, it prevented the use of this viral transfer in the present context. Cholinergic neurons innervating the urinary bladder, the target in this study, are dispersed in small ganglia in the pelvic region that are highly inaccessible and cannot be readily identified for injection in vivo. Hence, a genetic approach utilizing the Cre-*loxP* system was used. This system was originally introduced by the group of Rajewsky (Gu et al. 1993). It requires two independently generated genetically modified mouse strains. One of them expresses Cre (Causes recombination), a 38-kDa integrase physiologically encoded by bacteriophage P1, driven by a specific promotor. For the present study, a mouse line was chosen that expresses Cre under the control of the ChAT promotor, which shall be specific for cholinergic cells. Cre mediates site-specific recombination between specific sequences that are referred to as *loxP* (locus of crossover (x) in P1 bacteriophage) sites (Sharma and Zhu 2014). If Cre is expressed in a cell that carries two *loxP* sites not too far from each other in its genome, the DNA will be cut at these sites, the sequence between the *loxP* sites removed, and the DNA ligated again. Thus, the region flanked by the *loxP* sites will be removed from the genome. This can be used to delete a functional gene to generate a knockout mouse, but there are also approaches to activate a certain gene through this technique. This can be achieved by introducing a gene driven by a strong constitutive promotor but placing a STOP in front of the coding sequence to prevent expression. If this STOP is flanked by the *loxP* sites, Cre expression will result in loss of the STOP signal and, hence, activate expression of the previously silent gene. Here, a mouse line was chosen that carries such a construct with a “floxed” STOP in front of the ChR2-tdTomato fusion protein. Both mouse strains, the ChAT-Cre driver line, and the “floxed” ChR2-tdTomato line are commercially available and were obtained from the Jackson Laboratories, Sacramento. When these

lines are cross-bred, Cre shall be specifically expressed only in cholinergic cells, and this shall lead to ChR2-tdTomato expression exclusively in these cells.

Unfortunately, however, this is not always the case practically. First, Cre expression can occur spontaneously in cells even when the "specific" promotor is not activated. This is called "leaky Cre-expression". One of the possible reasons is a transient expression of the driver gene in early embryogenesis, but there are also other options (Sharma and Zhu 2014). This may lead to Cre-mediated STOP removal and, hence, ChR2-tdTomato expression in cells that are not cholinergic at the time of the investigation, i.e. false positive expression. Second, even if expressed in the correct target cell, Cre might not efficiently cut the DNA at the *loxP* site (Sharma and Zhu 2014), leading to "false negative" cells. This efficacy can vary from cell type to cell type (Sharma and Zhu 2014). Thus, it is common that, in mouse lines based upon this technology, there is both some ectopic expression (false positive) and not a fully complete expression in the cell type of interest (false negative). This technical limitation has to be taken into account, and the mouse model has to be carefully analyzed for ectopic and incomplete expression.

Therefore, expression of the ChR2-tdTomato was controlled in the urinary bladder and other peripheral organs with known cholinergic innervation by fluorescence microscopy, and parallel immunofluorescence with a green fluorophore was used to identify cell types and nerve fiber populations that express (or not express) ChR2-tdTomato. Expression outside nerve fibers ("ectopic") which may reflect false positive expression was very limited. It was observed only in a very small number of skeletal muscle fibers and, to an interindividual variable degree, in some smooth muscle cells including those of the detrusor muscle. It has to be noted that ACh synthesis is indeed not limited to neurons. Rather, there is a widespread non-neuronal cholinergic system comprising, but not limited to, several epithelial and immune cells (Wessler and Kirkpatrick 2008, Kummer and Krasteva-Christ 2014, Fujii et al. 2017). Indeed, skeletal muscle fibers have been reported to produce ACh, but in this case, it has been ascribed to the enzyme carnitine acetyltransferase rather than to ChAT (Tucek 1982). Thus, the very infrequent ChR2-tdTomato expression in skeletal muscle fibers may indeed represent "leaky Cre-expression" independent from the ChAT promotor. As the urinary bladder does not contain skeletal muscle fibers, this minor ectopic expression was irrelevant for our functional studies. As for smooth muscle cells, indication for ChAT-expression has been provided, mainly based upon ChAT-immunolabeling (Wessler and Kirkpatrick 2001)

which cannot be taken as final proof. Thus, the occasional ChR2-tdTomato expression seen in detrusor smooth muscle cells might reflect true activation of the ChAT promotor, but it also might due to leaky expression. In either case, this has to be considered when interpreting the functional data, and this will be further worked out in chapter 4.2.4.

There is also evidence for inefficient Cre-expression in non-neuronal cholinergic cells in our mouse model. ChAT-immunolabeling revealed cholinergic epithelial cells, most probably representing cholinergic tuft (=brush) cells (Schütz et al. 2015), that did not express ChR2-tdTomato. Similar cholinergic chemosensory cells also occur in the tracheal and laryngeal epithelium (Krasteva et al. 2011, Krasteva et al. 2015) but did also not show up in ChR2-tdTomato fluorescence. It thus appears to be likely that Cre-driven recombination was particularly ineffective in this cell type. In either case, this had no impact on the functional part of this study since, in the urinary tract, such cells are restricted to the urethra but are absent from the urinary bladder (Deckmann et al. 2014).

