Deciphering the properties of nicotinic acetylcholine receptors using α-Bungarotoxin

Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channel proteins activated by the binding of acetylcholine (ACh). Seventeen different nAChR subunits have been identified so far in mammals, including ten α (α1–10), four β (β1–4), γ, δ, and ε subunits.[1]

nAChRs play important physiological roles in the body, particularly in the brain, autonomic nervous system, and at neuromuscular junctions. They have also been implicated in various neurological diseases such as Alzheimer's and Parkinson's disease, substance dependence and addiction, depression, and some immunological disorders.[2] Given their potential roles in disease development and progression, nAChRs are important therapeutic targets for drug discovery endeavors. However, elucidating the function of particular nAChRs can be challenging due to the vast diversity of nAChR subtypes.

A variety of toxins from algae, plants, and animals target neuronal nAChRs by inhibiting the action of ACh at the receptor. These naturally occurring antagonists have been used extensively in research as a tool to explore the molecular mechanisms of ligand-binding at neuronal nAChR subtypes. One such toxin is α-Bungarotoxin, which is a 74 amino acid peptidyl toxin isolated from the venom of the banded krait snake, *Bungarus multicinctus*. In this literature review, we highlight three studies that utilize α-Bungarotoxin labeled with ^{125}I to study nAChR function and cell-surface expression.

Upregulation of nAChR binding following chronic cigarette smoke exposure

Researchers based at the University of California recently investigated the effects of chronic exposure to cigarette smoke constituents on nAChR radioligand binding levels in adult and adolescent rodents.[3] nAChR upregulation has been implicated as a contributing factor to tobacco dependence, so the team explored whether non-nicotine constituents could be contributing to some of the addictive properties of smoking. It is hoped these findings could aid the development of nAChR-based therapeutics for smoking cessation in the future.

In the first stage of their investigation, adolescent and adult rats were exposed to cigarette smoke extract (CSE) or nicotine daily for 10 consecutive days. The researchers then used quantitative autoradiography to measure receptor binding changes in three nAChR types, α4β2, α3β4, and α7, using ^{125}I-Epibatidine or ^{125}I-α-Bungarotoxin. They focused on brain areas involved in addiction and negative emotional states, including the amygdala nuclei and interpeduncular nucleus.

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For the α4β2 nAChR labeling, slides were incubated with 0.08 nM ^{125}I-Epibatidine. Of note, the team only analyzed brain areas containing at least 85% expression of α4β2 due to ^{125}I-Epibatidine's affinity for other nAChR types. Experimental conditions were identical for α3β4 nAChR labeling, except that 200 nM of the smoking cessation drug cytisine was added to the incubation solution to block binding to α4β2 nAChRs. To label α7 nAChRs, 5 nM of ^{125}I-α-Bungarotoxin was used under similar conditions.

Overall, analysis revealed that cigarette smoke constituents upregulate α4β2, α3β4, and α7 nAChR binding in both adult and adolescent rodents. In contrast, chronic nicotine exposure did not significantly increase binding in any brain region at either age under the study conditions.

Adolescents showed higher levels of ^{125}I-Epibatidine binding to α4β2 nAChRs than adults in the majority of brain regions analyzed. Notably, upregulation of α4β2 nAChRs in the medial amygdala was observed in adolescents, but not adults, following CSE treatment. CSE also increased α3β4 nAChR binding in the medial habenula and interpeduncular nucleus in both age groups. These two brain regions have previously been implicated in nicotine withdrawal.

For α7 nAChRs, adolescents showed higher levels of ^{125}I-α-Bungarotoxin binding than adults in a treatment-specific manner in the majority of the areas analyzed. Chronic CSE exposure led to an increase in α7 nAChR binding in the medial amygdala independent of age, and the central amygdala and lateral hypothalamus of adolescents. The latter two regions have been shown to mediate the shift to negative reinforcement and the negative emotional state of withdrawal, which is a key factor in dependence.

Commenting on their findings, the researchers said their study provides evidence that cigarette smoke constituents influence nAChR upregulation in an age-, nAChR type-, and region-dependent manner. The higher upregulation observed in adolescents in many brain regions suggests that nAChR pharmacology differs between adults and adolescents. However, both age groups were susceptible to the CSE-induced upregulation of nAChR binding. They concluded that a better understanding of this phenomenon is needed to aid the development of nAChR-based therapeutics for smoking cessation and to potentially improve efficacy and specificity for different age groups.

