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In vitro screening strategies for nicotinic receptor ligands

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This article is dedicated to Brian Jow who sadly passed away in its final stages of preparation. A talented scientist, loving father and husband, many will miss him.

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ABSTRACT

A common historical strategy to the discovery of nicotinic receptor ligands has involved the use of radioligand-binding assays for ligand identification in combination with two-electrode voltage clamp in *Xenopus* oocytes for electrophysiological characterization. More recently, higher-throughput methodologies have replaced these approaches to accommodate screening of large compound libraries and to provide increased capacity for electrophysiological profiling in mammalian cell lines. We, and others, have implemented cell-based screening assays using the fluorometric imaging plate reader (FLIPR^{TM}) for primary and lead optimization screening of nicotinic receptor agonists and positive allosteric modulators (PAMs). Using GH4C1 cells expressing the rat α7 nicotinic receptor, both acetylcholine and nicotine produced concentration-dependent elevations of intracellular calcium with EC_{50} values of 5.5 and 1.6 μM, respectively. PAM activity was robustly detected using the FLIPR assay; for example, the known α7 receptor PAM 5-hydroxyindole failed to directly activate the receptor but produced a leftward shift of the nicotine concentration–response curve in combination with a potentiation of the maximum evoked response to nicotine. Electrophysiological confirmation of agonist activity was achieved using the Dynaflow rapid perfusion system and patch clamp in the same GH4C1 cell expression system. Estimated EC_{50} values for acetylcholine-evoked currents in GH4C1/α7 cells were 55 and 576 μM for area-under-the-curve (AUC) and maximum peak height calculations, respectively. Similarly, PAM activity was confirmed using electrophysiological recordings while also allowing for the mechanistic discrimination of compounds, not possible using the FLIPR assay. Specifically, PAMs capable of slowing the rapid desensitization of α7 receptors to different extents were discernable in these studies. Further improvements in the capacity to screen compounds using electrophysiology has been achieved by implementation of high-throughput gigaohm quality recording systems such as the QPatch and PatchXpress where agonist EC_{50} values are highly comparable to those obtained using conventional manual patch clamp.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs), members of the neurotransmitter-gated ion channel superfamily [1], are involved in a wide variety of complex brain functions such as memory, attention and cognition as well as in pathological conditions such as Alzheimer's and Parkinson's disease, schizophrenia, epilepsy and nicotine addiction [2]. At least

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16 different genes code for nAChR subunits, which can assemble as pentamers in different combinations to form diverse nAChR subtypes. Unlike other nAChR subunits, α7 subunits efficiently assemble into functional homopentameric acetylcholine-gated nonselective cation channels when heterologously expressed in *Xenopus* oocytes or mammalian cells [3,4]. Electrophysiological studies revealed that these channels display highest permeability for Ca^{2+} and exhibit fast activation/inactivation kinetics [5].

Historically, industrial screening for identification of novel drugs acting on nAChRs was mainly based on the use of easy-to-automate radioactive displacement binding assays, which do not allow a distinction between agonists and antagonists [6]. Most of the first generation of nAChR agonists and antagonists has been identified employing such binding assays (for reviews see [7] and references therein) by screening compounds mainly designed by chemical modification of natural nAChRs ligands, such as nicotine, acetylcholine, epibatidine and anabaseine. For example, Abbott, Targacept and Sanofi-Aventis have all described series of nicotinic agonists and antagonists for either α7 and/or α4/β2 receptors with receptor affinities determined using a variety of radioligands, including [^{3}H]nicotine, [^{3}H]alpha-bungarotoxin and [^{3}H]epibatidine [8–11]. A significant disadvantage of binding assays is the need for radioisotopes such as ^{3}H and ^{125}I and the associated costs, safety hazards and environmental (e.g. disposal) problems. Also, because compounds identified in binding assays will typically reflect the mode of action of the ligand used, compounds with novel mechanisms of channel modulation cannot be identified. Thus, for HTS and lead optimization, ligand binding assays have largely been abandoned in favor of functional cell-based assays (see below).

A number of options are available for the cell-based functional assessment of α7 receptor ligand activity. These include radioactive and non-radioactive rubidium ion flux assays [12–14], other flux assays using ion specific fluorescent dyes for ions that are conducted by nAChRs such as K^+, Na^+ and Ca^{2+} [15–19] (although only Ca^{2+}specific fluorescence dyes have found widespread screening applications) or membrane potential sensitive fluorescent dyes [20–22]. For screening of α7 nAChRs that display a high conductivity for Ca^{2+} ions, Ca^{2+} flux assays represent the main HTS technology at present for which highly specialized fluorescence plate reader instrumentation (e.g. Fluorescence Imaging Plate Reader; FLIPR^{TM}) is available [23]. Since this technology allows not only end point measurements but also kinetic analysis with high-throughput, it is possible to identify agonists, antagonists and positive allosteric modulators (PAMs) by simple sequential compound addition protocols in a single experiment on the same microplate. We (see below) and others [24–29] have successfully employed FLIPR based calcium flux screening protocols for the initial identification of α7 receptor modulators in high-throughput screening and in subsequent support of lead optimization. In addition, we have successfully implemented a membrane potential fluorescent dye based screening assay for the α1 receptor subtype as an important selectivity assay.

Traditionally, compounds identified using radioligand-binding assays were subsequently evaluated using electrophysiological recording procedures, most commonly the two-electrode voltage clamp (TEVC) technique in the *Xenopus* oocyte expression systems [30–32]. More recently, many studies have been reported that utilize mammalian cell expression systems, thus, complementing the use of the same expression systems for cell-based higher-throughput screens as discussed above, instead of oocytes, and patch clamp techniques instead of TEVC [33,34]. GH4C1 cells have proven to be readily amenable to heterologous expression of α7 receptors [33] and upon further investigation, α7 receptors have been found to require the co-expression of ric-3, which coincidentally is endogenously expressed in both GH4C1 cells and oocytes [35].

All of the electrophysiological methods require some ability to bath apply ligand while recording the responses. In receptors such as the α7 nicotinic receptor, however, very fast desensitization occurs upon activation. To achieve the best responses, fast ligand application onto the cells is critical. One method used in several labs is the 'U-tube', a method used to switch flows very quickly across the cell being tested [36–39]. Ligand can also be applied by 'puffing' into slices [40], although dilution of the ligand concentration occurs, thus, obscuring the exact concentration. Cells in slices are generally identified by 'blind patch' [41] or DIC-IR optics [42], both of which can provide detailed information regarding the role of nicotinic receptors in synaptic transmission and plasticity [43].