With respect to nerve fibers, fluorescence microscopy of airways, digestive tract and urinary bladder revealed a dense innervation with ChR2-tdTomato-fluorescent fibers that matched in distribution and extent that what is known for cholinergic nerve fibers (Schäfer et al. 1988). Immunoreactivities to key proteins for cholinergic signaling, i.e. ChAT and VAChT (the latter investigated in the bladder only), colocalized with native ChR2-tdTomato-fluorescence. Although there was extensive colocalization, these signals did not always correlate 1:1. There are two likely reasons for this slight mismatch: 1) Incomplete efficiency of expression, as already discussed. Notably, this has also recently been described for another genetically based model to visualize ChAT expression (Brown et al. 2018). 2) The three proteins detected in fluorescence microscopy, i.e. the ChR2-tdTomato fusion protein, ChAT and VAChT, all have different subcellular distribution. ChR2 is a membrane protein, ChAT is a cytoplasmatic protein, and VAChT is a membrane protein of intracellular vesicles. This different subcellular localization will lead to incongruencies in the distribution of fluorescent signals. In case of very small (<1 µm) nerve fibers this might not drastically change the fluorescence pattern. In the case of neuronal cell bodies, however, this was clearly visible.

Particularly important, the catecholamine-synthesizing enzyme TH was not detected in ChR2-tdTomato-fluorescent fibers, so that stimulation of noradrenergic sympathetic fibers did not occur in our functional experiments. Colocalization with the neuropeptide SP, a member of the tachykinin family, was observed in the muscle layer of the

duodenum, but neither in the airways nor in the urinary bladder. This differential pattern of colocalization nicely mirrors previous immunohistochemical and functional studies demonstrating SP as a cotransmitter of ACh in enteric motoneurons (Qu et al. 2008), whereas it is contained within non-cholinergic, peptidergic sensory fibers in the airways (Xiao and Wu 2012, Tränkner et al. 2014) and urinary bladder (Moss et al. 1990, Mitsui and Hashitani 2013).

Altogether, the histological analysis of the urinary bladder and other peripheral organs validated the suitability of the newly generated mouse model for optogenetic studies of autonomic cholinergic neurons.

4.2 Contractile activity of the detrusor muscle of mice in response to exogenous stimuli

4.2.1 LED stimulation evokes detrusor contraction specifically in ChAT-ChR2 mice

To our knowledge, this is the first report providing data on organ bath experiments in mice with the expression of ChR2 in peripheral cholinergic nerve fibers. The control group of mice, expressing the only green fluorescent protein in cholinergic nerve fibers, was taken for comparison of the outcomes and exclusion of experimental side effects. Our results demonstrated that the application of LED stimuli with different impulses leads to the contraction of the urinary bladder only in ChAT-ChR2 mice. The response to EFS in our experiments was comparable for both groups, so we can conclude that the additional expression of ChR2 did not interfere with the functional properties of the cholinergic innervation of the urinary bladder.

To test our preliminary outcome about the unique specificity of the LED stimuli to the ChAT-ChR2 mouse strain, we used the same protocol for organ bath experiments as in control ChAT-GFP mice. The thermal effect in organ bath experiments should not be underestimated. Burdyga and Wray described in their experiments that warming of the tissue during organ bath experiments increases phasic contraction of the smooth muscle of the rat ureter. This effect was caused by prolongation of the Ca^{2+} transient that allows the myofilament to come closer to equilibrium with Ca^{2+} and increases force production (Burdyga and Wray 2002). In our experiments, however, the response to LED stimulation

was specific only for ChAT-ChR2 mice, and herewith, we could exclude a side effect, which might occur through thermal influence.

In our investigations, we observed a frequency-dependent increase in force in EFS, but not in our LED stimulation protocol. Applying pulsed LED stimuli at 32 Hz, we examined a decrease of contraction of the detrusor muscle. Meanwhile, EFS, on its turn led to a proportional increase of contractions as the frequency of stimuli raised. One likely explanation is that the pulse duration during the EFS was kept constant, whereas in LED protocol the pulse duration was adapted to equal on-off periods. In addition, other studies revealed inhibition of neuronal activity during high-frequency optical stimulation of ChR2-expressing neurons in the mouse brain (Witten et al. 2010, Anikeeva et al. 2012). These authors supposed that in most cases inhibition was produced by excitation of cholinergic or parvalbumin interneurons that have inhibitory effects on the neuronal fiber activity. Since there are no interneurons known in the detrusor bladder, this effect is unlikely to have occurred in our experiments. Liske and colleagues performed systematic analysis to determine if the frequency-dependent stimulation of ChR2 could achieve both excitation and inhibition. Their results demonstrated that the average force amplitude during LED stimulation decreased significantly with increasing light pulse frequency in Thy1-ChR2-EYFP mice (Liske et al. 2013).

4.2.2 Only a third of the contraction induced by cholinergic nerve fibers is mediated via muscarinic cholinergic receptors

An atropine-resistant response of the urinary bladder to electrical stimulation was first mentioned at the beginning of the 20th century (Langley 1911). Langley disclosed that the electrical stimulation of the urinary bladder was still effective in causing contraction after 10 mg of atropine sulfate. Hukinovic and colleagues observed that atropine, in the concentration of 0.1 µg/ml, reduced the effect of electrical stimulation in the rat urinary bladder by one third. They have reported that higher concentration of atropine had no further effect on reduction of contractility of the bladder (Hukinovic et al. 1965). Later studies also confirmed the resistance of the urinary bladder contraction to atropine treatment. For example, Brading and Williams in the early 90s compared the contractile responses of strips of rat and guinea-pig detrusor muscle to the effect of atropine treatment. They observed that atropine at 0.5 µM concentration did not show an obvious

effect either to guinea-pig nor rat detrusor muscle. However, an increased atropine concentration (3 µM) led to a 25% reduction of the contraction in both animal models (Brading and Williams 1990). Some other authors, for example, Elliot and colleagues, detected a diminution of the maximum contractile response by half after treatment with atropine the detrusor muscle of the rat (Elliot et al. 2000).

