Application Note

Activation and pharmacology of fast desensitizing nAChR α7 using specialized NPC-384 chips and fast pipetting speed on the SyncroPatch 384

Eurofins DiscoverX, USA.
Nanion Technologies GmbH, Munich.

Ion Channels:
nAChR α7/ric3

Cells:
HEK-293

Tools:
SyncroPatch 384

Summary

The nicotinic acetylcholine receptor (nAChR) is a member of the ligand-gated ion channel superfamily which includes $GABA_A$, $5HT_3$, NMDA and glycine receptors. It is a cation-permeable ion channel activated by the neurotransmitter acetylcholine and the natural alkaloid, nicotine. Neuronal nAChR are pentameric and functional channels are formed from a repertoire of nine α (α2 to α10) and three β subunits (β2 to β4). Most nAChR exist as heteromers with the stoichiometry 2α to 3β, however some α subunits function as homomers, these being α7 or α9 (for reviews see Refs. 1 & 2). nAChR have been proposed to play a role in many neurological disorders such as Alzheimer's disease, Parkinson's, schizophrenia and depression[1-4]. nAChR α7 are widely distributed in the mammalian brain including in the cerebral cortex, hippocampus, basal ganglia and cerebellum[4]. There is evidence that nAChR α7 play a role in cognition[5,6] and could be a potential therapeutic target in cognitive disorders such as Alzheimer's disease or schizophrenia[3,5,6].

Despite the therapeutic potential of targeting nAChR α7, studying this ion channel using patch clamp electrophysiology has faced numerous challenges due to the receptor's rapid desensitization, low ion channel conductance, and limited availability of selective ligands. To address these issues, recent innovations include the development of a stable nAChR α7/ric3 HEK cell line by Eurofins DiscoverX, which improves receptor expression and enables enhanced acetylcholine response with positive allosteric modulators (PAMs) such as PNU-120596. These advancements help overcome the challenges associated with receptor desensitization, allowing for more reliable assays. In addition, Nanion Technologies has introduced the NPC-384T F/M-STIM chip, engineered for the SyncroPatch 384 system to enhance the precision and speed of ligand applications critical for the reliable activation of fast-desensitizing ion channels including nAChR α7. Compared to standard chips, F/M-STIM chips deliver superior performance with higher seal resistance, greater current amplitudes, and faster response kinetics, while maintaining consistency in EC_{50} values for ligands such as acetylcholine, nicotine, and the PAM PNU-120596.

Results

HEK cells stably expressing nAChR α7 were captured to the standard NPC-384T chips and the newly developed F/M-STIM NPC-384T chips with excellent success rates (Figure 1A). In fact, the cells captured to the F/M-STIM chips with a slightly higher success rate than the standard chips. This was particularly apparent for the multihole chips with 4 (4X) or 8 (8X) holes per well. We used a fast pipetting speed and small

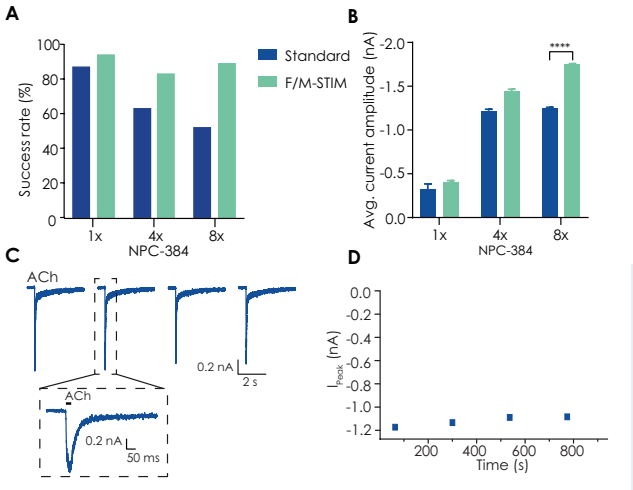


Figure 1: A *Success rate for cell capture for NPC-384T chips.* **B** *Average current amplitude of acetylcholine activated currents on standard or F/M-STIM NPC-384T chips.* **C** *Repetitive activation of nAChR α7 by ACh, currents were elicited at least 4 times in the same cell with similar peak amplitude. Inset shows enlarged example.* **D** *Corresponding timeplot of the experiment shown in* **C**.

volume of ligand to rapidly activate nAChR α7 and minimize exposure time down to 45 ms. When activated with 100 µM acetylcholine (ACh), current amplitude was consistently larger when using the F/M-STIM chips (Figure 1B), although this was only statistically significant for the 8X chips. When activated using 100 µM ACh repetitively four times in the same cell with approximately 4 mins recovery time in between each addition of ACh, peak current amplitudes were highly reproducible (Figure 1C,D). This gives us confidence that the cell line can be used for reliable pharmacology measurements on the SyncroPatch 384.