A more recent innovation for reasonably fast solution flow is the Dynaflow system (Cellectricon) [44–47]. While limited in its possible applications to dissociated cells, up to 48 solutions can be loaded into the plate, thus, enabling automated control of up to 24 test solutions and 24 buffer washes, or any combination desired. One of the newest methods to automate the electrophysiological measurement of nicotinic receptors is the application of the multi-channel high-throughput giga-ohm quality recording systems such as the QPatch and PatchXpress [48,49]. Experiments comparing PatchXpress with Dynaflow have been performed on α7 expressing GH4C1 cells. Currents appear similar in these two systems when stimulated with acetylcholine. While the utility of these systems for nicotinic receptors is still developing, the future looks very promising for this technology.

In combination, high-throughput approaches to calcium flux measurements using FLIPR and electrophysiological recordings are a highly effective strategy for the identification of α7 nicotinic receptor modulators. The majority of these molecules subsequently go on to show efficacy in animal models of cognition, sensory gating and neuroprotection.

2. Methods

2.1. *Measurements of calcium flux using FLIPR*

GH4C1 cells stably transfected with pCEP4/rat α7 nAChR were used as test system and maintained in poly-D-lysine coated flasks in F10 medium supplemented with 15% horse serum and 2.5% FBS, 1% penicillin-streptomycin, 200 mg/ml hygromycin B at 37 °C in a humidified 5% CO_2 incubator. Cells were seeded at a density of 20,000 cells/well (384-well format) and cultured for 48 h prior to experiment in the presence of 0.5 mM sodium butyrate. On the day of the experiment, cells were loaded with Fluo-4 AM for 30 min at 37 °C. After the dye was

washed, intracellular calcium fluxes in presence and absence of compounds were measured on FLIPR. Agonist responses were directly assessed while the effects of α7 nAChR PAMs were determined by first adding compound and subsequent challenge with an EC_{20} concentration of nicotine. Concentration–response data are expressed in terms of percent response relative to a maximum test concentration of nicotine (10 μM) while EC_{50} values were determined by nonlinear regression analysis using the GraphPad Prism software package.

2.2. Measurements of membrane potential using FLIPR

TE671 rhabdomyosarcoma cells were used as endogenous sources of muscle α1β1γδ [22] receptors, and SH-SY5Y neuroblastoma cells were used as an endogenous source of ganglionic α3 receptors. Cells were plated at a density of 50,000 cells/well (96-well format) 24 h prior to the day of the assay. For evaluation of compound-evoked changes in membrane potential, growth media was removed from the cells and membrane potential dye (Molecular Devices) loading solution, reconstituted in HBSS according to the manufacturer's instructions, was added to the wells. Plates were incubated for 60 min at 37 °C and then directly transferred to the FLIPR. Baseline fluorescence was monitored for the first 10 s followed by the addition of agonists and subsequent monitoring of fluorescence changes for up to 4 min. Agonist responses were normalized to a maximally effective concentration of acetylcholine for the estimation of EC_{50} values and (when studied) antagonists were added for 10 min at the end of the dye loading procedure but prior to transfer to the FLIPR.

2.3. Electrophysiology studies

GH4C1 cells stably expressing rat α7 nAChR were treated with 0.5 mM Na-butyrate added to the medium for 2 days before patch clamp recording. Patch pipettes had resistances of ~7 MΩ when filled with (in mM): 5 EGTA, 120 K-Gluconate, 5 KCl, 10 HEPES, 5 K_2ATP, 5 Na_2-phosphocreatine, 1 $CaCl_2$ and 2 $MgCl_2$. Cells were voltage clamped at −60 mV with a HEKA EPC-9 amplifier. To measure the activation and desensitization of α7 current, the Dynaflow (Cellectricon) perfusion system with 16- or 48-well chips was used. Different concentrations of acetylcholine and other α7 agonists were applied to cells in between washes with bath solution (Hanks' Balanced Salt Solution + 10 mM HEPES). Data were acquired and filtered at 1 kHz for 2-s episodes (500 ms bath, 500 ms agonist, 1000 ms wash) with a 10-s interval between episodes. Peak current amplitude and total charge (area-under-the-curve) were measured with the HEKA Pulse program. Concentration–response curves and EC_{50} values were plotted and calculated with Origin (MicroCal).

Acetylcholine-evoked currents were also measured using the automated electrophysiology platform PatchXpress 7000 A using the GH4C1/α7 cells. Cells were held at −60 mV and identical internal solution was used as in the manual patch clamp experiments. Sequential application of increasing concentrations of acetylcholine was applied to cells successfully achieving gigaohm seals and total charge measured as area-under-the-curve was compared to maximum peak current amplitude.

2.4. [^{3}H]Epibatidine binding studies

Membranes derived from GH4C1 cells stably expressing rat α7 nAChR were thawed, combined with binding buffer (50 mM HEPES, pH 7.4, 3 mM KCl, 70 mM NaCl, 10 mM $MgCl_2$), 5 nM [^{3}H]epibatidine (GE Healthcare; S.A. = 53 Ci/mmol) and test compound or 300 μM nicotine (for nonspecific binding) to achieve a final volume of 200 μl in a 96-well polypropylene plate. Following incubation at room temperature (23 °C) for 1 h, the samples were rapidly filtered over Unifilter GF/B filters using a Filtermate (Perkin-Elmer) and washed 5× with ice-cold binding buffer. Samples were processed and counted for radioactivity using a TopCount NXT (Perkin-Elmer). Competition binding curves were fitted with a four-parameter logistic model that defined IC_{50}s, and K_i values were calculated by the Cheng–Prusoff equation using the GraphPad Prism software package.