In the human bladder, atropine-resistant contraction is described 5 to 30 % in so-called "normal" bladders (Sjögren et al. 1982, Luheshi and Zar 1990, Tagliani et al. 1997). In 1999, Bayliss and colleagues analyzed characteristics of atropine-resistant contraction in the human detrusor. They concluded that humans with stable bladder (control group) do not have an atropine-resistant contraction at any electrical stimulation frequency and the group of patients undergoing transurethral prostate resection and urodynamically proved bladder instability according to ICS definitions have a frequency-dependent atropine resistance up to 20 % (Bayliss et al. 1999). Fry and colleagues published a detailed characterization of atropine-resistant contraction due to bladder disorders in a large number of human detrusor biopsies. They observed that patients with idiopathic detrusor overactivity have higher atropine-resistance (23.0 %) than patients with neurogenic detrusor overactivity (14.7 %) or bladder outlet obstruction (11.5 %). The results of the control group – patients with a stable and unobstructed bladder - were comparable with the study of Bayliss and colleagues and did not show atropine-resistant contraction (Fry et al. 2011). Striking results were achieved by Johal and colleagues. They observed a large proportion of atropine resistance in detrusor contraction from pediatric bladders compared with adult bladders (Johal et al. 2014). This conclusion could explain why many children are refractory to antimuscarinic therapy (Elmissiry et al. 2013).

We treated the detrusor muscle of ChAT-ChR2 mice with 2 µM atropine to block the muscarinic acetylcholine receptors of the muscular tissue and observed that atropine diminished the contractile force in organ bath in response to both LED stimulation and EFS. Interestingly, the atropine-resistant detrusor contraction induced by LED was the same for all frequencies and composed two-thirds of the pretreatment contraction. In contrast, during EFS the diminution of contraction after atropine treatment was frequency-dependent and was reduced by 5–21 %. In previous literature, we were not able to find sufficient information about isolated activation of postsynaptic parasympathetic neurons in the urinary bladder. Thus, we cannot truly compare our results of pure optogenetic stimulation of cholinergic nerve fibers with LED and data

obtained externally. Even so, in target-specific LED stimulation, reduction of contractility demonstrated a stronger response to atropine than it was seen in EFS. The blockade that we received by atropine in the EFS experiments was less than that reported in previous studies (Brading and Williams 1990, Elliot et al. 2000). Such discrepancy between our observations and the results of the previous studies can be explained by the duration of the impulse, individual properties of the organ bath and concentration of atropine.

The efficiency of atropine treatment was validated in our experiments by application of muscarine (10^{-5} M) to the organ bath, and this muscarine-induced contraction was fully abrogated. Muscarine was chosen instead of ACh since the latter also activates nicotinic receptors, and full blockade of the response to high doses of ACh requires both atropine and a nicotinic receptor blocker such as hexamethonium (Chesher and Thorp 1965).

The activation of parasympathetic postganglionic nerves releases ACh, which serves as a transmitter that mediates detrusor contraction via M2 and M3 subtypes of muscarinic receptors (Hegde and Eglen 1999). However, our data demonstrated that when cholinergic nerve fibers are selectively activated, a certain portion of the contraction is atropine-resistant. This outcome led us to the assumption of the existence of an additional transmitter or group of transmitters that are co-released besides ACh from cholinergic nerve fibers in the detrusor.

4.2.3 A purinergic transmitter is co-released with ACh from cholinergic nerve fibers in the detrusor muscle

The first evidence of non-cholinergic, non-adrenergic transmission in the detrusor was demonstrated in the seventies of the last century by Ambache und Zar (Ambache and Zar 1970). In their experiments, they demonstrated that the mucosa-free detrusor muscle of the guinea-pig bladder responds to electrical stimulation despite blockade of muscarinic receptors. In 1978, Burnstock and colleagues postulated that the atropine-resistant contraction in the guinea-pig bladder was triggered by ATP that is released from a neuronal pool (Burnstock et al. 1978).

Later studies supported this hypothesis not only in guinea-pig bladder but also in many other species, including pig, mouse, hamster, monkey, cat, and others (MacKenzie et al. 1982, Santicioli et al. 1986, Levin et al. 1990, Creed et al. 1994, Masuda et al. 1995, Pinna et al. 1998). Creed and colleagues came to the conclusion that two transmitters are

released from excitatory nerves due to a comparison of the reduction of the detrusor contraction during the EFS to different blocker cocktails. The results demonstrated that a cocktail of atropine and suramin reduces the contraction of rabbit's, guinea-pig's and monkey's (baboon or rhesus) detrusor more that atropine alone (Creed et al. 1994). Levin and his colleagues extensively studied lower urinary tract function in both the cat and the rabbit (Levin et al. 1980, Levin et al. 1981, Levin et al. 1986). They proposed the presence of purinergic nerves, which release ATP during the activation of cholinergic nerve fibers in the bladder. This conclusion was supported by the fact that ATP stimulates a contraction in the rabbit detrusor that has the same magnitude as the atropine-resistant contraction in EFS (Levin et al. 1990). The study of Pinna and colleagues provided clear evidence that the purinergic neurotransmitter ATP plays a key role in detrusor contraction. In their experiments in the hamster bladder, they were able to inhibit the EFS-induced detrusor contraction by atropine together with suramin by 90 % (Pinna et al. 1998).