Identifying positive allosteric modulators of the α7 nAChR

In another study, Waheed Shabbir (University of California) and colleagues set out to explore the modulatory effects of a panel of structurally related flavonoids on the function of α7 nAChRs using ^{125}I-α-Bungarotoxin.[4] Flavonoids are known to have anti-inflammatory, antioxidant, neuroprotective, and pro-cognitive effects, and several of these phytochemicals, such as genistein and quercetin, have previously been shown to act as positive allosteric modulators (PAMs) of

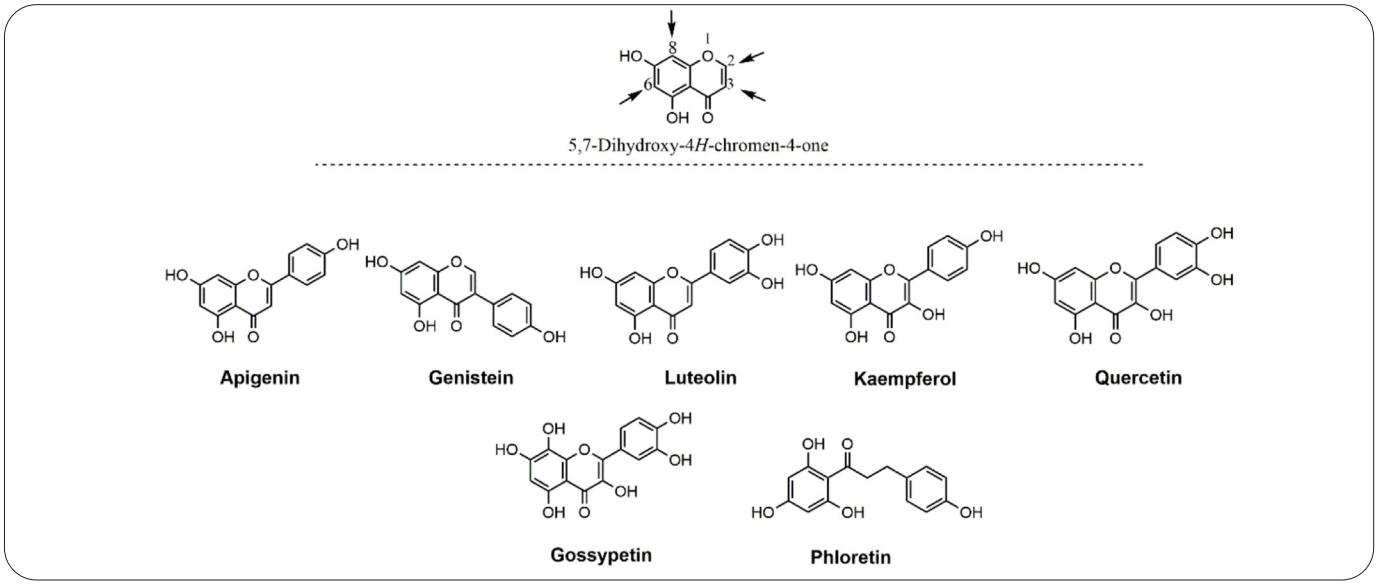

Figure 1: Features similarities between the tested polyphenol flavonoids as derivatives of 5,7-dihydroxy-4H-chromen-4-one.
Figure credit: Shabbir W, Yang K, Sadek B, Oz M (2020).[4]

the α7 nAChR receptor. Furthermore, several preclinical models have recognized α7 nAChRs as potential therapeutic targets for the treatment of pain, inflammation, various neurodegenerative diseases, and psychosis.

For the first stage of their investigation, the team assessed the stimulatory effects of 10 µM of apigenin on α7 nAChRs by measuring intracellular Ca^{2+} levels in Fluo-4 loaded SH-EP1 cells. Analysis revealed that apigenin alone had no effect on intracellular Ca^{2+} levels whereas addition of ACh rapidly increased Ca^{2+} concentrations. Interestingly, this positive modulatory effect was significantly diminished with increasing ACh concentrations. Similar effects were observed with the other flavonoids tested, with a potency order of genistein > gossypetin > kaempferol > luteolin, phloretin, and quercetin.

Once the researchers had determined that the flavonoids were potentiating the function of α7 nAChRs, they used radioligand binding experiments to investigate their effect on the binding of ^{125}I-α-Bungarotoxin at the receptor. This required the addition of 150 µL of cell suspension, 50 µL of ^{125}I-α-Bungarotoxin, and 50 µL of test compound to 96-well plates followed by 45 minutes incubation. Plates were then filtered through Unifilter GF/C plates (Revvity) washed, and the radioactivity bound to filters was counted in 50 uL of MicroScint 40 (Revvity) in a Packard TopCount scintillation counter.

