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REVIEW ARTICLE

Pharmacological methods for the preclinical assessment of therapeutics for OAB: an up-to-date review

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Abstract
Introduction Licenced oral pharmacotherapies for overactive bladder (OAB) act on muscarinic receptors or β_3-adrenoceptors. The search for new drugs to treat OAB that have novel mechanisms of action is very active, with the aim of discovering more effective and/or better tolerated agents.
Methods A literature review of the most frequently used pharmacological methods for the preclinical assessment of new agents aimed at treating OAB, such as isolated organ technique, electrophysiological techniques, radioligand binding assay, and animal models, was carried out. Novel potential developments based on recent knowledge of urothelial and neural mechanisms are also discussed.
Results The isolated organ technique, electrophysiological techniques, and the radioligand binding assay are very effective methods for the demonstration that a novel pharmacological target with a specific and high affinity binding site for a new drug is present in the bladder and its modulation regulates functions critical for the pathophysiology of OAB. Afterward, the new drug should be shown to be effective in animal models of OAB, although the translational value of these models is limited by a poor pathophysiological relationship with human OAB. Exciting novel perspectives focusing in particular on the theory of the mucosal–bladder network have recently opened new paths in the discovery and assessment of new therapeutics in this field.
Conclusions Available experimental models still play a central role in the appraisal of OAB therapeutics; however, their shortcomings and the paucity of very effective drugs indicate the need for new models that better reproduce the pathophysiological features of OAB. Some emerging lines of research show promise. A change of perspective in the future evaluation of putative drugs is required, especially in the light of the latest knowledge on the key role of the mucosal–bladder network and the brain–bladder neural pathways.

Keywords Overactive bladder syndrome · Isolated organ technique · Extracellular recordings · Patch clamp · Radioligand binding assay · Animal models

Abbreviations

BOO	Bladder outlet obstruction
CNS	Central nervous system
DO	Detrusor overactivity
EAE	Experimental autoimmune encephalomyelitis
DM	Diabetes mellitus
EFS	Electrical field stimulation
ICs	Interstitial cells
K_V7 channels	Voltage-dependent type 7 K^+ channels
LUT	Lower urinary tract
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NGF	Nerve growth factor
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide

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NVC	Non-voiding contractions
OAB	Overactive bladder
SHR	Spontaneous hypertensive rat
TTX	Tetrodotoxin
WKY	Wistar–Kyoto rats

Introduction

Overactive bladder syndrome (OAB) is a highly prevalent clinical condition defined as "urinary urgency, usually accompanied by frequency and nocturia, with or without urgency urinary incontinence, in the absence of urinary tract infection or other obvious pathology" [1, 2]. OAB is often, but not necessarily, associated with detrusor overactivity (DO), characterised by involuntary detrusor contractions during bladder filling in humans [3]. There is general agreement that the pathophysiology is multifactorial and four theories, myogenic, neurogenic, urotheliogenic and integrative have been put forwards [4, 5]. It has been hypothesised that the main alterations underlying OAB are DO, increased discharge of afferent fibres and/or abnormal afferent signal processing in the central nervous system (CNS). In turn, the increased activity of afferent fibres may be of myogenic origin, resulting from the coordination of the physiological detrusor "micromotions" (autonomous microcontractions stimulating the afferent activity during the filling phase), or be caused by mediator release from the urothelium or suburothelial interstitial cells (ICs) or afferent sensitisation induced by neurotrophins or other mediators [6]. On the other hand, the myogenic theory hypothesises that partial detrusor denervation causes smooth muscle supersensitivity and enhanced propagation of contractile signals, resulting in coordinated spontaneous detrusor contractions [5, 7]. Most of the successful pharmacotherapeutic ways of managing OAB act by inhibiting both the afferent output from the bladder and the efferent drive to the bladder, probably predominantly the latter [6]. Consequently, functional studies, mainly carried out using the in vitro isolated organ technique and electrophysiological extracellular recordings, have probably been the most significant so far for predicting clinically relevant molecules. The results of these studies were then validated in animal models of OAB, by investigating through cystometry the drug effects on DO, a surrogate marker, as OAB is diagnosed based on symptoms and consequently no animal model can reproduce the human disease.

Because of the absence of a real "cure" for OAB and several limitations of the available treatments, there has been a persistent need for new pharmacological therapies. The purposes of this paper are to carry out an updated review of classical pharmacological methods used for the preclinical assessment of novel therapeutics aimed at treating OAB patients, and to give an overview of future methodological developments in the light of new pathophysiological hypotheses.

Materials and methods

A systematic literature search of full, peer-reviewed, English-language papers published up to October 2015 was performed on MEDLINE employing both "MESH" and "free text" protocols and using the following search terms: pharmacological method, isolated organ technique, extracellular recording, patch clamp, radioligand binding assay, animal model AND overactive bladder OR urinary incontinence.

Results

The in vitro isolated organ technique

The isolated organ technique allows the primary pharmacological features of investigational drugs to be determined both qualitatively and quantitatively. Animal tissues are taken from laboratory animals that are healthy or that have been surgically or pharmacologically pre-treated, or that have particular genetic characteristics. Human tissues are generally by-products of surgical operations.

In vitro research with isolated organs can be carried out on organs in toto or more often on strips, segments or flat sheets obtained from them. These different types of preparations are generally incubated in appropriate physiological solutions; organs in toto may also be perfused through their arterial beds.