ATP is now recognized as a fast neurotransmitter released from nerve terminals. It has extracellular action via the ionotropic P2X receptors, which are ligand-gated cation channels, and metabotropic P2Y receptors, which are G protein-coupled receptors (Kennedy et al. 2013, Khakh et al. 2001). At this point, it should be discussed that ATP is not the only known purinergic ligand (Hoyle et al. 1990). The P2-purinoreceptors are also activated by adenosine 5-diphosphate (ADP). An adenine-dinucleotides that acts as a neurotransmitter is β-nicotinamide adenine dinucleotide (β-NAD) (Hoyle et al. 1990, Mutafova-Yambolieva et al. 2007). Stone was the pioneer author who described β-NAD-induced prejunctional inhibition of sympathetic neuromuscular transmission in the vas deferens of the rat (Stone 1981). Stone observed that β-NAD inhibits the contractile activity of the vas deferens of the rat by 40-42 %. Later, Burnstock and Hoyle concluded that β-NAD acts largely indirectly as P1–purinoreceptor agonist following its breakdown to adenosine by ectoenzymes. The results of their experiments demonstrated that β-NAD possesses concentration-dependent relaxations in the carbachol-contracted taenia coli. Also, they detected adenosine in all samples with β-NAD treatment. These results imply that β-NAD is broken down to adenosine or that β-NAD induces the release of adenosine from tissue (Burnstock and Hoyle 1985). Further, the studies of Smyth and colleagues identified the nerve-evoked release of β-NAD and its metabolic product cyclic adenosine 5'-diphosphoribose (cADPR) and adenosine 5'-diphosphoribose (ADPR) in vascular and

non-vascular smooth muscle preparations. These nucleotides probably regulate the release of norepinephrine at the vascular neuroeffector junction (Smyth et al. 2004). The same group of scientists observed the release of β-NAD by stimulation of postganglionic nerve terminals in the human detrusor smooth muscle in a TTX-sensitive manner. This observation led the authors to the conclusion that β-NAD can be a novel player in the neural control of the human bladder (Breen et al. 2006). But the release of β-NAD in this study was unaffected by guanethidine, and increased by capsaicin, these support the assumption that the β-NAD might be released from sensory nerves (Breen et al. 2006). This leads us to the conclusion that the contraction induced by the LED in bladders of ChR2 mice is more likely transmitted through ATP rather than β-NAD.

We used inhibitors of P2-purinergic receptors - suramin and PPADS - to block a potential purinergic component resulting from selective stimulation of cholinergic nerve fibers in the detrusor muscle. Suramin is a potent inhibitor of a Na^+-K^+-ATPase at an intracellular site, by interfering with the binding of ATP (Fortes et al. 1973). ATP acts as a transmitter and can cause either contraction or relaxation by the activation of P2X- or P2Y-purinoreceptors, respectively (Burnstock and Kennedy 1985, Dunn and Blakeley 1988). The first evidence of a reversible antagonistic effect of suramin on P2-purinoreceptors was obtained in mouse vas deferens by Dunn and Blakeley (Dunn and Blakeley 1988). They concluded that suramin can effectively inhibit the contraction of the vas deference provoked by α,β-methylene ATP. Later, Hoyle and colleagues showed a non-competitive antagonism of suramin to α,β-methylene ATP in detrusor strips of the guinea pig (Hoyle et al. 1990). Interestingly, the same study revealed concentration-dependent differential effects of suramin in the guinea-pig urinary bladder. The authors reported that suramin at a concentration of 1 μM increased the amplitude of contraction at low frequencies of EFS. At a concentration of 100 μM, it caused an inhibition of the contractile response only at frequencies of 4 to 32 Hz. The strongest inhibitory effect was observed at a concentration of 1 mM (Hoyle et al. 1990).

Lambrecht and colleagues were the first who reported PPADS as a P2X antagonist (Lambrecht et al. 1992). The results of their study demonstrated that PPADS selectively inhibits purinoreceptor-mediated response in the rabbit vas deferens. The same effect of PPADS was also shown in the rabbit urinary bladder (Ziganshin et al. 1993). Ziganshin and colleagues also described a concentration-dependent effect of PPADS in the rabbit detrusor muscle by EFS. At a concentration of 1 μM, PPADS inhibits a contraction at

lower frequencies. Between 10 and 30 μM, the inhibition of detrusor contraction was significant at all frequencies. In 1998, Pinna and colleagues compared the inhibitory effects of suramin and PPADS to the hamster urinary bladder. The results showed that the suramin at a concentration of 10^{-4} M and PPADS at 10^{-4} M give a similar degree of inhibition (Pinna et al. 1998).

Based on this data, we used a combination of PPADS and suramin because the literature review suggested that the inhibition of EFS-induced contractions by the mixture is slightly higher than that when drugs were applied separately (Undi et al. 2006, Shiina et al. 2007). Undi and colleagues described a stronger inhibition (approximately 70%) of contraction of human ileal tissue by both PPADS (50 μM) and suramin (100 μM) rather than when a single inhibitor was used (Undi et al. 2006). Similar results were also obtained by Shiina and colleagues. The contractile activity of the rectum of Japanese quails was strongly inhibited by combined application of suramin (50 μM) and PPADS (10 μM) (Shiina et al. 2007).

After the treatment of the urinary bladder with suramin and PPADS, contraction induced by LED was reduced up to two thirds in detrusor preparations taken from ChAT-ChR2 mice. These results serve as direct evidence of cotransmission of a P2X agonist (most likely ATP) and ACh in cholinergic nerve fibers in the urinary bladder of the mouse. In contrast to LED stimulation, the EFS-induced contraction was reduced by less than one third after application of suramin and PPADS. This differential effect reflects that EFS has a lack of target-specificity to cholinergic nerve stimulation of the detrusor and excites additional nerve fiber populations. It can be deduced from these findings that these additional, non-cholinergic fibers also contribute to neurally evoked detrusor contraction and that this component is largely non-purinergic. Comparing our EFS data with that of earlier EFS studies showed that the results reported herein are mostly consistent with our results achieved during the inhibition of electrical stimulated detrusor contraction with suramin and PPADS (Creed et al. 1994, Tong et al. 1997, Knight and Burnstock 2004). Still, Kennedy and coworkers described that suramin and PPADS suppressed the contraction slightly more in comparison to the previous studies. The peak response was inhibited by 50-60 % (Kennedy 2015).