As shown in Figure 2, the researchers observed no significant change in ^{125}I-α-Bungarotoxin binding in SH-EP1 cells preincubated with apigenin, which suggests that apigenin acts as a PAM of α7 nAChRs. Similar results were observed when they tested the effects of the other flavonoids on ^{125}I-α-Bungarotoxin binding. The researchers note that PAMs have shown promise as therapeutic agents due to their ability to maintain the temporal and spatial characteristics of the endogenous activation of the receptor and because they are usually more selective than agonists.

Since compounds that modulate α7 nAChR function may have potential significance for the treatment of pain, inflammation, and neurodegenerative disorders, the group concludes that apigenin and the structurally related flavonoids tested in this study all showed promising drug-likeness values and have potential for future drug discovery ventures to treat these conditions.

Studying the role of molecular chaperones in α7 nAChR expression

Another team of researchers from Northeastern University and the University of Minnesota explored the regulatory role of two molecular chaperones of α7 nAChR, Resistance to Inhibitors of Cholinesterase 3 (RIC3) and TMEM35A/Nicotinic Acetylcholine Regulator (NACHO), in α7 nAChR cell surface expression.[5] Molecular chaperones are a group of proteins that assist in the folding, assembly, and surface expression of proteins. While RIC3 and NACHO are known to be α7 nAChR chaperones, their precise role in receptor expression is still poorly understood.

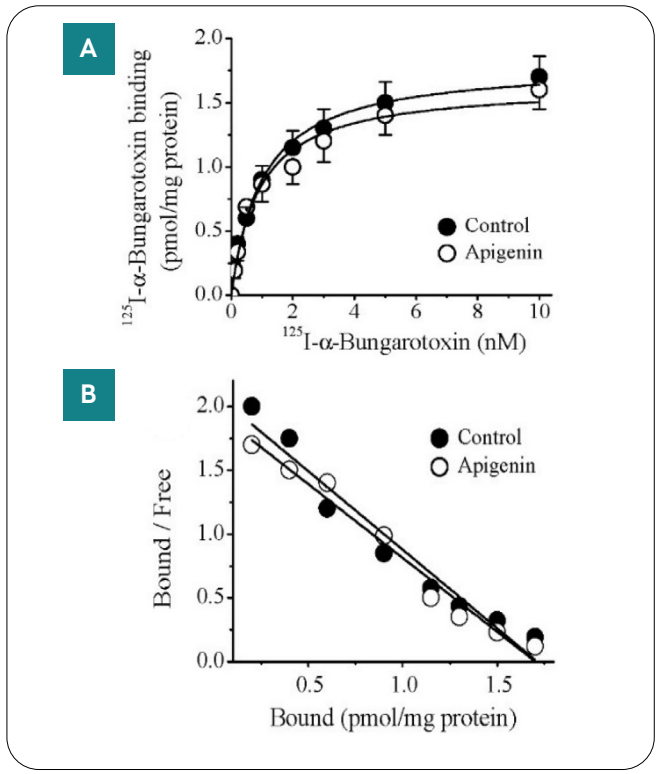

Figure 2: (A) The effect of apigenin on the binding saturation of ^{125}I-α-Bungarotoxin. Increasing concentrations of ^{125}I-α-Bungarotoxin are shown in X-axis as free ligand. SH-EP1 cells were incubated for 45 min with the indicated concentrations of ^{125}I-α-Bungarotoxin in the absence (filled circles) and presence (open circles) of apigenin (10 µM). Unlabeled bungarotoxin (3 µM) was added to incubation buffer to determine non-specific binding (n = 4–6) (B) Scatchard analysis, apigenin effects on saturation binding of ^{125}I-α-Bungarotoxin. Units are fmol/mg protein and fmol/mg protein/nM for x and y axis, respectively. Figure credit: Shabbir W, Yang K, Sadek B, Oz M (2020).[4]

The team first tested whether NACHO is required for surface expression of α7 nAChR by correlating the presence of NACHO in various primary and transformed cell types with their ability to bind ^{125}I-α-Bungarotoxin when the gene

encoding the α7 nAChR subunit *(chrna7)* was present. Binding was assessed by incubating cells with 10 nM ^{125}I-α-Bungarotoxin for three hours. They were then washed, lysed, and counted in a Packard Cobra gamma counter.

Analysis revealed that some cell lines demonstrated surface expression of α7 nAChR but did not express NACHO (Figure 3), suggesting that in some cells NACHO may not be required for the folding, assembly, and trafficking of the α7 nAChR.