The organs containing smooth muscle are those classically studied with this technique, but in addition to smooth muscle contractility, other functions, such as electrophysiological parameters or mediator release can be studied.

The basic requirement in the experimentation with isolated organs is to keep the tissues in a physiological state comparable with that in vivo. It is important to minimise the time that elapses between the sacrifice of the animal and the removal of the organ or between the surgical resection and the cutting of the human tissue specimen. Then, the organ or the tissue specimen should be immediately placed in an appropriate saline solution. Pioneering studies by Tyrode [8], Ringer [9] and Krebs and Henseleit [10] established the ionic compositions of these solutions. The Krebs solution is the most satisfactory for most mammalian preparations and that most frequently used. Typically, the preparations are made as soon as possible after the explant. Then, they are immersed in a fixed volume (generally between 2 and 20 ml) of the solution and contained in organ baths usually made of borosilicate glass (Fig. 1).

Smooth muscle contractility can be defined as the intrinsic ability of muscle cells to reduce their length or generate tension without changing length, although there is no commonly

agreed definition of detrusor muscle measurements. They can be studied in response to drugs or the stimulation of intramural neurons, usually using electrical field stimulation (EFS), performed by placing the preparations between parallel plates or inside ring electrodes (Fig. 1). Different detection methods of smooth muscle contractility may affect the response. The most frequently used are the isometric method, by which variations in tension of the preparation maintained at a constant length are measured, and the isotonic method, in which the tissue varies in length under a constant load (Fig. 1). Usually, the contractions, either length reductions or tension increases, are measured as peak amplitudes and expressed as percentages of a maximal contraction, to normalise data. If contractions induced by various experimental drugs have significantly different durations, then they are better measured as both peak amplitudes and areas under the curves.

In vitro detrusor contractility is studied more frequently by using muscle strips that can be obtained from animals or humans. Rodents are more often used, in particular rats and guinea-pigs. A detailed description of the bladder explant procedure and strip preparation from animals can be found in a previous review [11]. Bladder preparations with or without mucosa can be set up to investigate the influences of mucosal factors on smooth muscle contractility. When human detrusor is studied, the mucosal and serosal layers are generally removed to reduce strip thickness. Strips are mounted in organ baths and then subjected to an initial period of equilibration to the new conditions, usually lasting at least 1 h. When isometric conditions are adopted, the strips are stretched to 0.5–2 g (4.9–19.6 mN) at the beginning of the experiment and then periodically during the 60-min equilibration period. Initially, some researchers prefer to find the optimal tension to which each strip stretches by constructing length–tension curves [12]. Under isotonic conditions, detrusor strips passively relax under the load during the equilibration period. At the end of this period, the strips are generally stimulated to test smooth muscle and/or neuron viability. High K^+ concentrations are frequently used to initially evaluate smooth muscle contractility. They induce smooth muscle membrane depolarisation and consequent voltage-dependent L-type Ca^{2+} ($Ca_V1.2$) channel activation, Ca^{2+} influx and smooth muscle contractions. The latter can also be induced by

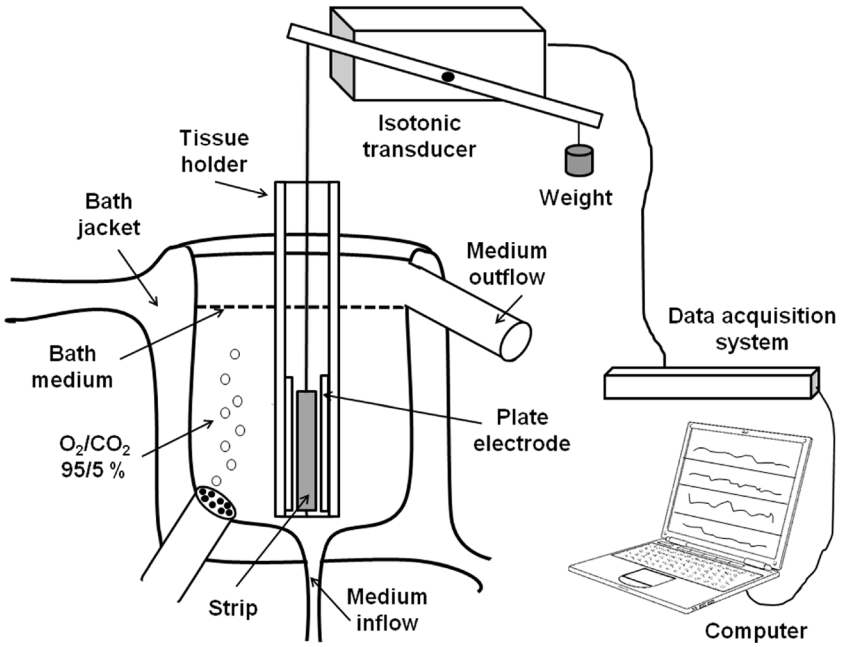

Fig. 1 Schematic diagram illustrating an isolated organ bath. Generally, the tissue is fixed at one end to a holder, mounted between parallel plate electrodes and attached at the other end to a transducer that converts changes in tension (isometric transducer) or length (isotonic transducer, under a constant weight; shown here in electric signals). The design of the organ bath should meet the following requirements: 1) a drug that is added to the bath medium does not have to spread out of it through the bath inlet; 2) the drug can be removed from the bath, after producing its effect, as efflux from an exit at the top rather than emptying and filling the bath; 3) the tissue must be kept in a constant environment (temperature, pH and O_2). The temperature can be maintained at a constant, typically 37 ° C, by immersion of the organ baths in a thermostatically controlled water bath or by the use of organ baths equipped with an external jacket (shown here), through which thermostated water is circulated. The solution that periodically replaces the incubation medium is preheated by means of a helix external or internal to the bath itself (not shown here). The degree of oxygenation is important to the viability of the tissue and often for the level of tone and spontaneous activity. The Krebs solution should be bubbled with a mixture of O_2/CO_2 95/5 % because otherwise the calcium carbonate precipitates. CO_2 is also fundamental because it lowers the pH of the Krebs solution at the physiological level of 7.4. Smooth muscle contractility is recorded on a computer using a data acquisition system