3. Results

The ability of the cell-based FLIPR calcium flux assay to detect both α7 agonist and PAM activity is shown in Fig. 1. The two-step addition protocol for determining the pharmacological activity of a compound is illustrated (Fig. 1A) with the test compound added prior to an EC_{20} concentration of nicotine, thus, allowing for the detection of agonist activity in the first addition or a PAM in the second addition. Fig. 1A further illustrates another key property of a PAM molecule, the absence of any direct stimulatory response when applied alone to the cells. Concentration-dependent responses are typically observed for the agonist activation, as illustrated for both nicotine and acetylcholine (Fig. 1B), allowing for estimation of EC_{50} values and intrinsic activities (or Emax relative to acetylcholine). In the FLIPR assay, EC_{50}s were determined as 1.6 and 5.5 μM, respectively, for nicotine and acetylcholine, with nicotine behaving as a full agonist in this system. A more detailed pharmacological characterization of a series of α7 agonists and antagonists is shown in Table 1, demonstrating a good agreement with published data and (most importantly for screening) confirming rank order of compounds. Further characterization of PAM activity can be achieved by determining the effect on the full concentration–response relationship for the agonist where, in the case of the known α7 PAM 5-hydroxyindole, both a leftward shift in the nicotine concentration–response, and potentiation of the maximum evoked response is observed (Fig. 1C).

In any drug discovery effort, it is critical not only to enable assays to evaluate compound activity on the primary target of interest, but also to enable assays on closely related targets, especially those that might represent a potential liability. Using the membrane potential sensitive fluorescent dye in combination with FLIPR we successfully developed an assay for monitoring compound activity on the α1β1γδ receptors in TE671 cells, and the α3 receptors in SH-SY5Y cells. Functional activity for representative agonists and antagonists is presented in Table 2 for α1β1γδ receptors in TE671 cells and Table 3 for α3 receptors in SH-SY5Y cells validating the appropriate pharmacology and rank order agreement with published data.

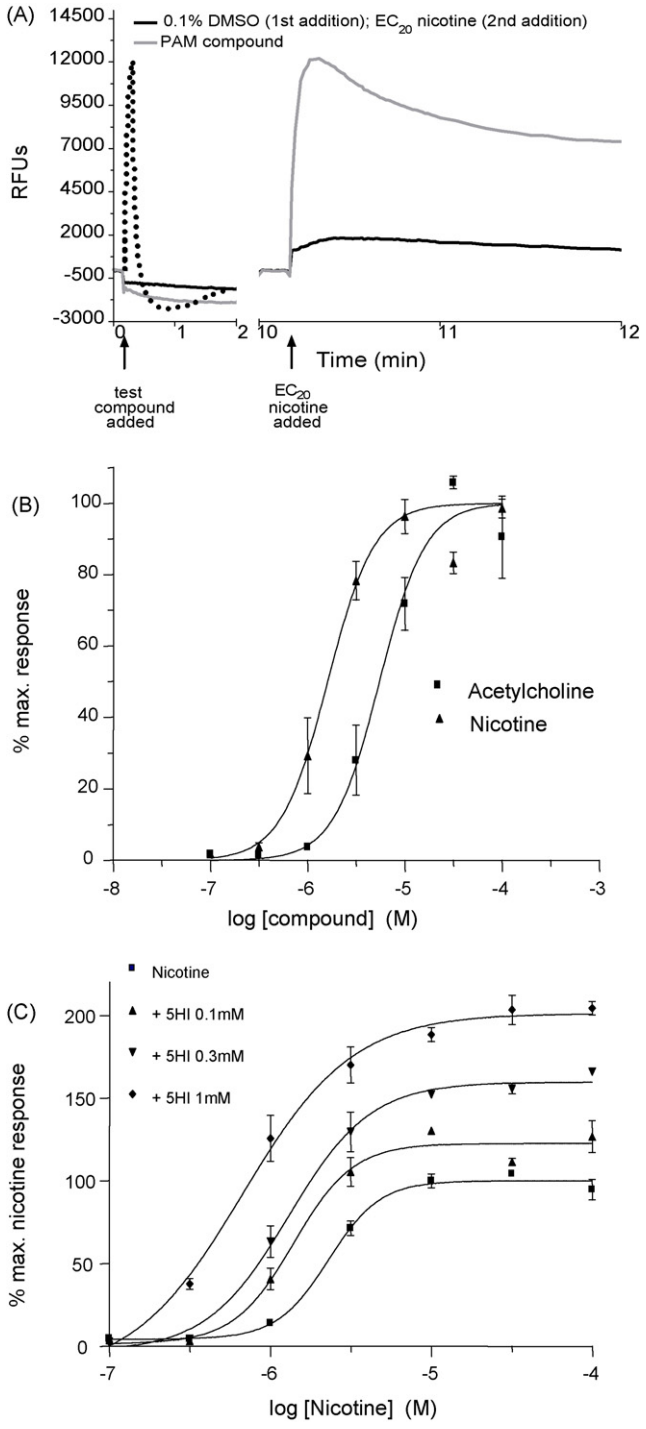

Fig. 1 – α7 nicotinic receptor agonist and PAM activity evaluated using the FLIPR and calcium flux measurements. (A) Two-step addition protocol illustrates the activity of an agonist in the first addition (dotted line), while a PAM is only revealed by the subsequent addition of a sub-maximal (EC_{20}) concentration of nicotine; PAM profile shown in solid grey line and effect of EC_{20} concentration of nicotine under non-potentiated conditions as black line. (B) Concentration-dependent responses to the agonists ACh and nicotine are expressed as a percentage of the maximum response to ACh. (C) Potentiation of the nicotine concentration–response in the presence of the PAM, 5-hydroxyindole. Maximum responses to nicotine were potentiated to 123, 160 and 201%, respectively, in the combined presence of 0.1, 0.3 and 1 mM 5-hydroxyindole. The EC_{50} for nicotine was shifted from 2.3 to 0.7 μM in the presence of 1 mM 5-hydroxyindole.

Confirmation of compound activity using an electrophysiological approach is a key next step, especially when using the less direct FLIPR calcium flux assay for initial compound profiling. Using manual patch clamp in GH4C1/α7 cells, the Dynaflow rapid perfusion system was found to be a reliable solution to the electrophysiological characterization of agonist and PAM activity. Currents evoked by sequential application of increasing concentrations of ACh to the same cell are illustrated (Fig. 2A) revealing a concentration-dependent response with the characteristic property of rapid desensitization, a hallmark feature of the α7 receptor. Quantification of evoked responses was compared using a total charge transfer, or an area-under-the-curve (AUC) measure (Fig. 2B), and maximum current amplitude, or a peak height measure (Fig. 2C). The AUC calculation represented a more sensitive index of compound potency than the peak height, as calculated in this example for ACh, with EC_{50} values of 55 and 576 μM, respectively. This greater sensitivity of the AUC calculation was observed universally for a broad range of α7 receptor agonist chemotypes (data not shown). Interestingly, nicotine (EC_{50}, AUC, 9.9 μM) exhibited partial agonist activity in the electrophysiological recordings (Fig. 2B, inset), yet appeared as a full agonist in the FLIPR studies. Results presented in Fig. 2B, and to a lesser extent Fig. 2C, also demonstrate a characteristic inverted U-shaped concentration–response relationship for ACh, which was also seen with other agonists (not shown). This behavior is likely attributed to both the rapid desensitization of the channels preventing accurate estimation of full activation, and the blocking or gating effect of ACh seen for α7 receptors at high concentrations [50–53].

