Nicotinic acetylcholine receptors in mitochondria: subunit composition, function and signaling

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Nicotinic acetylcholine receptors (nAChRs) of neuronal type not only mediate the fast synaptic transmission, but also modulate proliferation, cytokine or transmitter release and survival in both excitable and non-excitable cells. Recent studies clearly indicate that these receptors can stimulate intracellular signaling in ion-independent manner. Classically, the nAChRs were attributed exclusively to the cell plasma membrane. The present review provides evidence for the expression and functioning of nAChRs in intracellular organelles, specifically mitochondria. It is shown that mitochondria can express α7β2, α3β2, α3β4 and α4β2 nAChRs in a tissue-specific manner. Mitochondrial nAChRs are found in the outer membrane in conjunction with voltage-dependent anion channels and regulate the formation of mitochondrial permeability transition pore releasing pro-apoptotic substances like cytochrome c or reactive oxygen species. The nAChR signaling in mitochondria does not require the ion flow through nAChR ion channels. Instead, it can be stimulated by the binding of agonists, antagonists or nAChR-specific antibodies and engages intramitochondrial kinases, similar to those activated by plasma membrane nAChRs. Mitochondrial nAChRs follow, similar to plasma membrane nAChRs, biosynthetic post-translational pathways. In particular, one of the signals targeting them to mitochondria might be extra sialiation and fucosylation. Mitochondrial nAChRs form an additional line of defense for the cell survival, and their therapeutic targeting may be important for treating cancer and neurodegenerative diseases.

Keywords: Nicotinic acetylcholine receptor; mitochondria; apoptosis; cytochrome c; intramitochondrial kinases; glycosylation; neurodegeneration


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Introduction

Nicotinic acetylcholine receptors (nAChRs) were initially discovered in the neuro-muscular junctions and autonomic ganglia to mediate fast synaptic transmission and then in the brain to regulate transmitter release and to underlie nicotine dependence in smoking people [1]. During the last decade it has become clear that cholinergic regulation is universal and evolutionary ancient. The nAChRs were found in many animal non-excitable cells [2-4], in plants [5], and their prototype has been discovered in bacteria [6] to mediate vital cellular functions like proliferation, survival, adhesion and motility. However, traditionally, functional nAChRs were attributed exclusively to the cell plasma membrane, and their intracellular pool was regarded as immature biosynthetic precursors on the way to the membrane surface [7].
Structurally, nAChRs are homo- or heteropentamers composed of 10 α (α1 to α10), 4 β (β1 to β4), γ, δ, and ε subunits found in various combinations to form nAChR subtypes of different properties, functions and localization [8-9]. All of them belong to the family of ligand-gated ion channels opened by the binding of acetylcholine or other subtype-specific agonists. Homomeric α7 nAChRs are considered to be the most evolutionary ancient [10]; they are found in both neurons and non-excitable cells to control cell viability [11-12], motility [13], activation [14], as well as angiogenesis [15] and inflammation [16].

The nAChRs were traditionally classified as ionotropic receptors mediating rapid electrochemical signals; in contrast to muscarinic acetylcholine receptors, which were thought to be metabotropic and to be coupled to intracellular signaling cascades. However, many experimental data clearly indicate that such classification is not absolute. Cytoplasmic parts of nAChR subunits were found to be coupled to intracellular kinases to engage numerous signaling cascades resulting in cell proliferation, survival or cytokine production [17-19].

Experiments performed in our laboratory initially concerned the role of nAChRs in B lymphocyte development and activation. In the course of these studies, it was found that much more B lymphocyte precursors died by apoptosis in the bone marrow of α7/-/- mice compared to the wild-type counterparts [20]. Further studies demonstrated that the α7-selective agonist choline prevented the death of DT40 pre-B lymphoma cells caused by the mitochondria-specific apoptotic agent hydrogen peroxide, and its effect was withdrawn by the α7-specific competitive antagonist methyllycaconitine (MLA) [21]. These data for the first time attracted our attention to the potential role of α7 nAChRs in mitochondrial functioning and initiated a set of studies resulting in the discovery of mitochondrial nAChRs.