activating muscarinic receptors with, for instance, bethanechol or carbachol. When EFS is used, parameters are chosen so that neurons are selectively activated. EFS can be carried out at a constant voltage or current amplitude and pulse duration. Two main options are used: to stimulate at high voltage with a short pulse duration or at a submaximal current amplitude with a longer pulse duration. We use rectangular and bipolar pulses of constant current amplitude (submaximal: 120 mA) and pulse duration (1 ms). With these pulse parameters, smooth muscle cells are not activated and thus responses are nerve-mediated. EFS train duration can be very short to induce phasic responses at fixed time intervals or long to obtain tonic responses. With our EFS parameters, long train duration (2 min) EFS induces frequency-dependent human detrusor contractions in the frequency range of 0.125–16 Hz, which is the frequency range that can be generally used in experiments with animal detrusors [11]. The contractions of the human detrusor induced by the activation of postganglionic parasympathetic neurons are almost totally cholinergic [13, 14]. On the contrary, they have two components in a number of other species, particularly in rodents: a purinergic component [15], and a cholinergic component [11]. The neural nature of EFS-induced contractions can be demonstrated by abolishing nerve conduction through voltage-dependent Na^+ (Na_V) channel blockers [13, 14]. The most frequently used is tetrodotoxin (TTX). Similar contractions can be induced by directly activating the muscarinic receptors by receptor agonists. Under our conditions, detrusor contractions produced by EFS and bethanechol have similar maximal amplitudes, indicating that the maximal acetylcholine release induced by EFS can produce maximal muscarinic receptor activation. In addition, detrusor relaxations can be studied in vitro after precontracting the muscle strips with muscarinic agonists. By using this experimental setting, we have shown that blockers and activators of voltage-dependent type 7 K^+ (K_V7) channels contract or relax human detrusor strips respectively by acting at the muscular level [16].

Detrusor contractility can be also studied in vitro by using the whole bladder. In a guinea-pig model [16], the intravesical pressure is measured through a urethral catheter after filling the bladder with a baseline volume of 0.8–1.2 ml. A video imaging system is also set up to record bladder wall movements and to perform a multiple-point motion analysis [17]. A similar model has been described in the mouse [18]. In a more recent pig model [19], the bladder is perfused through an aortic catheter at a constant flow and maintained at a physiological temperature in a special double-jacketed organ bath. The intravesical pressure is recorded under isovolumetric conditions by a catheter inserted through the left ureter, whereas the right ureter is tied off. For image recording, markers (carbon particles) are placed on the bladder surface that allow the analysis of movements of the wall by appropriate software [19].

Electrophysiological techniques

Electrophysiological techniques are used to record afferent fibre activity and to study the role of ion channels in the regulation of smooth muscle contractility and neuronal, urothelial and IC function. Afferent activity is usually studied using extracellular recordings. The function of ion channels can be investigated in various cell types within the intact bladder wall or in cells isolated or cultured from the bladder by different recording methods, the most important of which are the techniques using sharp microelectrodes and the patch clamp. Sharp microelectrodes can be used to measure the membrane potential of single smooth muscle cells in intact pieces of bladder wall and constitute a method of studying the junction and action potentials in the detrusor [20–23].

Extracellular recordings

Afferent signals travel through Aδ and C fibres in the pudendal, hypogastric (sympathetic) and pelvic (parasympathetic) nerves and are transmitted to and processed by the spinal and supraspinal centres [24, 25]. Extracellular recording of afferent neuron activity can be carried out in vivo in anaesthetised animals [26] or in vitro, mainly on flat sheet preparations [27, 28]. In vivo recordings of pelvic nerve afferents are usually made at the dorsal root, whereas those of the hypogastric nerve are made at the nerve trunk [26]. The afferents coming from the bladder are generally identified by electrically stimulating the nerves at sites close to the bladder, or by bladder distension. This experimental setup allows the conduction velocity to be measured and thus the fibres to be classified as slow conducting (C fibres) or fast conducting (Aδ fibres). In vitro flat sheet preparations are obtained by opening the bladder longitudinally and pinning it down with the urothelium side up in a two-chamber organ bath. Afferent activity in response to mechanical stimuli, such as mucosal distortion, pressure, wall stretch or tension, or various chemicals, can be recorded by placing extracellular electrodes on thin nerve bundles close to the bladder [27] or in the hypogastric or pelvic nerve trunks [28]. Mucosal distortion and pressure are obtained by urothelial stroking or blunt probing respectively, with calibrated Von Frey nylon monofilaments at different force and duration values. These studies allowed the afferent neurons to be classified on the basis of their activation pattern and end location. Stretch-sensitive afferents that also respond to wall tension increases can be divided into two types, muscle and muscle–mucosal mechanoreceptors. Stretch-insensitive afferents are divided into three types, mucosal high- or low-responding and serosal mechanoreceptors. Muscle–mucosal and mucosal high-responding mechanoreceptors are chemosensitive too and the most probable afferents activated by mediators released by urothelial cells (urotheliogenic hypothesis) [29, 30]. On the other hand,

muscle mechanoreceptors are the most probable afferents stimulated by the active contractions originated by the synchronisation of the physiological "micromotions" in the overactive detrusor (integrative hypothesis) [31]. The in vivo measurement of the effects of an investigational drug on both single-fibre activities of the primary bladder afferent nerves and the number and amplitude of bladder micromotions, during constant filling cystometry and under isovolumetric condition, allow the relationships between micromotions and afferent activities to be evaluated in response to drug administration [32].