The present study is the first to provide direct evidence for cholinergic-purinergic cotransmission in the detrusor since tools for selective activation of cholinergic nerve fibers had not been available earlier. Also, selective disruption of postganglionic

parasympathetic cholinergic fibers, equivalent to chemical denervation of postganglionic sympathetic noradrenergic nerves with sympatholytics such as guanethidine or 6-hydroxydopamine, had not been achieving yet. Still, there is strong indirect evidence for cotransmission in postganglionic parasympathetic neurons at other organs, specifically salivary glands. Lundberg and colleagues observed that ACh and VIP are released from the parasympathetic nerve terminals in cat salivary glands. They described that during low-frequency stimulation, ACh is released and caused an increase in salivary secretion. VIP is released at high stimulation frequencies and causes vasodilatation of blood vessels. Also, they proposed that VIP acts as a neuromodulator and increases both the postjunctional effect of ACh in salivary secretion and the release of ACh from nerve varicosities via prejunctional receptors (Lundberg 1981). One of the first proposals that ATP and ACh might be cotransmitters in cholinergic nerves supplying the bladder was put forward by Hoyes and colleagues. They observed that the nerve terminals, supplying the smooth muscle of the bladder, have small dense-cored vesicles that contain a second transmitter, which is co-released with ACh during the activation of parasympathetic postganglionic nerve fibers (Hoyes et al. 1975). Further indirect evidence for purinergic-cholinergic cotransmission was reported by Mackenzie and coworkers. The authors observed that guinea-pig detrusor strips respond to EFS with a twitch-like rest contraction in the presence of atropine and guanethidine, but this contraction was abolished in the presence of botulinum toxin (Mackenzie et al. 1982). It was assumed that botulinum toxin specifically inhibits transmitter release from cholinergic nerve terminals (Carpenter 1978). Thus, Mackenzie and his coworkers suggested that the parasympathetic neurons supplying the smooth muscle of guinea-pig urinary bladder co-release in ACh with ATP or polypeptides. (Mackenzie et al. 1982). It is now clear, however, that botulinum toxin also inhibits to some extent noradrenergic postganglionic sympathetic nerve endings and peripheral terminals of sensory neurons (Rosetto 2018). Indeed, botulinum toxin is now used for the treatment of pain syndromes (Chiu et al. 2016, Park and Park 2017). Thus, botulinum toxin cannot be taken as a tool to selectively deactivate cholinergic neurons.

Cotransmission of ATP and noradrenaline is well studied in the sympathetic nervous system (Burnstock 2013). The first evidence that ATP is released from sympathetic nerve fibers was the demonstration that stimulation of sympathetic nerves led to release of tritium from taenia coli preincubated in [3H] adenosine. This converted to [3H]ATP (Su et al. 1971). In animal models, many pioneer experiments establishing sympathetic

cotransmission were performed in the vas deferens because of its highly dense innervation with sympathetic nerve fibers, mostly arising from the hypogastric ganglion (Hukinovic et al. 1961, Burnstock and Verkhratsky 2010). For example, Todorov and colleagues used high-performance liquid chromatography to monitor the release of ATP and noradrenaline (NA) during the stimulation of sympathetic nerves innervating the guinea-pig vas deferens. They discovered that the peak of NA release was at 40 s after the beginning of stimulation and remained constant, whereas the peak of ATP release was at 20 s after the start of stimulation and decreased despite ongoing contraction. These results were consistent with the phasic nature of the purinergic component that was described by Westfall and coworkers (Todorov et al. 1996, Westfall et al. 1978). The group of Westfall was the first to demonstrate that ATP provokes fast contraction of the vas deferens as a cotransmitter with NA released from sympathetic nerves producing a slower contraction. Later studies showed that the destruction of sympathetic nerves with 6-hydroxydopamine led to abolishing of the purinergic response, strongly suggesting that ATP was released from sympathetic nerve fibers and not separately from purinergic nerves (Kirkpatrick and Burnstock 1987). A more recent paper of Bank and coworkers described a purinergic component in the human vas deferens. This study demonstrated that the human vas deferens contracts in response to purinergic agonists and the P2 antagonist PPADS reduce EFS-evoked contraction up to 40 % (Banks et al. 2006).

Thus, depending on organ system, a P_2 receptor agonist, most likely ATP, can serve as a cotransmitter both in noradrenergic sympathetic neurons, as demonstrated earlier, and in cholinergic parasympathetic neurons, as validated in the present study for the mouse urinary bladder.