Notably, functional profiling of nicotine and acdetylcholine using the FLIPR assay generated EC_{50} values approximately 5–10 fold more potent than those obtained with the electrophysiological studies, a relationship that also was consistent for other α7 agonists from diverse chemotypes (data not shown). To explore this further we determined the α7 receptor affinities of both nicotine and acetylcholine using displacement of the high-affinity ligand [^{3}H]epibatidine from α7 binding sites in membranes prepared from GH4C1/α7 cells. Table 4 provides a comparison between the α7 receptor functional potencies and binding affinities for nicotine and acetylcholine. Both nicotine and acetylcholine exhibited binding affinities similar to the FLIPR functional potencies and approximately 5–10 fold more potent that the functional potency by electrophysiology. Comparing the binding affinity of eight reference agonists with their functional EC_{50} estimates determined using FLIPR generated a highly significant correlation ($r^2 = 0.95$) between the two [54].

In the case of compounds acting via a PAM mechanism the use of electrophysiological assessment for follow-up studies provides a powerful illustration of this approach, while also

Table 1 – Estimated EC_{50} and IC_{50} values for activation of α7 receptors in GH4C1 cells by reference α7 agonists and antagonists using the FLIPR calcium flux assay

	GH4C1, EC_{50} (μM)	*n*	EC_{50} (μM)[a]	EC_{50} (μM)[b]
(+)-Epibatidine	0.012 ± 0.002	7	0.17 ± 0.03	0.045
(−)-Nicotine	1.6 ± 0.24	22	2.0 ± 0.4	1.78
Acetylcholine	5.5 ± 0.16	6	17.0 ± 3.3	5.50
Choline	29.25 ± 9.17	4		54.95
DMPP	0.98 ± 0.51	6	5.4 ± 1.2	1.23
GTS21	2.92 ± 2.05	4		1.91
AR-R17779	0.27 ± 0.06	5		0.39
(−)-Cytisine	1.21 ± 0.13	5	10.0 ± 2.3	2.09
	IC_{50} (μM)			IC_{50} (μM)
Methyllycaconitine	0.00203 ± 0.00233	10		0.015
α-Bungarotoxine	0.012 ± 0.005	4		0.040

Total numbers of concentration–response curves (or assay plates) are indicated (*n*) performed in a minimum of three independent experiments.
[a] Comparative data from [19].
[b] Comparative data from [56] (data converted from pEC_{50}).

Table 2 – Estimated EC_{50} and IC_{50} values for activation of α1β1γδ receptors in TE671 cells by reference nicotinic agonists and antagonists using the FLIPR membrane potential assay

	TE671, EC_{50} (μM)	*n*	EC_{50} (μM)[a]
(−)-Cytisine	22.2 ± 3.6	5	77 ± 17
DMPP	0.29 ± 0.03	9	1.1 ± 0.4
(+)-Epibatidine	0.061 ± 0.013	9	0.27 ± 0.03
	IC_{50} (μM)		
α-Bungarotoxine	45.0 ± 21.2	8	

Total numbers of concentration–response curves (or assay plates) are indicated (*n*) performed in three independent experiments.
[a] Comparative data from [22].

highlighting one of the limitations of the FLIPR calcium flux assay. Since the FLIPR assay does not directly report the current passing through the channel, mechanistic insight into PAM activity using this method is lacking. Specifically, the FLIPR assay can only reveal the potentiating activity of a PAM without the ability to discern mechanistic information. Electrophysiological studies have clearly allowed for the discrimination of different PAM mechanisms (Fig. 3). In the example of PAM1 (Fig. 3A), the compound potentiated maximum responses to ACh, while simultaneously slowing the rapid desensitization normally observed with agonists. In comparison, PAM2 (Fig. 3B) potentiates ACh responses with a reduced effect on the desensitization kinetics, and also potentiated maximum responses to ACh (Fig. 3B, inset). Such mechanistic distinction was not determined via the FLIPR studies; both compounds could only be detected to enhance agonist-stimulated calcium flux.

A final, and evolving, advance in the electrophysiological characterization of compounds is the implementation of high-throughput and fully automated gigaohm quality recording systems such as PatchXpress and QPatch. We have found both the QPatch (manuscript in preparation) and PatchXpress (Fig. 4) able to reliably detect the rapidly desensitizing ACh-mediated current in GH4C1/α7 cells. Similar to manual recordings with the Dynaflow system, the AUC calculation was more sensitive for EC_{50} calculations; EC_{50} values for ACh in PatchXpress were estimated as 10.5 and >500 μM, respectively. Full integration of these automated systems in drug discovery efforts is predicted to have a large impact on nicotinic ligand discovery.

Table 3 – Estimated EC_{50} and IC_{50} values for activation of α3 receptors in SH-SY5Y cells by reference nicotinic agonists and antagonists using the FLIPR membrane potential assay

	SH-SY5Y, EC_{50} (μM)	*n*	EC_{50} (μM)[a]
(−)-Nicotine	8.0 ± 4.2	9	6.2 ± 0.2
(−)-Cytisine	9.3 ± 4.5	9	8.3 ± 2.2
DMPP	3.5 ± 1.0	9	4.6 ± 0.6
(+)-Epibatidine	0.0021 ± 0.0004	7	0.008 ± 0.0005
	IC_{50} (μM)		
Mecamylamine	1.7 ± 0.8	9	

Total numbers of concentration–response curves (or assay plates) are indicated (*n*) performed in three independent experiments.
[a] Comparative data from [22].

4. Discussion

In vitro screening strategies for nicotinic receptor ligands have evolved to take advantage of high-throughput cell-based assays of both calcium flux and membrane potential as illustrated here and by others [21,22,25–29,55]. This has provided the ability to profile multiple compound mechanisms, e.g. full and partial agonist, positive allosteric modulator, antagonist, in the same experiment and has enabled early screening of large compound libraries with subsequent support of lead optimization efforts. Combined with more direct measurements of compound activity using electrophysiology, both with rapid perfusion systems [44–47] and high-throughput gigaohm quality recording technology [48,49], this represents an efficient process for triaging compounds and selecting candidates for further testing in preclinical models of cognition, sensory gating and neuroprotection.