Patch clamp

The patch clamp technique involves electrical recordings from single ion channels or from the whole assembly of ion channels expressed in whole cells by recording the electrical currents carried by the ions. The currents flowing through single channels are to the order of 10^{-12} A (pA), corresponding to 10^{6}-10^{7} ions per second passing through each open channel [33].

Recording from single channels allows characterisation in terms of their unitary conductance, detailed analysis of the kinetics and the effects of drugs on them. This has been achieved by placing micropipettes on the cell surface cleaned enzymatically and measuring the current flow through the pipette. The need for a clean surface implies that this method can be applied mainly to isolated or cultured cells. Micropipettes have openings rather wider than conventional microelectrodes (about 0.5–1 μm) and, in contrast to the latter, are filled with normal saline. The greatest technical difficulty has been to establish an electric seal between the micropipette and the membrane. This problem has been solved by the observation that the electric seal between the pipette and the membrane can be improved by a factor of 100–1,000 when, once the contact has occurred, a slight suction is applied to the pipette. The electrical resistance across the seal is to the order of 10–100 gigaohms (GΩ) [34].

Starting from the configuration described above of the "cell-attached" pipette, other well-defined configurations may be obtained by simple mechanical manipulations that do not damage the seal (Fig. 2). The "cell-attached" pipette configuration has a less disturbing effect on the structure and environment of the cell membrane and voltage-dependent channels and ionotropic receptors can be studied in their normal ionic environment. As the pipette solution is in contact with only a small area of the membrane, ionic gradients can be established through it that would be harmful if the whole cell was exposed to them, and the interactions between receptors and ion channels located in different regions of the cell membrane can be investigated. The "patch clamp" technique is suited to the study of such interactions by applying receptor agonists in the bath and recording the flow of ions that pass through the area of the isolated membrane.

The "inside-out" configuration has been mainly used to study ion channels activated by Ca^{2+} or modulated by intracellular mediators. This is because in many preparations high-conductance Ca^{2+}-dependent K^{+} ($K_{Ca}1.1$ or BK) channels are activated in this configuration, unless the Ca^{2+} concentrations in the bath solution are kept low.

The "outside-out" configuration is particularly used to investigate the ion channels controlled by membrane receptors. The bath solution can be easily changed, allowing the analysis of the effects of different neurotransmitters or ions. The channels most studied with this method are those associated with nicotinic receptors, GABA and glycine.

In the "whole-cell" configuration, a conductance pathway with a very low resistance, is created between the cell and the pipette. When this configuration is established on a cell with a large diameter, it can be used to measure the membrane potential in the conventional manner (current–clamp conditions). When it is applied to cells with a small diameter, it provides the conditions to bring the membrane potential at the level of the pipette potential (voltage–clamp conditions) and to perform the dialysis of the diffusible internal contents of the cell. Kinetics and amplitudes of ionic currents can be evaluated with accuracy. Guinea-pig urinary bladder smooth muscle cells show a membrane voltage so homogeneous along their major axis under voltage–clamp conditions that currents as large as 1 nA can be precisely measured [35]. An inevitable consequence of the "whole-cell" configuration is the loss of ions, nucleotides and other diffusible constituents from the cytoplasm to the interior of the pipette [36]. The consequences are the alterations in receptor–channel coupling and in the features of some currents. Several techniques have been devised to achieve the electrical continuity between the cytoplasm and the pipette, at the same time minimising the dialysis. One of these techniques is the addition of the antifungal agent nystatin or the antibiotic mixture gramicidin to the pipette solution after the seal has been formed [37]. Nystatin and gramicidin spontaneously form channels selective for monovalent ions inside the membrane, and then lower the electrical resistance of the membrane patch to such a point that the cell can be brought to the pipette potential and thus currents flowing through the membrane can be measured. This configuration, referred to as "perforated patch", has the great advantage that the mechanisms regulated by second messengers in the cell remain unaffected during recording. On the other hand, an advantage of the "whole-cell" configuration is the possibility of modifying the internal solution of the cell by exchanging the contents of the cell and pipette [35]. This allows individual ionic currents to be effectively separated and the intracellular concentrations of second messengers to be changed, to study their effects on channels [38] or to incorporate into