**Structure, location and functions of nAChRs in mitochondria**

Mitochondria are responsible for cell bioenergetics, Ca\(^{2+}\) buffering and initiation of mitochondrial pathway of apoptosis. They are quite autonomous within the cell and, according to the endosymbiotic hypothesis, they were originated from ancient bacteria [22]. Structurally, they are composed of two membrane layers surrounding the intramitochondrial matrix. Ca\(^{2+}\) ions are accumulated in the matrix, the respiratory chain is located in the inner membrane, matrix and intermembrane space, while the outer membrane regulates the exchange between mitochondria and cytosol. During the last years, many receptors initially attributed exclusively to the cell plasma membrane have been found in mitochondria [23].

**Initial studies on mitochondrial nAChRs**

The presence of nAChRs in mitochondria has been being discussed since 1984, when Ronald Lukas published his seminal paper showing specific [22]I-α-bungarotoxin binding on mitochondria preparations from the rat brain [24]. Certain indirect data appeared to show that autoantibodies of patients with biliary cirrhosis recognized similar antigens in liver mitochondria and in nAChR-enriched membranes [25]. However, at that time the presence of classical plasma membrane receptors in intracellular organelles seemed absolutely incredible. Immunogold labeling of serial ultrathin sections from the rat hippocampus performed later demonstrated the α7-specific antibody binding to mitochondria. However, this was not confirmed by Western blotting and the authors did not take courage to state that α7 nAChRs are expressed in mitochondria [26]. Another line of evidence demonstrated the neuroprotective effect of nicotine against several toxic agents, like ethanol or streptozotozin [27-28]. The direct effect of nicotine on apoptosis-related pathways, including phosphorylation of the anti-apoptotic protein Bad, translocation of Bax and cytochrome c release through Erk-JNK-p38 and PI3K-Akt signaling pathways have also been reported [29-36]. Most of these effects, including the beneficial influence of nicotine on permeability transition pore, cytochrome c (cyt c) and apoptosis-inducing factor release, were attributed to the influence on mitochondria respiratory chain, which was shown to be receptor-independent [31], although in the study of Li et al. [27] the effect of ethanol on mitochondria membrane potential was attenuated by MLA. The important role of acetylcholine (ACh) for mitochondria functions, dynamics and movement within the cell has been demonstrated with the use of organophosphate-based pesticides, which inhibit acetylcholine esterase, thus increasing the concentration of ACh [32]. Therefore, numerous experimental data indirectly evidenced the presence of nAChRs in mitochondria and their involvement in regulating mitochondrial apoptosis pathway.

**Location and function of α7 nAChRs in mouse liver mitochondria**

Taking into account the suggested prokaryotic origin of mitochondria, we hypothesized that they could contain the most evolutionary ancient α7 nAChR. To prove this hypothesis, the overlapping staining of glioblastoma U373 cells with α7-specific antibody and the antibody against mitochondrial outer membrane translocase TOM22 or Mitotracker green, specific to the lipids of mitochondria outer membrane has been demonstrated [33]. Then we observed the binding of immunogold-detected antibodies specific to the extracellular domain of the α7 subunit with isolated mouse liver mitochondria in electron microscopy.
However, this binding was quite rare and non-convincing. The decisive data was obtained in immunosorbent sandwich assays, where the potential nAChR was captured from the mitochondria preparation with the antibody against the whole extracellular domain (1-208) of the α7 subunit and was further revealed with either biotinylated α7(179-190)-specific antibody or fluorescently labeled α-cobratoxin. Mitochondria of α7/−/− mice were used as negative controls. The results revealed positive binding of both the antibody and toxin with the wild-type but not α7/−/− mitochondria and with their outer but not inner membranes [34]. This data clearly demonstrated that α7 nAChRs were present in the outer membrane of mitochondria. Further experiments were undertaken to reveal the functions of this receptor in intracellular compartment.