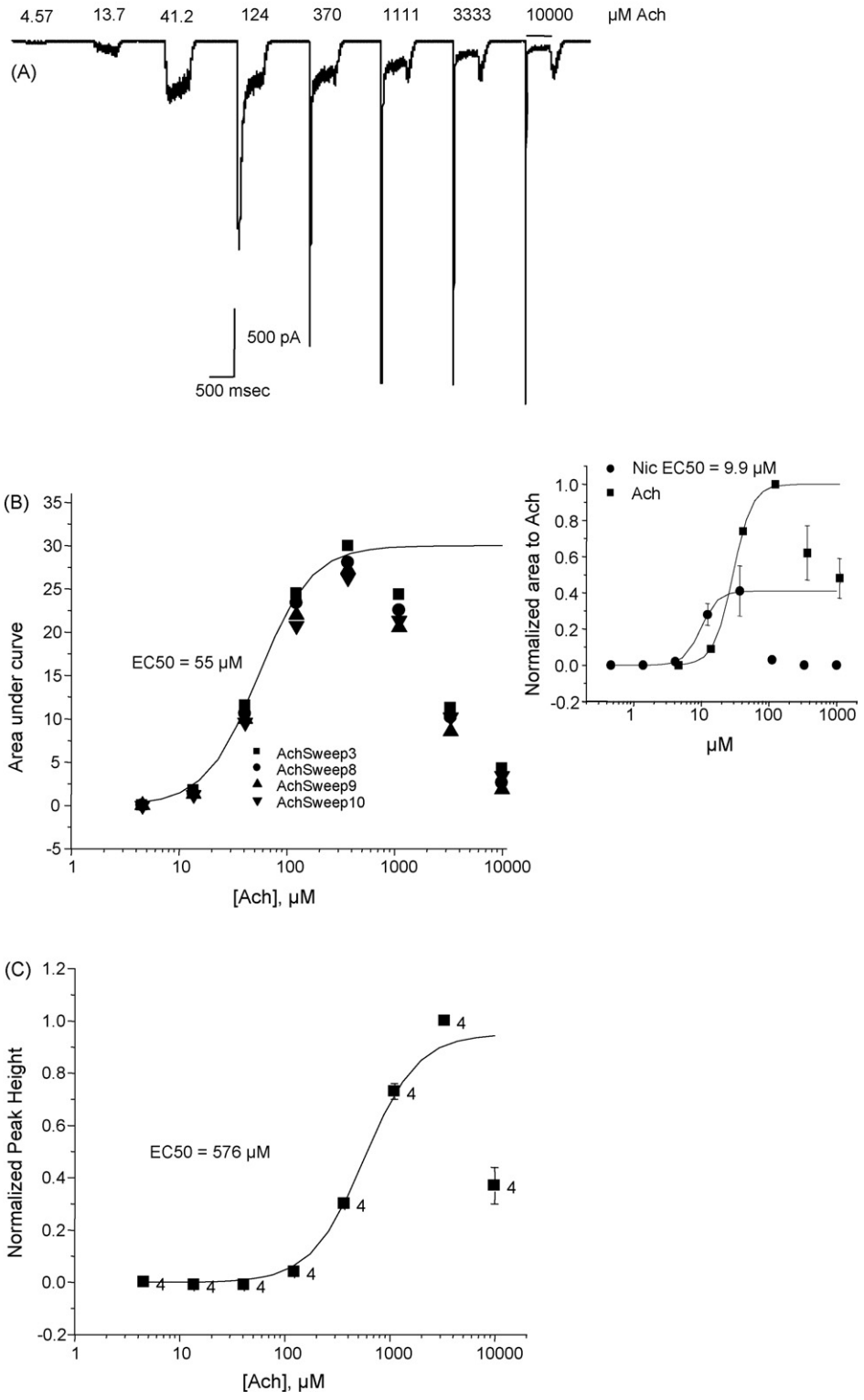

Fig. 2 – α7 nicotinic receptor agonist activity evaluated using whole-cell manual patch clamp electrophysiology and the Dynaflow rapid perfusion system. (A) Currents evoked by sequential application of ACh, at the indicated concentrations, to GH4C1/α7 cells were quantified using an AUC (B) and peak height (C) calculation yielding EC_{50} values of 55 and 576 μM, respectively. (B) (Inset) comparison of nicotine (solid circle) to acetylcholine (solid square) using whole-cell manual patch clamp revealing partial agonist activity.

Table 4 – Comparison of α7 receptor functional potencies using FLIPR and electrophysiological recordings with receptor binding affinity

	FLIPR, EC_{50} (μM)	Electrophysiology, EC_{50} (μM)	[^{3}H]Epibatidine, K_i (μM)
Nicotine	1.6	9.9	1.3
Acetylcholine	5.5	55	8.2

Electrophysiological recordings were performed using manual patch clamp with the Dynaflow perfusion system and data were calculated based on the area-under-the-curve. In all cases data are presented as means from at least three independent experiments.

It is important to emphasize the use of these assay platforms in an integrated manner since each approach has its own strengths and limitations. In the case of the affinity binding assay discussed here, the interaction with the specific binding site being probed, i.e., that of the agonist ligand epibatidine, can be assessed to determine overlap with this binding site and to allow elucidation of a structure-activity relationship (SAR) for the chemical series being assessed.