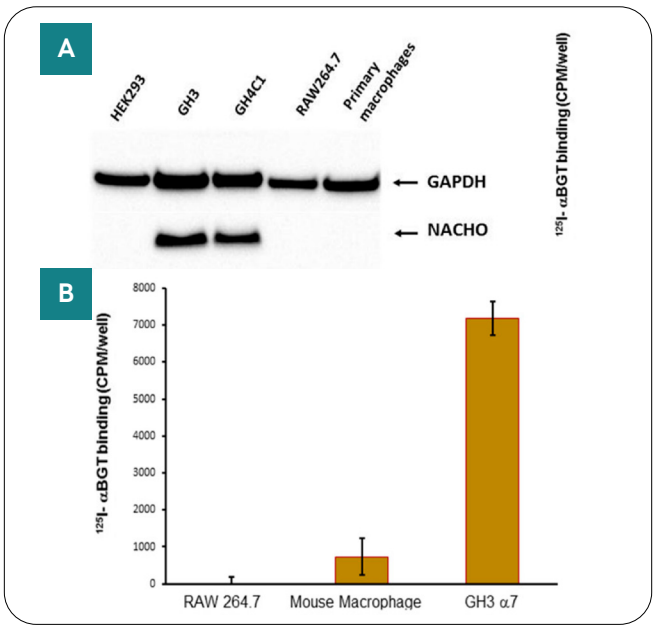

Figure 3: (A). Western Blot analysis of NACHO expression. GH3 and GH4C1 cells express endogenous NACHO, but mouse macrophage-like RAW264.7 cells, primary mouse macrophages, and HEK-293 do not. (B) *In vitro* ^{125}I-α-Bungarotoxin binding assessments. Primary cultured macrophages and GH3 cells transfected with rat chrna7 plasmid, but not RAW264.7 mouse macrophage-derived cells, showed ^{125}I-α-Bungarotoxin binding. Figure credit: Deshpande A, Vinayakamoorthy R, Garg B, Thummapudi J, Oza G, Adhikari K *et al.* (2020).[5]

Later in their study, the researchers generated ric3 and tmem35a KO mice and compared ^{125}I-α-Bungarotoxin binding between wild type and KO animal brain slices. The team notes that although knockout (KO) mice exist for the gene that codes for NACHO (tmem35a), there is a lack of animal models to determine the role of RIC3. In the present study, mouse brains were frozen, sectioned, and incubated for three hours with 5 nM ^{125}I-α-Bungarotoxin. Slides were then pressed against phosphor screens and processed on a Cyclone Phosphor Imager.

The autoradiographic analysis revealed that ric3 KOs showed subtle ^{125}I-α-Bungarotoxin binding changes across different mouse brain regions, whereas *tmem35a* KOs showed a complete loss of ^{125}I-α-Bungarotoxin binding (Figure 4). The researchers observed that these findings were inconsistent with effects observed *in vitro*, where RIC3 was shown to promote ^{125}I-α-Bungarotoxin binding to α7 nAChRs expressed in HEK cells, even in the absence of NACHO expression (Figure 5).