The isolated liver mitochondria were studied by fluorescent flow cytometry, which enabled gating them according to the size, granularity and binding of cardioliipin-specific dye acridine orange 10-nyl bromide. The levels of mitochondria membrane potential or intramitochondrial Ca²⁺ were studied with specific fluorescent dyes. It was found that mitochondria from mice, which chronically obtained nicotine with the drinking water, had significantly lower basic levels of membrane potential compared to those of control group. Pre-incubating mitochondria isolated from control mice with nicotine prevented dissipation of their membrane potential under Ca²⁺ addition, and this effect was strengthened by MLA. Moreover, mitochondria of mice intravenously injected with α7 nAChR-specific antibody demonstrated lowered membrane potential, which was not decreased further upon Ca²⁺ addition [35].

The mitochondria membrane potential is maintained by the gradient of ions penetrating mitochondria through various transporting complexes and channels. Taking into account the high permeability of α7 nAChRs to Ca²⁺, we suggested that this receptor might be one of the channels mediating Ca²⁺ entry into mitochondria. However, the data obtained demonstrated that, instead of facilitating Ca²⁺ accumulation in mitochondria, α7 nAChR agonists (choline, ACh or PNU-282987) prevented it. The effects were quite small (about 20%) and they were similar to that of DIDS (4,4’-disothiocyanato-2,2’-stilbene disulfonic acid) - the inhibitor of voltage-dependent anion channel (VDAC). VDAC is a main channel for Ca²⁺ exchange between the cytosol and the intermembrane space of mitochondria, one of the main components of the outer mitochondria membrane, and a component of mitochondrial pore formed upon the effect of apoptogenic factors to release apoptosis-inducing factors like cyt c [36-38]. We demonstrated that α7 nAChR interacts directly with VDAC in the outer membrane of mitochondria. This was established with ELISA sandwich assay where mitochondria membrane preparations were captured with the antibody against the α7 extracellular domain (1-208) and were revealed with VDAC-specific antibody or vice versa. Positive signals were obtained in both assays for the outer, but not inner, membrane mitochondria preparation [34]. Therefore, α7 nAChRs seemed to prevent Ca²⁺ accumulation in mitochondria by affecting VDAC.

Apoptogenic doses of Ca²⁺ (9-90 µM) or H₂O₂ (0.5 mM) induced cyt c release from isolated liver mitochondria; that was significantly decreased by the VDAC inhibitor DIDS, demonstrating the involvement of VDAC in mitochondria permeability transition pore (mPTP) formation. Similarly, cyt c release could be attenuated by α7 nAChR agonists such as choline, ACh or PNU-282987, and their effects were more or less efficiently prevented by MLA [34]. This data allowed us to make an important conclusion: that α7 nAChRs interacting with VDAC in the outer membrane of mitochondria regulate mitochondrial pore formation and cyt c release.

Multiple subtypes and subunit composition of mitochondrial nAChRs

To reveal if α7 is the only nAChR subtype expressed in mitochondria we extended the range of subunit-specific antibodies used in the sandwich assay with the outer membranes of liver mitochondria. It was found that, in addition to α7, the preparation contained α3, α4 and β2 subunits. Moreover, α7 subunits appeared to be combined with β2 ones to form heteromeric α7β2 nAChRs. This was shown by the sandwich ELISA assay where the receptor was captured with an α7-specific antibody and revealed with a β2-specific antibody. The signal disappeared after the samples were pre-treated with 2% SDS demonstrating the non-covalent intersubunit interaction. A definite increase of α3 subunits and β4 subunits was observed in mitochondria of α7/−/− mice; the absence of β2 subunits was compensated by β4 ones. It was concluded that, in addition to α7β2 nAChRs, the liver mitochondria contained α3β2 and α4β2 subtypes, and that the lack of either the α7 or β2 subunit was compensated with α3β4 nAChRs. The spectrum of nAChR subtypes expressed in mitochondria appeared to be tissue specific: liver mitochondria contained mainly α7 nAChRs and less α3 and α4 subunits, brain mitochondria contained more α4-containing nAChRs, while lung mitochondria contained mostly α3β4 nAChRs [39]. It seems that all (or most of) nAChR subtypes expressed in a given tissue can be targeted to mitochondria. This suggestion was further confirmed by data from Sergey Grando’s laboratory showing...
immunoprecipitation of α3, α5, α7, α9, α10, β2 and β4 subunits from keratinocyte mitochondrial proteins [40].