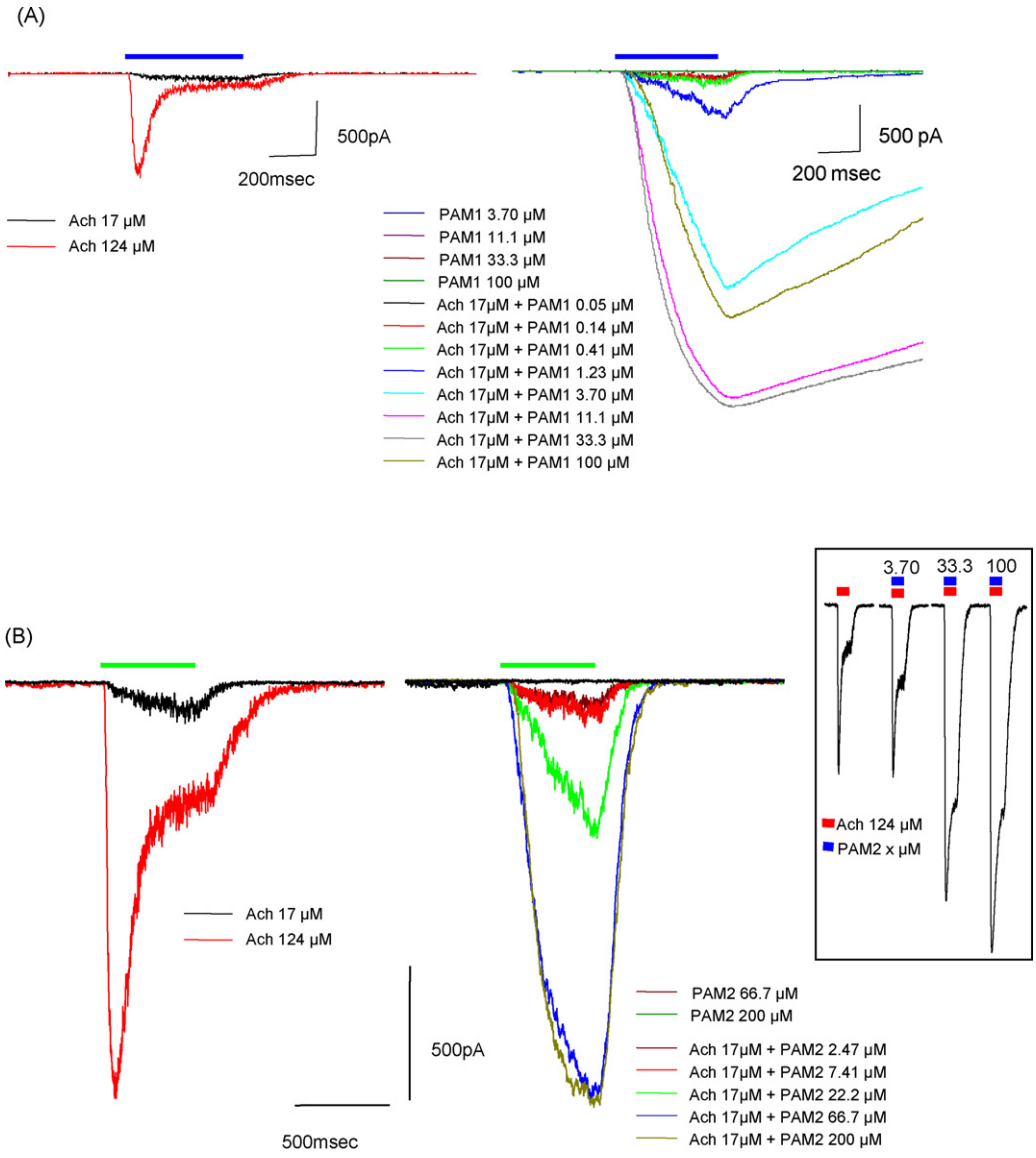

Fig. 3 – α7 nicotinic receptor PAM activity evaluated using manual patch clamp electrophysiology and the Dynaflow rapid perfusion system. (A) Left panel indicates responses to EC_{20} (17 μM) and EC100 (124 μM) concentrations of ACh illustrated for reference, while right panel shows the effect of PAM1 to potentiate the maximum current produced with an EC100 of ACh, combined with a slowing of the rapid desensitization. Currents are overlaid for comparison purposes. (B) PAM2 potentiates the EC_{20} responses to ACh only to a magnitude comparable to the EC100 response observed for ACh and has a less prominent effect on the desensitization kinetics. (B) (Inset) effect of PAM2 on the response evoked by a maximally effective concentration of ACh.

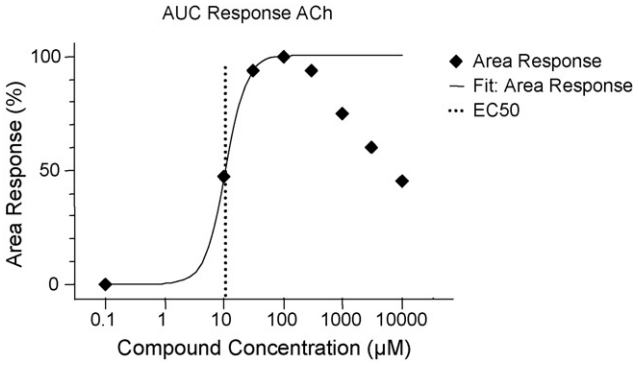

Fig. 4 – α7 Nicotinic receptor agonist activity evaluated using high-throughput patch clamp electrophysiology with the PatchXpress system. Currents were evoked by sequential application of increasing concentrations of ACh, producing an EC_{50} of 10.5 μM based upon an AUC calculation.

Moreover, the determination of the binding affinity can be used in concert with the functional assays to determine relationships between receptor affinity and functional activity. The main limitations of the receptor binding assay is the ability to probe only the binding site of the radiolabed ligand used and the lack of ability to determine novel mechanisms of action such as allosteric modulation. Consequently, compounds acting via different mechanisms are best revealed using functional assays of receptor activation.

The two main approaches described here for the functional assessment of α7 receptor activation, FLIPR and electrophysiology, provide for an efficient and comprehensive assessment of compound activity. In the case of the FLIPR assay, the high-throughput afforded by this technology allows for an initial assessment of large numbers of compounds, for both agonist and positive allosteric modulator activity. Interestingly, and despite the fact that α7 receptor mediated calcium flux in GH4C1 cells occurs via both the α7 receptor directly and indirectly through voltage-gated calcium channels [56], we found the FLIPR assay to be well correlated with the receptor binding affinity for agonists, allowing this functional assay to also be used for determination of SAR for agonist chemical series. On the other hand, positive allosteric modulators discovered in the FLIPR assay failed to displace epibatidine binding (data not shown) suggesting an interaction with novel binding sites and the need for new radioligand development to enable assessment of these sites. Finally, although the FLIPR assay successfully identifies positive modulators, it fails to provide any mechanistic insight for these agents, and poorly discriminates partial agonist activity, as revealed for nicotine in the current analysis.

