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EXPRESSION OF NICOTINIC ACETYLCHOLINE RECEPTOR SUBUNIT MRNA IN MOUSE BLADDER AFFERENT NEURONS

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Abstract—Nicotinic acetylcholine receptors (nAChR) influence bladder afferent activity and reflex sensitivity, and have been suggested as potential targets for treating detrusor overactivity. Mechanisms may include indirect effects, e.g. involving the urothelium, and direct action on nAChR expressed by afferent neurons. Here we determined the nAChR repertoire of bladder afferent neurons by retrograde neuronal tracing and laser-assisted microdissection/reverse transcriptase polymerase chain reaction (RT–PCR), and quantified retrogradely labelled nAChRα3-subunit-expressing neurons by immunohistochemistry in nAChR α3β4α5 cluster enhanced green fluorescent protein (eGFP) reporter mice. Bladder afferents distinctly expressed mRNAs encoding for nAChR-subunits α3, α6, α7, β2–4, and weakly α4. Based upon known combinatorial patterns of subunits, this predicts the expression of at least three basically different subunits of nAChR – α3*, α6* and α7* – and of additional combinations with β-subunits and α5. Bladder afferents were of all sizes, and their majority (69%; *n* = 1367) were eGFP–nAChRα3 positive. Immunofluorescence revealed immunoreactivities to neurofilament 68 (NF68), transient receptor potential cation channel vanilloid 1 (TRPV1), substance P (SP) and calcitonin gene-related peptide (CGRP) in eGFP–nAChRα3-positive and -negative neurons. For each antigen, all possible combinations of colocalisation with eGFP–nAChRα3 were observed, with eGFP–nAChRα3-positive bladder neurons without additional immunoreactivity being most numerous, followed by triple-labelled neurons. In conclusion, more than one population of bladder afferent neurons expresses nAChR, indicating that peripheral nicotinic initiation and modulation of bladder reflexes might result, in addition to indirect effects, from the direct activation of sensory terminals. The expression of multiple nAChR subunits offers the potential of selectively addressing functional aspects and/or sensory neuron subpopulations.

Key words: bladder afferent neurons, nicotinic acetylcholine receptor, RT–PCR, retrograde neuronal tracing.

INTRODUCTION

Neural control of micturition is complex involving supraspinal pathways, spinal cord centres, and peripheral autonomic and sensory neurons (Fowler et al., 2008). Dysregulation of these circuits causes major health problems among which, numerically, overactive bladder (OAB) is one of the most prevalent, affecting several million men and women worldwide (Irwin et al., 2006). It is characterised by symptoms during the bladder-filling phase such as urgency, frequency and nocturia (Abrams et al., 2002). While its aetiology still remains not fully understood, recent evidence suggests that it involves an increased release of acetylcholine (ACh) during urine storage from neuronal and/or non-neuronal sources, directly or indirectly activating bladder sensory afferents (Andersson, 2011). In line with this concept, bladder sensory neurons express muscarinic acetylcholine receptor (MR) subtypes M2R, M3R, and M4R (Nandigama et al., 2010), intravesical administration of the MR agonist oxotremorine elicits concentration-dependent excitatory and inhibitory effects on reflex voiding and afferent activity (Kullmann et al., 2008; Matsumoto et al., 2010), and muscarinic inhibitors are first-line treatment for OAB symptoms (Andersson, 2011).

Cholinergic effects, however, are not solely mediated by MRs. Nicotinic acetylcholine receptors (nAChR) not only mediated fast synaptic transmission in bladder parasympathetic ganglia but also influence bladder afferent activity and reflex sensitivity (Masuda et al., 2006, 2009; Salas et al., 2007; Kontani et al., 2009). Mechanisms may include indirect effects such as regulating mediator release from the urothelium which expresses multiple subunits of nAChR (Beckel et al., 2006; Bschleipfer et al., 2007; Beckel and Birder, 2012), and direct action on nAChR expressed by afferent

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Abbreviations: ACh, acetylcholine; BMG, β2 microglobulin; CGRP, calcitonin gene-related peptide; DiI, 1,1′-dioctadecyl-3,3,3′, 3′-tetramethylindocarbocyanine perchlorhydrate; DRG, dorsal root ganglia; eGFP, enhanced green fluorescent protein; FB, Fast Blue; MR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; NF68, neurofilament 68; OAB, overactive bladder; PFA, paraformaldehyde; RT–PCR, reverse transcriptase polymerase chain reaction; SP, substance P; TRPV1, transient receptor potential cation channel subfamily V member 1.

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neurons. Accordingly, nAChR have also been suggested as potential targets for treating detrusor overactivity (Masuda et al., 2006). In general, subpopulations of sensory neurons do express nAChR subunits (Genzen et al., 2001; Lips et al., 2002; Haberberger et al., 2004; Mao et al., 2006), and nAChR expression is quantitatively altered in dorsal root ganglia (DRG) at segmental levels (L5-S2), where bladder afferent neurons are located, in mice subjected to experimental bladder outlet obstruction (Bschleipfer et al., 2012). In a study focusing upon the roles of nAChR in the micturition reflex in rats, first recordings of nicotine induced ionic currents in isolated bladder-specific DRG neurons and their sensitivity to the general nAChR antagonist mecamylamine have been reported (Masuda et al., 2006). Beyond that, direct proof of nAChR expression by bladder afferent neurons and in particular their subunit characterisation are lacking.