α7 nAChRs are traditionally being regarded as homopentamers. However, several lines of evidence indicate that α7 subunits can combine with other (structural) subunits to form heteromeric nAChRs. In 1999, Palma et al. [41] showed that α7 subunits can co-assemble with β3 subunits to form a pentamer, which appeared on the cell membrane, but was not activated by ACh. Later, Khiroug et al. [42] demonstrated that α7 can co-assemble with β2 to form a functional ion channel with kinetic properties slightly different from those of homomeric α7 pentamers. PC-12 cells were shown to express more than one form of α7 nAChR [43]. Kinetic characteristics of MLA-blocked receptors found in electrophysiological experiments suggested the presence of heteromeric α7-containing nAChRs in the submucosal nerve plexus of the guinea-pig [44]. α7 and β2 mRNAs were found to be co-expressed in almost all cholinergic cells and in the majority of GABAergic neurons in the brain [45]. Heteromeric α7β2 nAChRs were further identified in the mouse and human forebrain [46-47] and a model predicting receptor function that is based on stoichiometry and position of β2 subunits within the α7β2 nAChR has been suggested [48]. The determined presence of α7β2 nAChRs in mitochondria may indicate that special kinetic and pharmacological properties of this heteromeric nAChR subtype (low amplitude of agonist-evoked currents, lower sensitivity to agonists, slow whole-cell current kinetics) mostly correspond to the mode of cholinergic regulation in these intracellular organelles.

The mechanism of nAChR functioning in mitochondria

According to the data of sandwich ELISA, all mitochondrial α nAChR subunits were coupled to VDAC and, therefore, could be expected to influence mitochondrial pore formation and cyt c release. This was indeed shown with the micromolar ACh concentrations able to activate α3β2 and α4β2 but not α7-containing nAChRs [39]. To understand the mechanism of nAChR involvement in regulating mitochondrial functions, we suggested that the initial signal could be the cation flow through the nAChR ion channel affecting intramitochondrial signaling machinery. However, it was found that cyt c release could be attenuated not only by nAChR agonists, but also, less efficiently, by competitive antagonists like MLA (50 nM, for α7 nAChRs), conotoxin MII (1 nM, for α3β2 nAChRs), dihydro-β-erythroidine (DHβE, 1 μM, for α4β2 nAChRs) and even by α7-specific antibodies [49]. The positive allosteric modulator (PAM) PNU-120596, which presumably prolongs the open state of the α7 ion channel [50], did not improve the effect of agonists but slightly attenuated cyt c release itself. Similarly, the release of reactive oxygen species from isolated mitochondria, which also occurs through the mitochondrial pore, could be attenuated by either ACh (1 mM) or MLA (10 nM) [49]. This data clearly indicated that the nAChR-mediated ion flow was not critical for attenuating cyt c or reactive oxygen species release. Instead, certain changes in the receptor molecule induced by the agonist, antagonist, allosteric modulator or even antibody binding seemed to be sufficient to stimulate the mitochondrial nAChR signaling.

The nAChR pentamer is highly flexible and capable of significant molecular movements. The agonist binding is followed by a gating process that involves a large reorganization of the receptor mediated by two distinct quaternary transitions: a global twisting and a radial expansion/contraction of the extracellular domain [51]. In addition, three allosteric binding sites have been identified in the extracellular domain of the α7 subunit. One of them is located near the N-terminal α-helix of the extracellular domain, another one is found in a preexisting intrasubunit pocket opposite the agonist binding site and the third site is located at a pocket right below the agonist binding site. Ligating these sites can induce substantial conformational changes in the nAChR molecule [52].