In the case of the electrophysiological recordings to assess receptor activation, the main limitation is clearly in the throughput of compounds that can be studied. Although initial attempts to use automated electrophysiology platforms in this regard appear promising, it is anticipated that even these automated systems will never achieve the capacity supported by the FLIPR. The strength of the electrophysiological approach lies in its information rich nature as demonstrated by the ability to discern mechanistic insight into the action of positive modulators and to reveal partial agonist activity of nicotine, relative to the efficacy observed with acetylcholine. An unexpected finding in the electrophysiological recordings, and potential limitation, was the right shifted nature of compound potency compared with both the receptor binding affinity and FLIPR functional assessment of agonists. As discussed above, the rapid desensitization of the α7 receptor, and the direct block observed with higher concentrations of agonists, likely places some limitations on the accurate assessment of compound potency using this method.

Use of the integrated approach described here provides a strategy for the discovery of novel α7 nicotinic receptor modulators targeted toward clinical development with the ultimate goal being the introduction of new and effective therapeutics for schizophrenia and Alzheimer's disease. No approach can be used in isolation of the others given the merits and limitations of each discussed.

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REFERENCES

[1] Lindstrom J. Neuronal nicotinic acetylcholine receptors. Ion Channels 1996;4:377–450.
[2] Dani J. Overview of nicotinic receptors and their roles in the central nervous system. Biol Psychiatry 2001;49.
[3] Couturier S, Bertrand D, Matter J, Hernandez M, Bertrand S, Millar N, et al. A neuronal nicotinic acetylcholine receptor subunit (alpha 7) is developmentally regulated and forms a homo-oligomeric channel blocked by alpha-BTX. Neuron 1990;5.
[4] Quik M, Choremis J, Komourian J, Lukas RJ, Puchacz E. Similarity between rat brain nicotinic *a*-bungarotoxin receptors and stably expressed a-bungarotoxin binding sites. J Neurochem 1996;67:145–54.
[5] Peng X, Katz M, Gerzanich V, Anand R, Lindstrom J. Human a7 acetylcholine receptor: cloning of the a7 subunit from the SH-SY5Y cell line and determination of pharmacological properties of native receptors and functional a7 homomers in *Xenopus* oocytes. Mol Pharm 1993;45:546–54.
[6] Terstappen GC. Ion flux and ligand binding assays for analysis of ion channels. In: Clare JJ, Trezise D, editors. Expression and Analysis of Recombinant Ion Channels. Weinheim: Wiley-VCH; 2006. pp. 165–186.
[7] Jensen A, Frolund B, Liljefors T, Krogsgaard-Larsen P. Neuronal nicotinic acetylcholine receptors: structural revelations, target identifications and therapeutic inspirations. J Med Chem 2005;48:4705–45.
[8] Bencherif M, Bane AJ, Miller CH, Dull GM, Gatto GJ. TC-2559: a novel orally active ligand selective at neuronal acetylcholine receptors. Eur J Pharmacol 2000;409:45–55.
[9] Gopalakrishnan. Soc Neurosci Abs 2006:325.8.
[10] Hauser. Soc Neurosci Abs 2004:435.13.