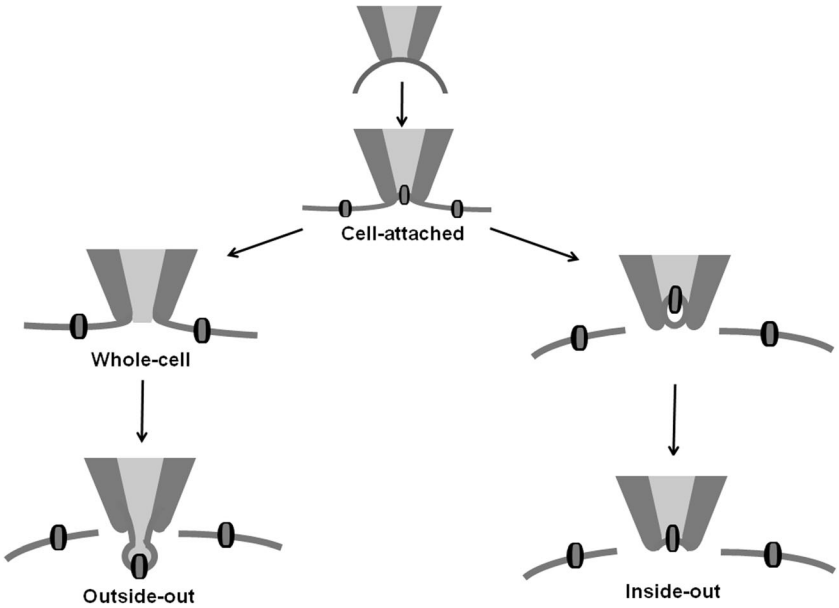

Fig. 2 Diagram showing the different configurations of the patch clamp technique. Starting from the "cell-attached" configuration, the breaking of the membrane by means of a short pulse of strong suction or voltage establishes an electrical connection between the pipette and the cell interior (*top left*). The cell–pipette complex is well insulated from the bath medium (R > 10 GΩ). This configuration, called "whole-cell", is very similar to a conventional membrane penetration with a microelectrode, but is more sensitive. Pulling away the pipette from the "cell-attached" configuration, an intermediate stage is produced in which a closed vesicle is sealed inside the pipette tip. The vesicle is not suitable for electrical measurements; however, brief air exposure breaks the outer half, giving rise to the configuration called "inside-out" (*bottom right*). This name has been given because the cytoplasmic surface of the membrane ("*inside*") is not in contact with the solution contained in the pipette, but with the external solution ("*our*") contained in the bath. The complementary configuration, called "outside-out", can be obtained when the pipette is withdrawn from the "whole-cell" configuration (*bottom left*)

the cell fluorescent probes to investigate intracellular transduction pathways [39].

The radioligand binding assay

The radioligand binding technique aims to evaluate the binding of a ligand to its recognition site on a pharmacological target and are independent of the determination of the subsequent stages. The radioligand binding assay allows us to establish whether a certain tissue or cell possesses pharmacological targets with recognition sites specific for the ligand and to determine the binding characteristics, such as the constant of affinity, receptor density and kinetic constants of the reaction [40].

This technique requires the use of radiolabelled molecules. The isotopes most commonly used for this purpose are the ^{3}H and ^{125}I. The radiolabelled ligand must have high affinity, which is the basis of the sensibility of the assay, high receptor selectivity and high specific activity, which represents the amount of radioactivity per molar unit of the compound [41, 42].

The preparation is more frequently made using tissue suspended in appropriate buffer solutions through successive stages of homogenisation and centrifugation. The various steps have the purpose of purifying the preparation, freeing the homogenate from endogenous substances and enzymes that may interfere. The most commonly used buffer solutions are the Na^{+}-K^{+} phosphate (inorganic) and Tris (tris-hydroxymethyl-aminomethane) (organic) buffers. Particular attention must be paid to the presence of ions, which can change the affinity of the ligands, and the pH value, which is typically maintained between 7 and 8 [41, 42].

The fundamental principle of the technique of binding assay is to incubate the tissue homogenate suitably diluted with a radioactive ligand (Fig. 3). The binding reaction is terminated by a procedure to separate the free ligand from the ligand–receptor complex, such as filtration or centrifugation. At the end of the separation, the filter or the pellets will contain the ligand–receptor complex (Fig. 3).

During incubation, the formation of the ligand–receptor complex takes place in accordance with the law of mass action. With [L] indicating the concentration of the ligand, [R] the concentration of the receptor and [LR] the concentration of the ligand–receptor complex, the reaction can be represented as follows: $[L] + [R]\,K_1 \leftrightarrow K_2[LR]$, with K1 and K2 the constants of association and dissociation respectively. After a few minutes to half an hour, this reaction reaches the equilibrium phase or steady state, depending on K_1 and K_2, at which the specific binding is maximum.

There are three main types of experiments in binding assays. In saturation experiments (Fig. 3), starting from the concept that a tissue preparation contains a defined number of