4.2.4 Potential sources of residual LED-induced detrusor contraction under combined muscarinic-purinergic receptor blockade

Our results demonstrate that approximately 20% of the LED-induced contraction remained under combined cholinergic muscarinic and purinergic inhibition. This was not due to a non-specific effect of LED illumination on detrusor activity, as LED had no effect in controls expressing a fluorescent protein (eGFP), but not ChR2, in cholinergic nerve fibers. There are several possible explanations for this residual contractile effect (Fig 4.1):

1. Direct and indirect involvement of nicotinic cholinergic receptors on the detrusor contraction

2. Co-release of another, non-purinergic transmitter

3. Ectopic expression of ChR2

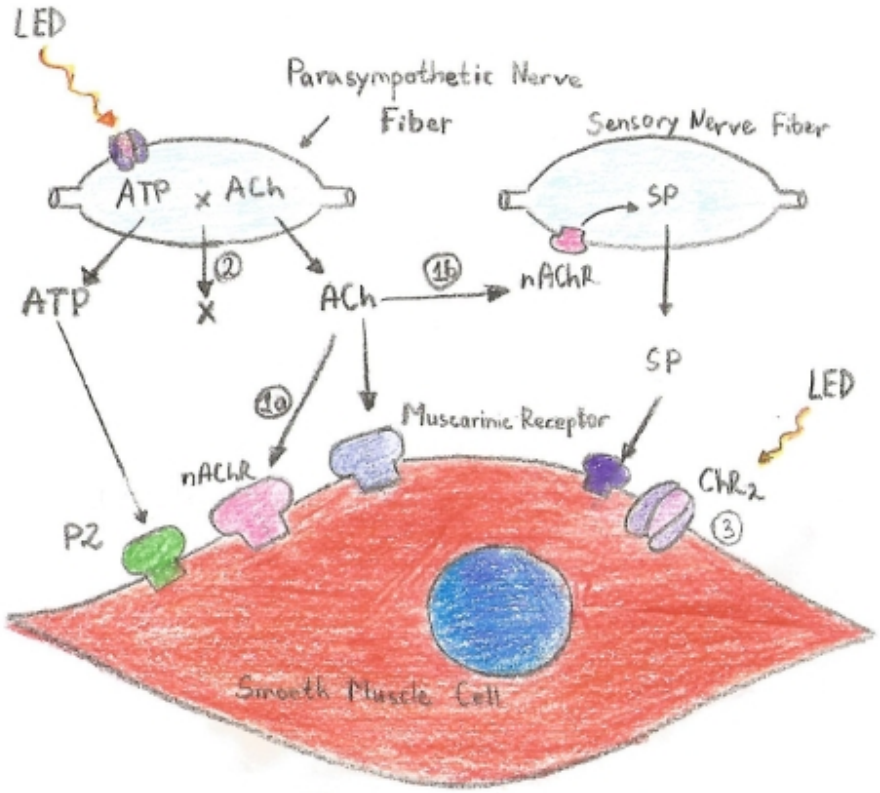


Figure 4.1 Schematic showing of possible pathways explaining the residual LED-induced contraction of detrusor after the muscarinic, purinergic blocker cocktail. 1a. Direct activation of nAChR through ACh; 1b. Indirect activation of nAChR; 2. Co-release of another, non-purinergic transmitter; 3. Ectopic expression of ChR2

Direct and indirect involvement of nicotinic cholinergic receptors on detrusor contraction

It is established that the main mechanism of nerve-evoked urinary bladder contraction involves the release of ACh acting on muscarinic receptors and of ATP acting on purinergic receptors (Burnstock 2014). Nicotinic receptors are not thought to directly mediate contraction of the normal bladder muscle (Gomez-Amaya et al. 2015) but still, contribute to contraction evoked by ACh given in high doses in organ bath experiments (Chesher and Thorp 1965). It is known that autonomic ganglia, including bladder intramural parasympathetic ganglion cells, express numerous types of nicotinic receptors. Deficiency of α3, β2 and β4 subunits of nicotinic acetylcholine receptor (nAChR) in mice leads to dysfunction of the bladder (De Biasi et al. 2000). However, there is insufficient information on the mechanism of how nAChRs induces bladder smooth muscle contraction (Fincan et al. 2016). Previously, it was discussed that ACh facilitates its own release through the effect on prejunctional nicotinic receptors (Vizi et al. 1989). Furthermore, Vizi and colleagues observed that atropine, a muscarinic blocker, had no effect on the release of ACh evoked by physostigmine at the skeletal neuromuscular junction (Vizi et al. 1989). Another evidence of a crucial role of nAChR in the regulation of bladder function was demonstrated by Gallagher and colleagues. They reported that neuronal nAChRs mediate fast synaptic transmission between preganglionic and postganglionic bladder neurons yielding detrusor muscle contraction (Gallagher et al. 1982).

A recent publication of Gomez-Amaya and coworkers demonstrate the expression of α1 nicotinic receptor subunits on the smooth muscle in the mongrel hound dog urinary bladder reinnervated by somatomotor fibers. They observed that, under this experimental condition, activation of this receptor leads to a more rapid increase in bladder pressure than the activation of muscarinic receptors (Gomez-Amaya et al. 2015). Another evidence of direct participation of nicotinic receptors in detrusor contraction was published by Fincan and colleagues. They observed that nicotine significantly enhanced EFS induced contractile responses, and these responses were largely inhibited with mecamylamine (Fincan et al. 2016). Earlier, Vural and coworkers published interesting results showing that nicotine, a non-specific nAChR agonist, increased the contractile responses of the rabbit urinary bladder strips elicited by the EFS in a dose-dependent manner (Vural et al. 2007).

On the other hand, nAChR might indirectly increase the release of various neurotransmitters following nerve stimulation in different tissues (Todorov et al. 1991, Yokotani et al. 2001). Todorov and colleagues verified the presence of prejunctional nicotinic receptors on noradrenergic nerve terminals in the vas deferens of the guinea-pig. They observed that nicotinic receptor agonists promoted the release of [3H]-NA and this stimulation-evoked release was reduced by hexamethonium (Todorov et al. 1991). Vural and colleagues supposed that nicotine may increase the EFS-induced contractile response, possibly by facilitating co-neurotransmitter release from nerve terminals via activation of nAChRs in rabbit bladder. According to their experiments, neither nitric oxide nor prostaglandins are involved in this process, since neither N-nitro-L-arginine methyl ester hydrochloride nor indomethacin, a non-selective cyclooxygenase inhibitor, affected the nicotine-induced contraction. Additionally, they proposed that nicotine evokes, by acting on the neuronal nAChRs, the co-release of ACh and ATP from postsynaptic parasympathetic nerve terminals in the rabbit bladder. This proposal arose from the observation of amplification of EFS-induced contractile responses after application of nicotine in the presence of either atropine or α,β-methylene ATP. However, hexamethonium, a nicotinic blocker, did not show any effect on stimulation in the absence of nicotine, indicating that nAChRs are not involved in physiological activation of the bladder (Vural et al. 2007).