In addition to the direct effect on cell membrane potentials, nAChR activation triggers multiple intracellular signaling cascades. A signaling pathway downstream the α7 nAChR, involving JAK2/STAT3 and NF-κB, has been implicated in the anti-inflammatory effect of nicotine. Activation of STAT3, via either JAK2 and/or PI3K, through a single (JAK2/PI3K/STAT3) or two convergent cascades (JAK2/STAT3 and PI3K/STAT3), was necessary for nicotine-induced interleukin-1 receptor-associated kinase M expression [53]. Neuroprotective effects of nicotine in cultured cortical neurons were mediated by the α7 nAChR and PI3K-Akt signaling pathway [54]. Activation of α7 nAChRs linked to the Jak2/PI3K/Akt cascade induced the antioxidant enzyme HO-1 to provide neuroprotection in SH-SY5Y cells [11]. It was suggested that α7 nAChR stimulates the Src kinase family, which activates PI3K to phosphorylate Akt, which subsequently transmits the signal to up-regulate Bcl-2 and Bcl-x [19].

Numerous data indicate that the nAChR-initiated signaling can be either ion-dependent or independent. Experiments performed with the α7 nAChR, which contained a point mutation (α7E260A) controlling the exceptional Ca2+ ion conductance of this receptor, demonstrated that Ca2+ ions were required for controlling the expression of several pro-inflammatory cytokine and chemokine RNAs but were

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not critical for inflammatory cell recruitment to the blood in response to LPS administration \[55\]. In other experiments, neuroprotection of SH-SY5Y cells against okadaic acid-induced neurotoxicity mediated by α7 nAChRs was independent of Ca\(^{2+}\) and involved the intracellular signaling pathway Janus Kinase-2/Phosphatidylinositol-3-kinase(P1\(\kappa\)K)/Akt. Moreover, when Ca\(^{2+}\) entry was promoted through the α7 nAChR by using the α7-selective PAM PNU-120596, the observed protection was lost \[56\]. Furthermore, it was shown that the positive allosteric modulation of α7 nAChRs elicited by PNU-120596 could lead to overloading of intracellular Ca\(^{2+}\) and neuronal cell death \[57\]. Therefore, α7 nAChRs can be implicated in both ionotrophic and metabotropic signaling. The latter is thought to be triggered by conformational changes occurring in the nAChR molecule upon the binding of specific ligands. For example, the so-called "silent agonist" NS6740, which selectively induces prolonged desensitization of α7 nAChRs, possessed significant dose- and time-dependent antinoceptive activity in the chronic constrictive nerve injury model for neuropathic pain \[58\]. Application of α7-specific antagonists such as MLA or α-bungarotoxin induced intracellular Ca\(^{2+}\) rise in non-excitatory cells like leukocytes or monocytes \[59-60\]. In both cases, the role of nAChRs was to regulate intracellular signaling of either T-cell antigen-specific receptor or purinergic P2X7 receptors obviously in an ion-independent manner. Experiments of Chernyavsky et al. \[61\] demonstrated the existence of two-component signaling system coupling the ionic events and protein kinase signaling cascades downstream the α7 nAChR in skin keratinocytes. The Raf/MEK1/ERK1/2 cascade up-regulating α2-integrin was activated by both Ca\(^{2+}\)-dependent recruitment of Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) and Ca\(^{2+}\)-independent activation of Ras. Likewise the P1\(\kappa\)K-mediated activation of Rho kinase was elicited by both Ca\(^{2+}\) entry-dependent involvement of CaMKII and Ca\(^{2+}\)-independent activation of Jak2. According to one of the hypotheses, heteromeric nAChRs are specialized in rapid electrochemical signal transduction, while homomeric α7 nAChRs can be implicated in both ionotrophic and metabotropic signaling \[62\]. It seems that mitochondrial α3β2, α4β4 and α7β2 nAChRs regulate mitochondrial pore formation in an ion-independent manner. However, this does not exclude the possibility that they can function as ion channels for other, yet non-identified purposes.