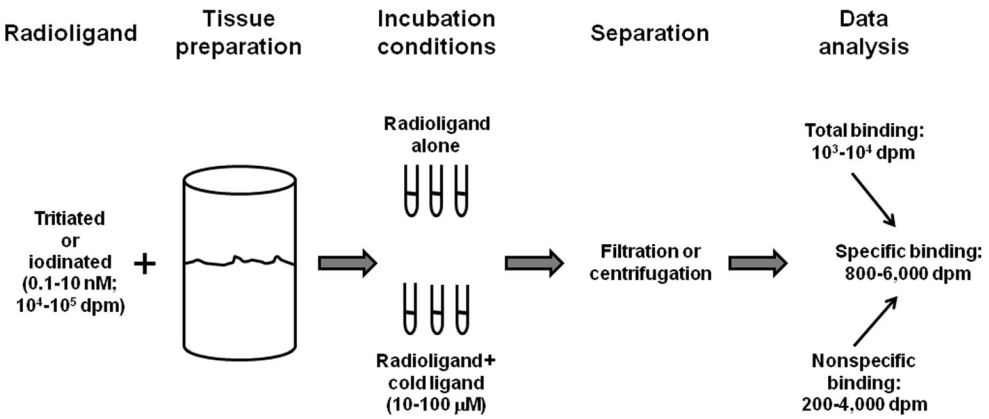

Fig. 3 Schematic workflow representing the conducting of a radioligand binding saturation experiment. Increasing concentrations of a radiolabelled ligand and a tissue preparation, more frequently a purified tissue homogenate, are made. Then, a fixed amount of tissue preparation is incubated with each concentration of radiolabelled ligand, in the absence or presence of an excess of unlabelled ligand that allows the assessment of the radioactivity bound non-specifically and, in contrast to the total binding, allows the specific binding to be obtained. The incubation is performed at an optimal temperature and for a correct time, which must be evaluated from time to time by means of kinetic studies on the reaction of binding. At the end of the incubation, the free and the bound ligands are separated by filtration or centrifugation and counted in a β- or γ-counter, depending on the radioisotope (for ^{3}H or ^{125}I respectively). The specific binding is calculated from the difference between the total and the nonspecific binding and is plotted on the y-axis with the corresponding total ligand concentration on the x-axis. The numbers are only examples. *dpm* disintegrations per minute

receptors, increasing the concentration of the labelled ligand, the specific binding will be saturated. Thus, [LR], i.e. the complex formed by the radiolabelled ligand (L) bound to the receptor (R), which gives the bound radioactivity (B), is determined at the time of the equilibrium of the reaction and a rectangular hyperbola is obtained on a graph with the concentration of the total radioligand on the x-axis and the bound radioactivity (B) on the y-axis. In kinetic experiments, [LR] is determined as a function of time. The actual speed with which the complex [LR] is formed, is given by the difference between the rate of formation of [LR] from [R] and [L] and the rate of dissociation of [LR] into [L] and [R], which can be expressed by the following equation: $d[RL]/dt = K_1 x[L]x[R] - K_2 x[RL]$. Upon reaching equilibrium, the rate of formation of RL is null, i.e. $d[LR]/dt = 0$. The determination of the constants of association and dissociation rates occurs through mathematical processing of the data obtained by measuring the binding at various times during incubation.

In inhibition experiments, the binding assay has the aim of assessing the ability of different drugs to compete with the labelled ligand for the binding site, i.e. [LR] is determined in relation to the increase in the concentration of an unlabelled ligand. It is useful to determine the IC_{50}, defined as the drug concentration that inhibits the specific binding of the labelled ligand by 50 %, by building a semi-logarithmic dose–response curve, which can also be transformed through various mathematical procedures [40].

Animal models

The assessment of new compounds in animal models usually aims to prove, using the in vivo cystometry technique, their ability to dose-dependently decrease the resting intravesical pressure and the frequency/amplitude of non-voiding bladder contractions (NVCs), increase bladder capacity, intervoid interval, and volume voided per micturition, without affecting the micturition pressure, threshold pressure and residual volume [43]. NVCs are increases in intravesical pressure during cystometry that are not associated with the release of fluid [43]. They are an index of abnormal response in bladder storage similar to that observed in human DO; this latter is defined as "the occurrence of involuntary detrusor contractions during filling cystometry" [1]. However, non-voiding intravesical pressure increases are a normal feature of the filling phase in some animal species and the features (such as amplitude or frequency) that they must have to qualify as NVCs are not defined [43]; thus, many experimental reports are subjective in distinguishing the true presence of "overactivity". In addition, whether or not this "overactivity" in animals is involuntary obviously cannot be ascertained [43]. Other outcomes are represented by the reduced collagen deposition in the bladder wall, the inhibition of bladder micromotions and mechanosensitive bladder afferent activity.

The model of bladder outlet obstruction (BOO) has been designed to reproduce the pathophysiological mechanisms that develop in patients with BOO due to benign prostatic hyperplasia. Detrusor hypertrophy follows, to overcome the obstacle to the flow and eventually DO often develops [44]. The obstruction is generally obtained by placing a ring or a ligature around the urethra. This latter can be loose, so that the obstruction becomes increasingly larger as the animal grows up. This model has been tested in the mouse [45], rat [46], guinea-pig [47], rabbit [48] and pig [49].

The ischaemic model consists of the generation of bladder ischaemia by balloon endothelial injury of the iliac arteries in the rabbit [50] and rat [51]. It has been shown that a moderate bladder ischaemia, defined as a 40 to 60 % decrease in bladder blood flow, induces DO and increases in vitro contractions in response to muscarinic receptor activation, whereas severe bladder ischaemia, defined as a greater than 60 % decrease in bladder blood flow, produces impaired detrusor contractility [50].