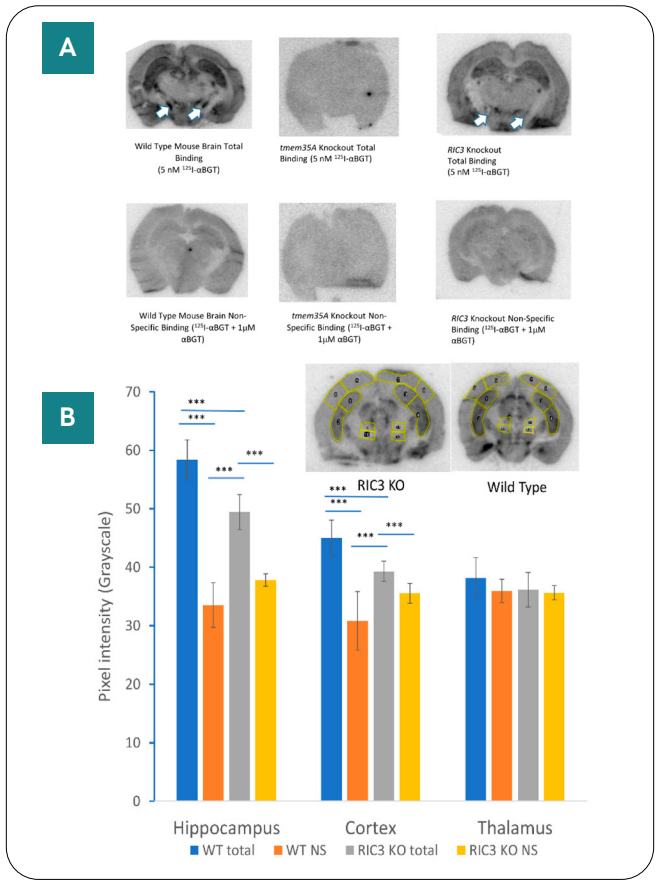

Figure 4: A) Autoradiographic comparison of ^{125}I-α-Bungarotoxin binding between wild type and KO animal brain slices. Top row shows total binding for wild type (left), tmem35a KO (middle) and ric3 KO (right) brain sections. The bottom row shows corresponding non-specific binding. There was no specific binding in tmem35a KO, and significant loss of binding in specific brain structures in the ric3 KO brains (arrows). (B) Autoradiographic analysis of ^{125}I-α-Bungarotoxin binding using ImageJ. Significant loss of toxin binding was observed in the hippocampus and cortex of the ric3 KO compared to the corresponding structures in wild type (WT) animals (Specific binding is the difference between total binding and non-specific [NS] binding). The insets show typical sections and the areas used for analysis over two sections per condition (N = 8 areas per brain region, with a medial and lateral area for each brain side times two sections). This analysis was done on one experiment comparing one animal per condition since the two experiments performed so far were done using different batches of ^{125}I-α-Bungarotoxin with different specific activities and slightly different exposure times and are not easily comparable. Error bars represent standard deviations. *** $p > 0.001$, (** $p < 0.01$, * $p < 0.05$) by single factor ANOVA. Figure credit: Deshpande A, Vinayakamoorthy R, Garg B, Thummapudi J, Oza G, Adhikari K *et al.* (2020).[5]

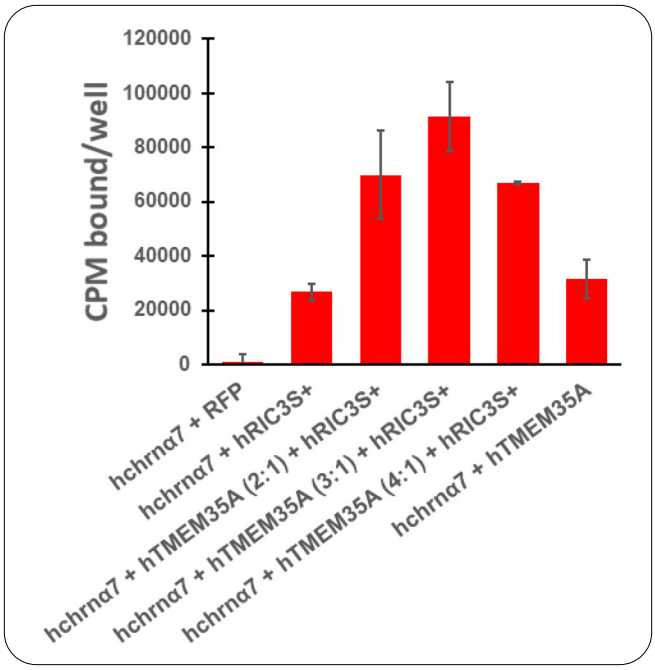

Figure 5: The absence of NACHO in HEK cells has no effect on the ability of RIC3 to promote surface human α7 nAChR expression, and the effects of the two chaperones are synergistic when expressed together. Binding assays in 24-well plates were performed. Total cDNA in transfections was constant, with hchrna7 DNA (0.15 µg/well) that was equaled the sum of htmem35a and hric3 cDNA or RFP DNA (0.15 µg/well RFP DNA in transfection controls). The ratio of three parts htmem35a cDNA to one part hric3 cDNA (e.g., 0.11 µg htmem35a and 0.04 µg hric3/well) produced the highest surface α7 nAChR expression in HEK cells. In all four experiments, the combined effects were more than additive. In experiments where RIC3 or NACHO was the only chaperone, surface α7 nAChR expression was comparable between these two chaperones as shown. Figure credit: Deshpande A, Vinayakamoorthy R, Garg B, Thummapudi J, Oza G, Adhikari K *et al.* (2020).[5]

Because the *in vitro* data shows a different picture of chaperone action compared to *in vivo* studies, the researchers concluded that additional regulatory factors are likely involved in the *in vivo* expression of α7 nAChRs.

Conclusion

Given their potential role in various neurological conditions, there is growing interest in the development of drugs that modulate nAChR functions. However, research into nAChRs has been hampered by the diversity of these receptors and complex expression patterns. Here we highlighted three research papers that aimed to elucidate the role and function of various nAChRs utilizing radiolabeled snake venom, α-Bungarotoxin.

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