Once we had confirmed that success rates were improved and current amplitudes were larger when using the NPC-384 F/M STIM chips, we investigated whether the EC_{50} for the activating ligands acetylcholine (ACh) or nicotine were changed. Using multihole chips with 4 holes per well (4X) we activated nAChR α7 with increasing concentrations of either ACh or nicotine. Each well received a single concentration of the test ligand concentration, followed by a maximum activation concentration and the concentration response curves were calculated across multiple wells, each normalized to its own full activation response. Figure 2 shows the concentration response curve for nAChR α7 by ACh using either standard or F/M-STIM NPC-384T chips. The concentration response curves overlaid exactly with almost identical EC_{50} values of 103 µM (standard; n = 100 wells) and 108 µM (F/M-STIM; n = 135 wells). The EC_{50} values are also in excellent agreement with the literature[7,8]. Figure 3 shows the same experiment using nicotine as the ligand. The concentration response curves for nicotine activation of nAChR α7(Figure 3) for standard and F/M-STIM chips also overlay almost exactly with almost identical EC_{50} values of 34 µM (standard; n = 146 wells) and 32 µM (F/M-STIM; n = 145 wells). These EC_{50} values are also in excellent agreement with published data[8,9].

To further characterize nAChR α7, we investigated the effect of the inhibitor methyllycaconitine (MLA) and the positive allosteric modulator (PAM) PNU120596. MLA is a natural toxin found in the seeds of the plant *Delphinium brownii*. It has been shown to be much more potent on neuronal nAChR α7 than muscle nAChR[10] and is a useful pharmacological tool to distinguish certain subtypes of native nAChR. We activated nAChR α7 using 100 µM ACh and found that MLA blocked nAChR α7 responses with an IC_{50} of 2.6 nM (Figure 4; n = 317 wells), consistent with the literature[11].

Given the interest in nAChR α7 as a potential therapeutic target to improve cognitive performance and treat neurodegenerative diseases such as Alzheimer's, we investigated the positive allosteric modulation of PNU-120596, a specific modulator of α7 containing receptors[12], on nAChR α7 responses. Figure 5 shows the concentration response curve for PNU-120596 potentiation of nAChR α7 activated by 100 µM ACh. The concentration response curve is very steep with a Hill coefficient of 7.25 revealing an EC_{50} of 874 nM (n = 284 wells). This is in excellent agreement with the literature[12].

The results shown here demonstrate the robust performance of the NPC-384T F/M-STIM chips for recording nAChR α7. Due to the precise positioning of the patch clamp aperture directly underneath the inlet of the pipette, coupled with the fast application speed, we can rapidly and consistently activate these fast desensitizing ion channels. Modulating compounds can be either preincubated and co-applied with the ligand, or co-applied without preincubation, giving flexibility for investigating mode of action. All experiments were performed at room temperature, but the experiments could be performed at elevated temperature to investigate what effect this may have on the modulation properties of compounds on nAChR, as has been previously demonstrated[13]. Increasing temperature to 40°C can have a profound effect on the modulatory effects of PNU120596, being almost ineffective at 40°C[13] compared with the large modulatory effect at room temperature (20°C)[13], which could have signifcant implications for drug discovery of PAMs targeting nAChR α7.

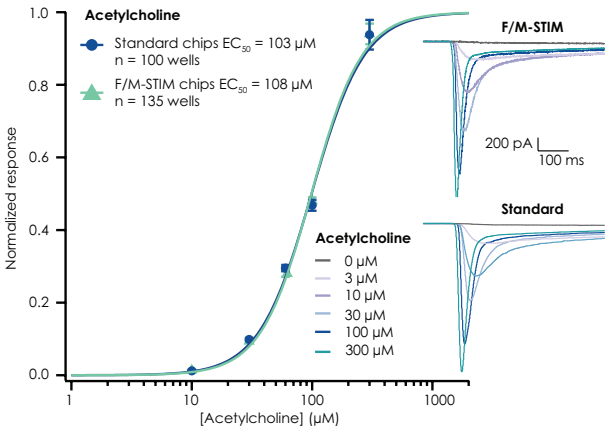
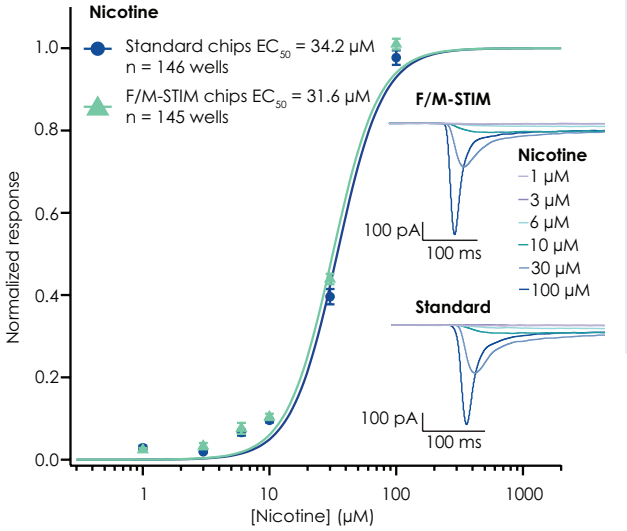