The OAB/DO models produced by injuries or experimentally induced disorders of the CNS include those resulting from bladder denervation, spinal cord injury or transection, or cerebral infarction, and the experimentally induced models of Parkinson's disease and multiple sclerosis. Bladder denervation can be obtained using sacral rhizotomy in the rat [52] or cat [53], or ablation of the pelvic ganglia in the rat [54], producing parasympathetic pre- or postganglionic denervation respectively. Spinal cord injury or transection induces DO and detrusor sphincter dyssynergia primarily by hyperreflexia in humans [55] and in animal models (mainly the rat and the cat) [56]. Hyperreflexia results in part from the abolition of the descending inhibitory control from higher centres and mainly from nerve plasticity, with nociceptive C-fibres assuming an important functional role in the afferent limb of the highly active spinal micturition reflex pathways that underlies DO [25, 57]. Indeed, desensitisation of C-fibres has a therapeutic effect on two-thirds of patients with a spinal cord lesion and detrusor hyperreflexia [58]. Some CNS diseases are associated with neurogenic DO, in particular stroke, Parkinson's disease and multiple sclerosis [59–61]. A commonly used animal model of stroke is that produced in the rat by the occlusion of the middle cerebral artery, causing infarction in the ipsilateral frontoparietal cortex and putamen, which is important for micturition control [62]. In this model, DO develops, with a reduction in bladder capacity and an increase in urination frequency [63, 64]. Neurogenic DO, urgency and frequency are observed in 45–93 %, 33–54 % and 16–36 % of patients affected by Parkinson's disease respectively [60]. The most frequently used experimental models of Parkinson's disease are those obtained by systemic treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine injection into the substantia nigra pars compacta [65]. Monkeys treated with MPTP develop DO [66], which is alleviated by dopamine D_1 receptor agonists [67] or the concurrent activation of dopamine D_1 and D_2 receptors [68]. DO is detected in approximately 60 % of patients with multiple sclerosis [61]. The most common animal model of multiple sclerosis is experimental autoimmune encephalomyelitis (EAE) produced by active immunisation with either myelin-derived proteins or peptides in adjuvant or by passive transfer of activated myelin-specific CD4+ T lymphocytes [69].

Chemical agents can be instilled in the bladder to induce inflammation and/or hypersensibility. The most frequently used substances are acetic acid and citric acid. It is thought that these acids might activate afferent C-fibres, thus creating a condition of increased sensory activity, similar to that thought to occur in the OAB [70]. These treatments reduce bladder capacity and compliance and increase bladder motility. Much less inflammation and urothelial damage is associated with C-fibre activation with citric acid compared with acetic acid. This evidence may be important in making the model more similar to the human pathological condition, in which no inflammation is detectable at the histopathological examination [71]. Other substances used with this aim are capsaicin, hydrochloric acid, protamine sulphate, turpentine and xylene [72].

Animals with induced or genetic metabolic abnormalities are other models of OAB/DO. Hyperlipidaemia is induced by feeding the rats with a high fat content diet, such as 1 % cholesterol for 8 weeks [73]. The animals show shorter voiding intervals and smaller functional bladder volumes on cystometry and a higher tension amplitude of the spontaneous contractions of bladder strips with regard to the control group [70]. DO and smooth muscle hypertrophy have been shown in another study [74]. Atherosclerosis with consequent ischaemia and the increase in purinergic transmission are putative pathophysiological mechanisms [72].

Bladder dysfunction is frequently found in patients with diabetes mellitus (DM) [75] and the OAB is diagnosed in approximately 35 % of type 2 DM patients [76]. Various rodent models of type 1 or 2 DM are available [77]. The most frequently used type 1 DM model is that induced by streptozotocin, a drug producing pancreatic β-cell destruction. Mice or rats treated with streptozotocin develop OAB during an early phase (<9 weeks) and atonic bladder in a late phase (>12 weeks) [77]. Many monogenic or polygenic models of type 2 DM are also available, including Zucker diabetic fatty rats and mice with homozygous deficiency of leptin or its receptor [77].

Animal models of storage dysfunctions obtained by genetic modifications, mainly knockout models, have been developed in recent years. The most important are the knockout mice for neuronal nitric oxide synthase (nNOS) [78, 79], $K_{Ca}1.1$ (BK) channels [80] and uroplakin II or IIIa [81, 82]. Knockout mice for nNOS show hypertrophic and dilated bladders, probably because of impaired urinary outlets, and increased urinary frequency [78]. However, these findings are only in part consistent with those of another study, in which bladder capacity was greater in nNOS knockout mice compared with wild-type mice, but no differences in voiding and motor responses of bladder strips were observed between the two groups [79]. In BK knockout mice increased spontaneous and evoked muscle contractions and urination frequency were observed [81]. Uroplakins are particles produced by the urothelial cells,

existing in four major isoforms, that assemble in heterodimers. These latter form the plaques covering the urothelium that are the basis of urothelial barrier function. Continuous cystometry in conscious, freely moving mice has revealed NVCs and an increase in mean spontaneous activity, indicative of DO, in uroplakin II and uroplakin IIIa knockout mice, and increases in intermicturition and micturition pressures in uroplakin II knockout mice relative to wild-type mice [82].

Alterations in bladder function have also been observed in the spontaneous hypertensive rat (SHR), a genetic model of hypertension. SHRs show a voiding frequency higher than their control rats, the Wistar Kyoto (WKY) rats [83]. The hyperactive voiding is associated in SHR rats with an increased adrenergic innervation and elevated nerve growth factor production by smooth muscle cells compared with WKY rats [83]. In vivo continuous cystometric analysis showed that SHRs have a lower bladder capacity and micturition volume and increased amplitude of NVCs compared with WKY rats [83]. In addition, reduced adrenergic inhibitory control over bladder strip contractions induced by nerve stimulation has been observed in SHRs compared with WKY rats [84]. However, central mechanisms also seem to be responsible for bladder overactivity in SHRs, inasmuch as the intrathecal administration of doxazosin was able to reduce the amplitude of NVCs [84].

Discussion and future directions

The classical pharmacological methods continue to be of utmost importance in the preclinical appraisal of novel compounds, but the pros and cons of these methods need to be well known by researchers.