According to recent studies, mitochondria contain their own set of protein kinases involved in regulating many internal mitochondrial processes \[63\]. To reveal which kinases are engaged in mitochondrial pore formation, we applied a set of kinase inhibitors to affect cyt c release from isolated mitochondria. It was found that Ca\(^{2+}\) or H\(_2\)O\(_2\) stimulated cyt c release through different signaling mechanisms: Ca\(^{2+}\) engaged CaMKII and PKC, while the effect elicited by H\(_2\)O\(_2\) was mediated through Src kinase and PKC. Inhibiting these kinases under the effect of Ca\(^{2+}\) or H\(_2\)O\(_2\) affected mitochondria similarly to the α7-specific agonist PNU-282987. Therefore, α7 nAChR signaling could inhibit CaMKII- and Src-dependent pathways. In contrast, inhibiting the P1\(\kappa\)K was sufficient to stimulate cyt c release from isolated mitochondria without any apoptogenic stimuli. PNU-282987 blocked the effect of the P1\(\kappa\)K inhibitor wortmannin and restored the level of Akt kinase phosphorylation (that follows P1\(\kappa\)K activation) decreased by either Ca\(^{2+}\) or wortmannin. This data clearly indicated that α7 nAChRs attenuated mPTP formation by activating the intramitochondrial P1\(\kappa\)K/Akt pathway \[69\].

To evaluate the relative effect of cholinergic signaling on CaMKII-, Src- or P1\(\kappa\)K-dependent pathways in mitochondria and to discriminate the pathways engaged by different nAChR subtypes, we applied selective subtype-specific ligands (agonists and antagonists) in combination with apoptogenic agents stimulating different signaling pathways in mitochondria (Ca\(^{2+}\), H\(_2\)O\(_2\) or wortmannin). The data obtained indicated that the main role of α7β2 nAChRs is to activate the mitochondrial P1\(\kappa\)K/Akt pathway (74%) and much less to inhibit the CaMKII and Src-kinase pathways (6% and 9%, respectively). In contrast, the influence of the α3β2 and α4β2 nAChRs on P1\(\kappa\)K activity was much weaker (48% and 37%, respectively), although the inhibition of CaMKII and Src-kinases mediated by these receptors was stronger than that elicited by α7β2 nAChRs (20 to 37%). Therefore, diverse nAChR subtypes affected intramitochondrial kinase-dependent signaling pathways in different ways and with distinct efficiency \[39\]. The involvement of intramitochondrial P1\(\kappa\)K and Src in mPTP opening stimulated with H\(_2\)O\(_2\) and its regulation by α7- and β4-containing nAChRs was also shown in studies by Chernyavsky et al. \[64\].

**Mitochondrial nAChRs as therapeutic targets for the treatment of oncological and neurodegenerative disorders**

The data obtained clearly indicate that mitochondrial nAChRs form an additional line of cell defense against various pathogenic agents and inflammation. Their functional and potential therapeutic significance concerns, first of all, two main pathologies: cancer and neurodegenerative disorders like Parkinson’s and Alzheimer’s diseases.

Nicotine and its metabolites have been shown to promote tumor growth. Nicotine stimulated cell proliferation and inhibited apoptosis in colon cancer cell lines through α7 nAChRs, causing increase in the expression of P1\(\kappa\)K and
P-Akt/Akt ratio as well as in the expression of PKC, ERK1/2, survivin, and P-Bcl2 (Ser70) [17]. Nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), formed by nitrosation of nicotine, stimulated activation of α7 nAChR-related c-Src and PKCζ resulting in increased migration and invasion of cancer cells [65]. The proliferation of airways epithelial cancer cells and pancreatic cancer cells were shown to be under the control of α7-nAChRs, while breast cancer cells and colon cancer cells were regulated by α9 nAChRs [66]. It was also reported that consuming nicotine decreased the efficiency of chemotherapy by increasing the malignant cell survival [67]. Discovery of mitochondrial nAChRs revealed that nicotinic regulation of cell survival and death is more complex than originally thought, because it involves signals emanating from both cell membrane and mitochondrial nAChRs [68]. Since nicotine is lipophilic, it easily penetrates into the cytosol and can directly affect mitochondria. It was shown that nicotine efficiently abolished cisplatin-promoted mitochondria translocation of Bax and the release of cyt c [29]. Importantly, malignant cell transformation was accompanied by an increase in the mitochondrial nAChRs quantity, explaining the increased viability and resistance to apoptosis of cancer cells [69]. Therefore, cancer therapy, aimed to kill the malignant cells, should take into account the cholinergic regulation in mitochondria.