[11] Lippiello P, Letchworth SR, Gatto GJ, Traina VM, Bencherif M. Ispronicline: a novel alpha4beta2 nicotinic acetylcholine receptor-selective agonist with cogntive enhancing and neuroprotective properties. J Mol Neurosci 2006;30: 19–20.
[12] Lukas RJ, Cullen MJ. An isotopic rubidium ion efflux assay for the functional characterization of nicotinic acetylcholine receptors on clonal cell lines. Anal Biochem 1988;175:212–8.
[13] Terstappen GC. Functional analysis of native and recombinant ion channels using a high-capacity nonradioactive rubidium efflux assay. Anal Biochem 1999;272:149–55.
[14] Terstappen GC. Nonradioactive Rb^+ efflux assay and its applications in drug discovery and development. Assay Drug Dev Technol 2004;2:553–9.
[15] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.
[16] Kasner SE, Ganz MB. Regulation of intracellular potassium in mesangial cells: a fluorescence analysis using the dye PBFI. Am J Physiol 1992;262:462–7.
[17] Minta A, Kao JP, Tsien RY. Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. J Biol Chem 1989;264:8171–8.
[18] Minta A, Tsien RY. Fluorescent indicators for cytosolic sodium. J Biol Chem 1989;264:19449–57.
[19] Quik M, Philie J, Choremis J. Modulation of alpha7 nicotinic receptor-mediated calcium influx by nicotinic agonists. Mol Pharmacol 1997;51:499–506.
[20] Epps DE, Wolfe ML, Groppi V. Characterization of the steady-state and dynamic fluorescence properties of the potential-sensitive dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($Dibac_4(3)$) in model systems and cells. Chem Phys Lipids 1994;69:137–50.
[21] Lu Q, Lin S, Dunlop J. Membrane potential based assays for ion channels and electrogenic transporters. Handbook of assay development in drug discovery 2006;5:343–356.
[22] Fitch R, Xiao Y, Kellar K, Daly J. Membrane potential fluorescence: a rapid and highly sensitive assay for nicotinic receptor channel function. Proc Natl Acad Sci USA 2003;100:4909–14.
[23] Schroeder KS, Neagle BD. FLIPR: a new instrument for accurate high-throughput optical screening. J Biomol Screen 1996;1:75–80.
[24] Broad LM, Zwart R, Pearson KH, Lee M, Wallace L, McPhie GI, et al. Identification and pharmacological profile of a new class of selective nicotinic acetylcholine receptor potentiators. J Pharmacol Exp Ther 2006;318:1108–17.
[25] Eisele J, Bertrand S, Galzi J, Devillers-Thiery A, Changeux J, Bertrand D. Chimeric nicotinic-serotonergic receptor combines distinct ligand binding and channel specificities. Nature 1993;366:479.
[26] Hurst RS, Hajos M, Raggenbass M, Wall TM, Higdon NR, Lawson JA, et al. A novel positive allosteric modulator of the alpha7 neuronal nicotinic acetylcholine receptor: in vitro and in vivo characterization. J Neurosci 2005;25: 4396–405.
[27] Mazurov A, Hauser T, Miller CH. Selective alpha7 nicotinic acetylcholine receptor ligands. Curr Med Chem 2006;13:1567–84.
[28] Roppi VE, Wolfe ML, Berkenpas MB. Methods and Compositions for Measuring Ion Channel Conductance. 2000.
[29] Wishka D, Walker D, Yates K, Reitz S, Jia S, Myers J, et al. Discovery of N-[(3-R)-1-azabicyclo[2.2.2]oct-3-yl]furo[2,3-c]pyridine-5-carboxamide, an agonist of the alpha7 nicotinic acetylcholine receptor, for the potential treatment of cognitive deficits in schizophrenia: synthesis and structure–activity relationship. J Med Chem 2006;49:4452–536.
[30] Maskell PD, Speder P, Newberry NR, Bermudez I. Inhibition of human alpha 7 nicotinic acetylcholine receptors by open channel blockers of *N*-methyl-D-aspartate receptors. Br J Pharmacol 2003;140:1313–9.
[31] Meyer EM, Tay ET, Zoltewicz JA, Meyers C, King MA, Papke RL, et al. Neuroprotective and memory-related actions of novel alpha-7 nicotinic agents with different mixed agonist/antagonist properties. J Pharmacol Exp Ther 1998;284:1026–32.
[32] Nirthanan S, Charpantier E, Gopalakrishnakone P, Gwee MCE, Khoo H-E, Cheah L-S, et al. Candoxin, a novel toxin from Bungarus candidus, is a reversible antagonist of muscle (alphabetagammadelta) but a poorly reversible antagonist of neuronal alpha 7 nicotinic acetylcholine receptors. J Biol Chem 2002;277:17811–20.
[33] Virginio C, Giacometti A, Aldegheri L, Rimland JM, Terstappen GC. Pharmacological properties of rat [alpha]7 nicotinic receptors expressed in native and recombinant cell systems. Eur J Pharmacol 2002;445:153–61.
[34] Virginio C, Graziani F, Terstappen GC. Differential inhibition of rat [alpha]3* and [alpha]7 nicotinic acetylcholine receptors by tetrandrine and closely related bis-benzylisoquinoline derivatives. Neurosci Lett 2005;381:299–304.
[35] Williams ME, Burton B, Urrutia A, Shcherbatko A, Chavez-Noriega LE, Cohen CJ, et al. Ric-3 promotes functional expression of the nicotinic acetylcholine receptor alpha7 subunit in mammalian cells. J Biol Chem 2005;280:1257–63.
[36] Albuquerque EX, Pereira EF, Braga MF, Alkondon M. Contribution of nicotinic receptors to the function of synapses in the central nervous system: the action of choline as a selective agonist of alpha 7 receptors. J Physiol Paris 1998;92:309–16.
[37] Alkondon M, Albuquerque EX. A non-alpha7 nicotinic acetylcholine receptor modulates excitatory input to hippocampal CA1 interneurons. J Neurophysiol 2002;87:1651–4.
[38] Alkondon M, Pereira EF, Eisenberg HM, Albuquerque EX. Choline and selective antagonists identify two subtypes of nicotinic acetylcholine receptors that modulate GABA release from CA1 interneurons in rat hippocampal slices. J Neurosci 1999;19:2693–705.
[39] Alkondon M, Pereira EF, Eisenberg HM, Albuquerque EX. Nicotinic receptor activation in human cerebral cortical interneurons: a mechanism for inhibition and disinhibition of neuronal networks. J Neurosci 2000;20:66–75.
[40] Henderson Z, Boros A, Janzso G, Westwood AJ, Monyer H, Halasy K. Somato-dendritic nicotinic receptor responses recorded in vitro from the medial septal diagonal band complex of the rodent. J Physiol 2005;562:165–82.
[41] Liu Z-W, Yang S, Zhang Y-X, Liu C-H. Presynaptic alpha-7 nicotinic acetylcholine receptors modulate excitatory synaptic transmission in hippocampal neurons. Sheng Li Hsueh Pao Acta Physiol Sin 2003;55:731–5.
[42] Zaninetti M, Tribollet E, Bertrand D, Raggenbass M. Nicotinic cholinergic activation of magnocellular neurons of the hypothalamic paraventricular nucleus. Neuroscience 2002;110:287–99.
[43] Mann EO, Greenfield SA. Novel modulatory mechanisms revealed by the sustained application of nicotine in the guinea-pig hippocampus in vitro. J Physiol 2003;551:539–50.
[44] Olofsson J, Pihl J, Sinclair J, Sahlin E, Karlsson M, Orwar O. A microfluidics approach to the problem of creating separate solution environments accessible from macroscopic volumes. Anal Chem 2004;76:4968–76.
[45] Pihl J, Karlsson M, Chiu DT. Microfluidic technologies in drug discovery. Drug Discov Today 2005;10:1377–83.

[46] Pihl J, Sinclair J, Sahlin E, Karlsson M, Petterson F, Olofsson J, et al. Microfluidic gradient-generating device for pharmacological profiling. Anal Chem 2005;77: 3897–903.
[47] Sinclair J, Olofsson J, Pihl J, Orwar O. Stabilization of high-resistance seals in patch-clamp recordings by laminar flow. Anal Chem 2003;75:6718–22.
[48] Mathes C. QPatch: the past, present and future of automated patch clamp. Expert Opin Ther Targets 2006;10:319–27.
[49] Tao H, Santa Ana D, Guia A, Huang M, Ligutti J, Walker G, et al. Automated tight seal electrophysiology for assessing the potential hERG liability of pharmaceutical compounds. Assay Drug Dev Technol 2004;2: 497–506.
[50] Papke RL. Estimation of both the potency and efficacy of alpha7 nAChR agonists from single-concentration responses. Life Sci 2006;78:2812–9.
[51] Papke RL, Meyer E, Nutter T, Uteshev VV. Alpha7 receptor-selective agonists and modes of alpha7 receptor activation. Eur J Pharmacol 2000;393:179–95.
[52] Papke RL, Porter Papke JK. Comparative pharmacology of rat and human alpha7 nAChR conducted with net charge analysis. Br J Pharmacol 2002;137:49–61.
[53] Uteshev VV, Meyer EM, Papke RL. Activation and inhibition of native neuronal alpha-bungarotoxin-sensitive nicotinic ACh receptors.. Brain Res 2002;948:33–46.
[54] Kowal. Soc Neurosci Abs. 2006.
[55] Broad LM, Zwart R, Pearson KH, Lee M, Wallace L, McPhie G, et al. Identification and pharmacological profile of a new class of selective nicotinic acetylcholine receptor potentiators. J Pharmacol Exp Ther 2006;318:1108–17.
[56] Feuerbach D, Lingenhohl K, Dobbins P, Mosbacher J, Corbett N, Nozulak J, et al. Coupling of human nicotinic acetylcholine receptors alpha 7 to calcium channels in GH3 cells. Neuropharmacology 2005;48:215–27.