The isolated organ technique has several advantages:

1. It is the most complex system in which it is possible to accurately control the concentration of the drug at the level of the receptor compartment that allows us to calculate the pharmacodynamic parameters of the drug itself.
2. It is the simplest unit capable of giving rise to responses comparable to those that occur in vivo.
3. It allows the detection of the primary response induced by a drug that would be very difficult to isolate in in vivo experiments.

However, it does also have some limitations, such as:

1. Differences compared with the in vivo responses due to the absence of hormonal influences, nerve reflex responses and other pathophysiological factors, or, because the drugs can induce in vivo effects in other tissues, either in the same organ (e.g. afferent nerves) or at distant sites (e.g. CNS), that affect the primary response.
2. Omission of adverse effects due to drug actions at other sites in vivo.
3. OAB has multiple contributory pathogenetic mechanisms and some of them may lie outside the bladder [4, 5]

Electrophysiological techniques may give precious information in the discovery process of OAB therapeutics; however, they have similar limitations to those described above.

The obvious limitation of radioligand binding techniques is that they cannot provide any functional information on the drug–target interaction. However, when functional studies are also carried out, it is possible to compare the parameter values of the functional and biochemical drug–receptor interactions (e.g. EC_{50} and K_D), so that some significant aspects of the target (e.g. the existence of spare receptors) can be assessed.

Although studies carried out on animal models are a necessary step in validating pathophysiological hypotheses and in testing new treatments before proceeding with human studies, their translational value could be questioned. In fact, although human OAB has a symptom-based definition [1], it is necessary to use urodynamic (e.g. DO) or behavioural outcomes as surrogate markers in animals. Indeed, based on the clinical definition [1], no animal model of "OAB" currently exists [72]. This carries inevitable problems, because bladder contractions during filling are normal in some animal species and DO is not a specific marker of OAB in humans [3]. Furthermore, OAB is generally idiopathic, and is not caused by the overt neuropathies and lesions contemplated in most models [29, 72].

In the recent years, the urotheliogenic origin of the OAB has also been proposed based on much evidence of the key role of the urothelial cells as part of a peripheral control mechanism located in the bladder mucosa involving ICs and peripheral neurons [29, 30]. ICs form a two-dimensional network in close association with the suburothelial sensory nerves and urothelium [30]. Various imbalances within this "mucosal-bladder network" have been identified as being significant contributors to the generation of OAB [4]. The main functional properties of urothelium include the expression of receptors for neurotransmitters released from efferent and sensory nerves, and the ability to release several signalling molecules [29, 30, 85]. Alterations in these signalling mechanisms have been found in OAB patients and the urothelium is thought to mediate, totally or partly, the effect of many drugs [29, 30, 32, 85, 86]. The function of ICs is unclear, although they may operate by integrating signals and responses in the bladder wall, propagating and modulating spontaneous contractions, or acting as a control step in bladder sensory function [87, 88].

The in vitro isolated organ, electrophysiological techniques and the radioligand binding assay can be applied in studies investigating the role of the mucosal layer in normal bladder

function and in the pathophysiological mechanisms of OAB, such as studies on the contractility of strips with and without mucosa, electrophysiological studies on urothelial ion channels or the characterisation of receptors in urothelial cells. Animal models have also been used to probe urothelial responses to injuries of the urothelium itself, urethra or CNS [29]. However, experimental models reproducing urothelial abnormalities would be of utmost importance in the development of new therapeutic agents. A major challenge is to determine which of several abnormally regulated mucosal signalling pathways is the most important in inducing bladder dysfunction [29]. Recently, innovative transgenic animals with alterations specifically targeting the urothelium (urothelial restriction or conditional transgenics) have been developed to test the hypothesis that urothelial dysfunction can drive altered bladder function [29]. These models may also pave the way to genetic therapy for OAB. Interesting examples of urothelially restricted transgenic mouse models are that of stable nerve growth factor (NGF) over-expression [89] and β1-integrin knockout mice [90].

Furthermore, interest is growing in non-voiding detrusor contractions in animals and humans and their link with bladder afferent activity [18, 91]. For instance, β-adrenoceptor activity has been associated with micromotions and bladder afferent activity investigating the effect of mirabegron [32, 92, 93]. Ongoing studies on the modulation of micromotions will offer new perspectives for the treatment of LUT dysfunctions.

Another exciting novel perspective focuses on the hypothesis that OAB might have a cerebral origin and that a disruption of the brain–bladder network might be its main underlying cause [94]. Furthermore, the damage of the cerebral white matter has been recognised as a frequent feature in older OAB patients [95]. Therefore, future research should also focus on the assessment of CNS damage and dysfunction underlying this disorder. In this regard, the development of animal models with similar CNS alterations is of utmost importance [95]. The characterisation of the neural correlates of the normal and pathological bladder storage function using functional imaging tools in animals may offer new methods of drug discovery in this field [96].

Conclusions

The classical pharmacological techniques remain essential methods in the preclinical assessment process of new drugs for OAB/DO, in particular for those drugs that act on receptors, channels or transporters, constituting part of a certain functional pathway. Many animal models that mimic one or more aspects of the OAB are available and are of great value for testing investigational therapeutics. The identification of specific dysfunctions of the mucosal bladder network and conditional transgenics will offer new, exciting perspectives for the disease-orientated evaluation of novel compounds.

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Compliance with ethical standards

Conflicts of interest None.

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