Figure 2: *Concentration response curves for nAChR α7 activation by acetylcholine using standard NPC-384T and F/M-STIM chips. The CRCs overlay exactly and the EC_{50} for acetylcholine is unchanged. 4X chips were used in this example. Average raw current traces at different concentrations of ACh are shown in the inset for F/M-STIM and standard NPC-384T chips.*

Figure 3: *Concentration response curves for nAChR α7 activation by nicotine using standard NPC-384T and F/M-STIM chips. The CRCs overlay exactly and the EC_{50} for nicotine is unchanged. 4X chips were used in this example. Average raw current traces at different concentrations of nicotine are shown in the inset for F/M-STIM and standard NPC-384T chips.*



Conclusions

We have overcome challenges associated with investigating nAChR α7 by obtaining the nAChR α7/ric3 cell line from Eurofins DiscoverX which improves expression levels of nAChR α7 and coupling this with fast ligand addition on the SyncroPatch 384 and using specially designed NPC-384T chips. The F/M-STIM chips for the SyncroPatch 384 improved success rates for cell capture and led to increased peak current amplitudes. Using this combination we could reliably and repetitively activate nAChR α7-mediated responses and investigate pharmacology including activation by ligands ACh and nicotine, inhibitor MLA and PAM PNU-120596 with IC_{50} and EC_{50} values in exact agreement with the literature.
The results clearly indicate that the combination of the HEK nAChR α7/ric3 cell line, the high throughput automated patch clamp system (SyncroPatch 384), the NPC-384T F/M-STIM chips and the fast speed of addition can be reliably used to investigate activation properties and pharmacology of this challenging ion channel target. We have optimized the solution addition parameters to achieve fast activation using an pipette addition speed of 110 µl/s, and minimized exposure time using a small 'puff' of ligand and rapidly removing the solution from around the cell. This ensures reliable pharmacology of activators, inhibitors and positive allosteric modulators of the nAChR α7 receptor and highlights the suitability of the SyncroPatch 384 to record challenging and fast desensitizing ligand-gated ion channels.

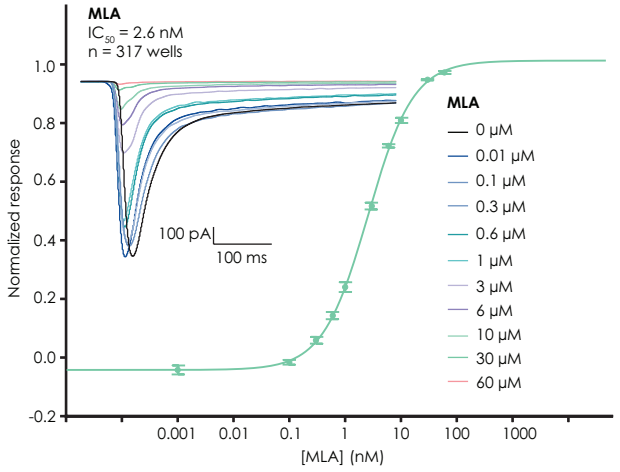


Figure 4: *nAChR α7 receptors were blocked by MLA. Concentration response curve for block of nAChR α7 by MLA using 4X F/M STIM chips. nAChR α7 was activated by 100 µM ACh and blocked by increasing concentrations of MLA. Average raw current traces at different concentrations of MLA are shown in the inset for F/M-STIM NPC-384T chips.*