We here addressed this issue in mice by a combination of retrograde neuronal tracing, immunohistochemistry, and laser-assisted microdissection/reverse transcriptase polymerase chain reaction (RT–PCR). Neuronal nAChR are hetero- or homopentamers composed of several subunits (α2–7, α9–10 and β2–4) with different properties depending on subunits composition (Lukas et al., 1999; Wu and Lukas, 2011; Dash et al., 2012). Given that the majority of nAChR in sensory neurons contains the subunits α3 and β4 (Mao et al., 2006), we employed a BAC transgenic mouse line with enhanced green fluorescent protein (eGFP) driven by the nAChR α3β4α5 cluster [Tg(Chrna3-eGFP)] (Frahm et al., 2011) for retrograde tracing experiments to identify such bladder afferent neurons and to characterise them further by immunohistochemistry with marker antibodies for subsets of sensory neurons. We choose to subject retrogradely labelled bladder sensory neurons to laser-assisted microdissection with subsequent RT–PCR instead of nAChR immunostainings to circumvent specificity problems reported for most currently available antibodies against nAChR subunits on respective gene-deficient mice (Herber et al., 2004; Moser et al., 2007). Sensory neurons identified by retrograde labelling were further characterised for the expression of several markers, including the transient receptor potential vanilloid subtype 1 (TRPV1) as a marker of a large population of sensory neurons responding to nociceptive stimuli including those that project to projecting to the bladder (Hwang et al., 2005), calcitonin gene-related peptide (CGRP) and substance P (SP) as neuropeptides expressed by peptidergic nociceptors (Basbaum et al., 2009), and neurofilament-light (NF68) as a structural marker for fast-conducting sensory neurons (Goldstein et al., 1991; Lawson et al., 1993).

EXPERIMENTAL PROCEDURES

Animals and retrograde neuronal labelling

Experiments were performed on 8 adult C57/BL6 mice and nine eGFP–nAChRα3 transgenic mice (BAC transgenic mice with eGFP driven by the nAChR α3β4α5 cluster; (Frahm et al., 2011) kept under standard laboratory conditions. Care and handling of mice were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the experiments approved by the appropriate governmental authorities.

For labelling afferent neurons supplying the bladder, mice were anaesthetised by an intraperitoneal injection of atropine (0.05 mg/kg; Atropinsulfat B. Braun®, B. Braun Melsungen AG, Melsungen, Germany), ketamine (100 mg/kg, Ketalar®, Park Davis, Barcelona, Spain), and xylazine (15 mg/kg, Rompun®, Bayer AG, Leverkusen, Germany). The bladder was exposed by a midline incision, and 3 µl of tracer (either DiI [1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorhydrate, Molecular Probes Europe, Leiden, The Netherlands; 0.25‰ in *N*,*N*′-dimethylsulphoxide] or Fast Blue [FB; Polyscience, Inc., Warrington, PA, USA]) was injected using a Hamilton microsyringe (26-gauge needle) at different sites in the muscle wall of the bladder. Leaking dye at the injection site was removed by using a cotton swab. The incision was closed, and the mice were allowed to recover from anaesthesia.

Tissue processing

Four days after the tracer injection, mice were sacrificed by the inhalation of an overdose of isoflurane, and L6-S1 DRG were dissected. For laser-assisted microdissection, DRG from C57/Bl6 mice were mounted in OCT compound in cryomolds, snap-frozen in melting isopentane, and stored in liquid nitrogen until sectioning. For immunohistochemistry, retrogradely labelled eGFP–nAChRα3 transgenic mice were sacrificed by isoflurane inhalation and transcardially perfused with heparin-containing rinsing solution (Forssmann et al., 1977), followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Dissected DRG were additionally fixed in 4% PFA for another 2 h, rinsed repeatedly in 0.1 M phosphate buffer, and then placed overnight in 18% sucrose in 0.1 M phosphate buffer. Thereafter, tissues were mounted in cryomolds in OCT compound, frozen in liquid nitrogen, and stored at −20 °C until sectioning. For RT–PCR analysis of whole ganglia and organs, DRG at levels L6-S1, brain and lymph nodes, the latter two serving as positive controls, were dissected and treated with lysis buffer (RLT-buffer; Qiagen, Hilden, Germany).

Laser-assisted microdissection

These experiments were performed on DRG from C57/BL6 mice subjected to retrograde labelling with DiI. This fluorophore was chosen since our microscope set-up for laser-assisted microdissection is not equipped with a filter set for FB detection. Laser-assisted microdissection was performed as described in our previous studies (Nandigama et al., 2010). Ten-µm thick DRG cryosections were mounted on 1-mm polyethylene naphthalate membrane-covered slides (Catalogue No. 1440–1000; PALM Microlaser Technologies GmbH, Bernried, Germany). Retrogradely labelled DiI-positive cell bodies were identified in epifluorescence illumination. Thirty cell profiles of DiI-positive as well as DiI-negative cell bodies were harvested by laser microdissection and pressure-catapulting technology (PALM Microlaser Technologies). Dissected DiI-negative cell bodies served as controls. Dissected cell bodies catapulted into the cap of a microfuge tube were treated with lysis buffer (RLT-buffer; Qiagen, Hilden, Germany). All the samples were immediately frozen in liquid nitrogen.

RNA extraction and RT–PCR

Whole L6-S1 DRG, brain and lymph nodes were homogenised by using a ball mill (Mixer Mill MM300; Retsch GmbH, Haan, Germany) and total RNA was isolated using the Qiagen