We here addressed the potential influence of nAChRs on optogenetically induced detrusor contraction in ChAT-ChR2 bladders by applying mecamylamine, a general nAChR antagonist. Our results demonstrate that mecamylamine did not affect the LED-induced contractile responses resulting from selective stimulation of cholinergic nerve fibers. Thus, the residual contraction seen after combined muscarinic and purinergic receptor blockade is not caused by ACh acting upon nAChR.

Co-release of another, non-purinergic transmitter

Another explanation for the residual contraction of LED-induced cholinergic contraction of the detrusor after cholinergic-purinergic blocker cocktail might be another cotransmitter that is released from cholinergic nerve fibers, for example, peptides (Hoyes et al. 1975). Meini and Maggi provided the first evidence for the existence of a capsaicin-sensitive component in the atropine-resistant non-adrenergic non-cholinergic contraction induced by EFS in the rat urinary bladder. This effect, however, was ascribed to tachykinin release from sensory nerve terminals (Meini and Maggi 1994) and, hence, does

not represent peptidergic cotransmission from cholinergic neurons. Benko and colleagues also observed a remaining EFS-induced contraction after atropine, suramin, and PPADS pretreatment, but this was independent from capsaicin-sensitive (i.e. peptidergic sensory) nerve endings and the transmitter background remained unclear. However, the same study revealed that capsaicin-induced contraction has myogenic, but not neurogenic origin due to the resistance of capsaicin-induced contraction to tetrodotoxin and ω-conotoxin GVIA (Benko et al. 2003). Other early studies negated the possibility of cholinergic-peptidergic cotransmission in the detrusor, due to the lack of the similarity of mimicry of neuropeptide-stimulated contraction to the fast atropine-resistant contraction of the detrusor (Mackenzie and Burnstock 1984, Callahan and Creed 1986). Collectively, there is currently no other transmitter being identified that might serve as an additional cotransmitter in cholinergic-purinergic bladder innervation.

Ectopic expression of ChR2

It finally has to be considered that ChR2 has been ectopically expressed outside cholinergic nerve fibers. Indeed, we observed occasional tdTomato expression in patches of lung alveolar cells, very rarely in skeletal muscle fibers in larynx and tongue, and, although not regularly and to a varying degree from animal to animal, in detrusor smooth muscle cells. Then, contraction might be induced by the activation of ChR2 in the smooth muscle cell membrane through LED stimulation. Park and colleagues have generated a ChR2 mouse line that expresses ChR2 in the detrusor smooth muscle. In this model, they assessed the depolarization, induced by ChR2 activation in the isolated detrusor after LED stimuli. The results show that illumination at 473 nm immediately induces an inward current in the smooth muscle cells with an initial peak followed by quick relaxation to a plateau level (Park et al. 2017). Hence, this interindividual variable and minor ectopic expression of ChR2 in smooth muscle cells in our experimental model may well explain the small residual contractile response seen after combined muscarinic and purinergic receptor blockade.

4.3 The future role of optogenetics in the understanding of the lower urinary tract

LUT possesses an essential role in storing and voiding of urine. Highly complex neural circuits involving supraspinal, spinal and peripheral mechanisms, coordinate the coaction

of smooth and striated muscle, hereby enabling the functioning process of micturition (Fowler et al. 2008). Often, storage disorder component of LUT is associated with overactive bladder, defined symptomatically as the OAB, or urodynamically as detrusor overactivity (Abrams et al. 2003). Existing pharmacological treatment might have initial good response rates but are not effective in all patients (Andersson 2016). The mechanisms of effect of the antimuscarinic therapy, that improve the symptoms, and therefore are considered to be the first-line treatment of OAB, remain undiscovered. The common opinion that antimuscarinic drugs may reduce the ability of the detrusor to contraction via the blockage of the muscarinic receptors of the efferent nerve fibers of the detrusor muscle is still under debate (Yamaguchi 2010). Further studies are needed in the future, to a better understanding of the function of peripheral neuronal circuits in the bladder sensation and contraction, before the initiation of the targeted pharmacological therapy. Our study is the first describing the successful application of optogenetics in the neuromodulation of isolated cholinergic neural circuits of postganglionic parasympathetic neurons in the detrusor smooth muscle. The application of optogenetics is a promising new field in the research of neurologic diseases and may be extended to other cell populations contributing to the regulation of micturition. Up to date, optogenetic investigations of micturition centers and the central peripheral neural circuits, controlling the lower urinary tract, are lacking. Application of the optogenetics will help to modulate the mechanisms that underlie sensory transduction of pelvic and hypogastric bladder afferents or help to understand the role of sub-urothelial sensory processing in IC and in the modulation of the spontaneous activity of the detrusor smooth muscle. Another field where optogenetic could have clinical relevance is the treatment of OAB or atone bladder instead of electrical sacral neuromodulation. By the application of different opsins in the urinary bladder, control of the contractile behavior may be achieved through the on-off switch of the LED stimuli (Parker et al. 2017). All of these will provide new opportunities leading to the development of novel treatments for bladder dysfunctions.