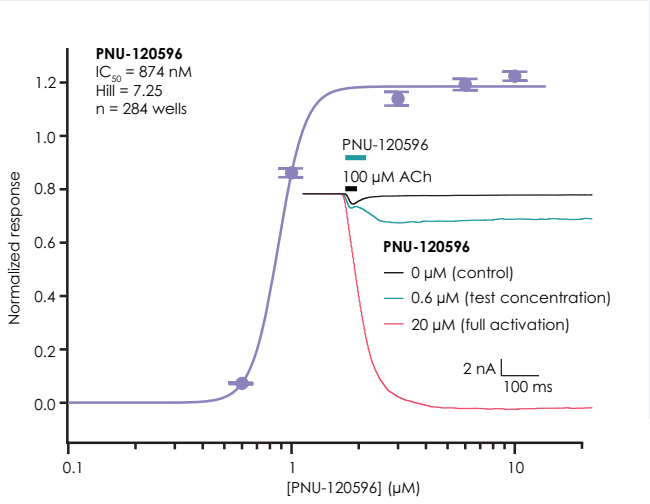


Figure 5: *Concentration response curve for potentiation of nAChR α7 by PNU-120596. nAChR α7 was activated by 100 µM ACh and potentiated by co-application of increasing concentrations of PNU-120596. A full activation of nAChR α7 by 20 µM PNU120596 was used to normalize the CRC. The CRC was very steep with Hill coefficient of 7.25. Raw traces from an example cell are shown for potentiation by 600 nM PNU-120596.*

References

1. Colquhoun, D., *et al*., 2003. in Burger's Medicinal Chemistry, Vol II Drug Discovery and Drug Development, Chapter 11, pp. 357-405. Ed. Abraham, D., J. Wiley, New York.
2. Albuquerque, E.X., *et al*., 2009. Physiol Rev. 89: 73–120. doi:10.1152/physrev.00015.2008.
3. Araki, H., *et al*., 2002. Jpn. J. Pharmacol. 88: 133 – 138. doi: 10.1254/jjp.88.133.
4. Hurst, R., *et al*., 2013. Pharmacol. & Therapeut. 137: 22–5. doi: 10.1016/j.pharmthera.2012.08.012.
5. Leiser, S.C., *et al*., 2009. Pharmacol. & Therapeut. 122: 302 - 311. doi: 10.1016/j.pharmthera.2009.03.009.
6. Pohanka, M. 2012. Int. J. Mol. Sci. 13: 2219-2238. doi: 10.3390/ijms13022219.
7. Andersen, N., *et al*., 2013. PNAS. 110 (51): 20819 - 20824. doi: 10.1073/pnas.1315775110.
8. Virginio *et al*., 2002. Eur. J. Pharmacol. 445: 153–161. doi: 10.1016/S0014-2999(02)01750-8.
9. Fu, X.W., *et al*., 2008. Am. J. Resp. Cell & Mol. Biol. 41: 94-99. doi: 10.1165/rcmb.2008-0352OC.
10. Ward, J.M., 1990. FEBS Letter. 270: 45-48. doi: 10.1016/0014-5793(90)81231-C.
11. Wonnacott S and Barik J (2007) Nicotinic ACh Receptors. Tocris Rev 28. https://resources.tocris.com/pdfs/archive/pdf_downloads/nicotinic_ach_receptors_review.pdf.
12. Hurst, R.S., *et al*., J. Neurosci. 25(17): 4396–4405. doi: 10.1523/JNEUROSCI.5269-04.2005.
13. Milligan, C., & Möller, C. 2013. In: Ion Channels: Methods and Protocols, Methods in Molecular Biology, Chapter 13, pp. 171 - 187. Ed. Gamper, N. Springer Science+Business Media LLC.

Methods

Cells

HEK-293 cells expressing nAChR α7/ric3 (CYL3097) were kindly provided by Eurofins DiscoverX.

Electrophysiology

Whole cell patch clamp recordings were conducted according to Nanion's standard procedure for the SyncroPatch 384 employing ligand puff activation approach and NPC-384T F/M-STIM chips. Nanion's internal solution was CsF based (08 3008), external recording solution was Nanion's standard external solution (08 3001) with 3 mM $CaCl_2$.

For activation pharmacology experiments, a small volume (5 µl) ACh or nicotine was stacked inside the pipette with 5 µl wash solution and rapidly applied to the cell at a speed of 110 µl/s and then 40 µl solution was rapidly removed from each well. Cells were held at a continuous holding potential of -80 mV. For inhibitor and potentiator experiments, nAChR α7 was activated 3 times with 100 µM ACh as described above to establish a stable baseline and then co-applied with either the inhibitor or the PAM. In all experiments a single test concentration of activator, inhibitor or PAM was added to each well and for activator and PAM experiments, a full activation with maximum concentration of ligand or PAM was performed at the end of the experiment which was used for normalization. CRCs were constructed and fit with a Hill equation to calculate EC_{50} and IC_{50} values using DataControl 384.

Nanion Technologies GmbH I Ganghoferstraße 70a, 80339 Munich, Germany
+49 89 2190 95-0 I info@nanion.de I www.nanion.de