5 Summary

Parasympathetic nerve fibers control the contraction of the bladder musculature. Only a third of the contraction induced by their general activation through EFS is inhibited by antagonists of muscarinic ACh receptors. The remaining contraction is predominantly mediated via purinergic receptors. However, it is unclear whether purinergic ligands are released together with ACh from the same nerve fibers, or from a separate fiber population. We have clarified this question in a transgenic animal model with an optogenetic approach. Here, a light-sensitive channel, ChR2, is expressed in cholinergic neurons so that they can be selectively excited by light at a wavelength of 460 nm.

A mouse line expressing a ChR2-dtTomato fusion protein under control of the ChAT promotor was generated by crossing the strains B6N.129S6(B6)-Chat$^{tm2(cre)Lowl}$/J and B6;129-Gt(ROSA)26Sor$^{tm1(CAG-COP4*E123T*H134R,-tdTomato)Gfng/J}$. ChR2-dtTomato expression in the urinary bladder and reference organs was characterized by immunofluorescence microscopy. Detrusor contractions were measured in an organ bath in response to EFS and LED (460 nm) stimulation. As a control group, we used a ChAT-eGFP mouse strain that does not express ChR2.

Immunofluorescence microscopy revealed expression of the ChR2-dtTomato fusion protein in cholinergic (ChAT- and VAChT-positive), but not other (noradrenergic, peptidergic sensory) nerve fibers in the urinary bladder and other peripheral organs (e.g. airways and duodenum). EFS led to a frequency-dependent contraction in all strains. LED stimulation evoked a contraction of the urinary bladder only in the ChR2-expressing mouse strain so that thermal effects were excluded. Atropine completely inhibited the muscarine-induced contraction of the bladder but reduced the LED-evoked contraction of the detrusor only by 35%. The application of purinergic receptors antagonists, suramin and PPADS, reduced the LED-induced contraction of the detrusor by 60%. Even after combined cholinergic muscarinic and purinergic inhibition, approximately 20% of the LED-induced contraction remained.

For the first time, this optogenetic approach proves cholinergic-purinergic cotransmission in the detrusor by releasing at least two transmitters from the same nerve fiber. The remaining contraction after combined blockade of muscarinic cholinergic and purinergic (P2) receptors may result from a minor ectopic expression of ChR2 in the detrusor muscle or another transmitter system, which can be clarified by further investigations using this model.

6 Zusammenfassung

Parasympathische Nervenfasern steuern die Detrusorkontraktion. Eine vollständige Inhibition der cholinergen Übertragung hemmt die durch generelle Nervenstimulation (elektrische Feldstimulation) induzierte Kontraktion nur zu einem Drittel. Die verbleibende Kontraktion wird vorwiegend über purinerge Rezeptoren vermittelt. Unklar ist jedoch, ob die purinergen Liganden gemeinsam mit ACh aus den gleichen Nervenfasern freigesetzt werden, oder aus einer separaten Faserpopulation. Wir haben diese Frage in einem transgenen Tiermodell mit optogenetischem Ansatz geklärt. Hierbei wird ein lichtsensitiver Kanal, ChR2, in cholinergen Neuronen exprimiert, sodass diese selektiv durch Licht einer Wellenlänge von 460 nm erregt werden können.

Eine Mauslinie, die ein ChR2-tdTomato-Fusionsprotein unter der Kontrolle des ChAT-Promotors exprimiert, wurde durch Kreuzung der Stämme B6N.129S6(B6)-Chat$^{tm2(cre)Lowl/J}$ und B6;129-Gt(ROSA)26Sor$^{tm1(CAG-COP4*E123T*H134R,-tdTomato)Gfng/J}$ generiert. Die ChR2-dtTomato Expression wurde in der Harnblase und Referenzorganen durch (Immun)fluoreszenzmikroskopie charakterisiert. Detrusor-Kontraktionen wurden im Organbad nach EFS und Lichtstimulation (LED, 460 nm) gemessen. Als Kontrollgruppe wurde ein ChAT-eGFP-Mausstamm ohne ChR2-Expression verwendet.

Die Immunfluoreszenz zeigte eine Expression des ChR2-tdTomato-Fusionsproteins in cholinergen (ChAT- und VAChT-positiv), aber nicht anderen (nordrenergen, peptiderg-sensorischen) Nervenfasern in der Harnblase und anderen peripheren Organen (z.B. Atemwege, Duodenum). Eine elektrische Stimulation aller Fasern führte in beiden Mausstämmen zu einer frequenzabhängigen Kontraktion. Die optische Stimulation durch LED-Lichtfaser kontrahierte ausschließlich ChR2-exprimierende Blasen, so dass thermische Effekte ausgeschlossen werden konnten. Atropin inhibierte vollständig die durch Muskarinzugabe induzierte Kontraktion, führte aber nur zu einer Verminderung der LED-evozierten Kontraktion um 35 %. Die gemeinsame Zugabe von Antagonisten purinerger Rezeptoren (Suramin und PPADS) reduzierte den LED-Effekt um 60 %. Auch nach gleichzeitiger Gabe von Atropin, Suramin und PPADS verblieb ein Resteffekt von 20 %.

Dieser optogenetische Ansatz weist erstmals eine cholinerg-purinerge Kotransmission im Detrusor durch Freisetzung zumindest zweier kleinmolekularer Transmitter aus der gleichen Nervenfaser nach. Die verbleibende Kontraktion nach Blockade cholinerger muskarinischer und purinerger (P2) Rezeptoren kann durch eine geringe ektope Expression des ChR2 in einzelnen glatten Muskelfasern oder auch durch ein weiteres Transmittersystem hervorgerufen werden, was durch weiterführende Untersuchungen in diesem Modell geklärt werden kann.

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8 Declaration

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