In contrast, therapy of neurodegenerative disorders aims to support the viability of brain cells. Recent studies demonstrate a clear link between neurodegeneration and inflammation, which often precedes the development of cognitive symptoms [70]. The anti-inflammatory and neuroprotective role of α7 nAChRs is well documented [16, 71-72]. The new data appear to show the impact of mitochondrial nAChRs in neuroprotection.

Neurodegeneration upon Alzheimer’s disease is accompanied and stimulated by accumulation of extracellular senile plaques formed by pathologically processed and oligomerized β-amyloid (Aβ1-42). Oligomeric aggregates of Aβ1-42 were shown to interact with α7 nAChRs with high affinity and to stimulate the α7-dependent signaling pathway resulting in internalization to intracellular compartments including mitochondria [73-74]. Nicotine markedly reduced Aβ(1-42)-induced neuronal death in rat cortical neuronal culture, attenuated mitochondrial AIF and cyt c release and caspase 3 activation [75]. In other studies, nicotine prevented apoptosis of PC-12 cells by blocking Aβ1-42-induced mitochondrial release of cyt c [72] and suppressed H₂O₂-induced apoptosis of brain astrocytes via the mitochondrial pathway through α7 nAChR stimulation [76].

Our recent data demonstrate that chronic inflammation induced in mice by regular injections of bacterial lipopolysaccharide (LPS) resulted in the decrease of α7 nAChRs and accumulation of pathological Aβ(1-42) in both the whole brain and brain mitochondria, that made mitochondria more susceptible to the apoptogenic effect of Ca^{2+} and less susceptible to regulation by α7-specific agonist PNU-282987 [77-78]. Similar effects were observed in mice immunized with the recombinant extracellular domain of the α7 subunit and possessing significant titers of α7(1-208)-specific antibodies in the blood. Mice either treated with LPS or immunized with α7(1-208) during 5 months demonstrated significant decline of episodic memory that is one of the first symptoms in Alzheimer’s disease. These data suggest that targeting nAChRs expressed in mitochondria may be a novel therapeutic strategy for the treatment of neurodegenerative disorders.

Carbohydrate content as a possible targeting signal of newly synthesized nAChRs to mitochondria

The discovery of mitochondrial nAChRs is the first example of these receptors functioning in intracellular compartment. However, many important questions still remain unanswered. It is to be investigated if mitochondrion is the only intracellular location of functional nAChRs. In addition, it is not clear which signal(s) target newly synthesized nAChRs to mitochondria instead to plasma membrane. The nAChR is a glycoprotein where the main N-linked carbohydrate component is attached to an Asn residue located in the extracellular domain in the course of post-translational modifications [79]. To elucidate the post-translational biosynthetic pathway of mitochondrial nAChRs, we compared the carbohydrate residues attached to the α7 nAChR expressed either in the plasma membrane or in mitochondria. For this purpose, plasma membranes and mitochondria were obtained from the rat brain according to conventional procedures [80-81] and were lyzed in detergent-containing buffer to be further analyzed in lectin-antibody sandwich assay. The immunoplates were coated with α7(179-190)-specific capture antibody, the plasma membrane or mitochondria preparations (additionally treated with 2% SDS to destroy any complexes of nAChR with other proteins) were applied at 70 µg/ml and the bound α7 nAChRs were revealed with a set of biotinylated lectins specific to different carbohydrate residues, including fucose-specific lectin from Laburnum anagroids (LABA) and sialic acid-specific agglutinin from Sambucus nigra (SNA) (kind gift of Dr. V. Antonyuk from The Institute of Cell Biology, Lviv, Ukraine), as well as galactose-specific peanut agglutinin (PNA), N-acetylgalactosamine-specific wheat germ agglutinin (WGA), and lectin from Artocarpus integriorfolia (Jacalin) specific to O-linked glycans. The bound lectins
were revealed with Streptavidin-peroxidase conjugate and o-phenylenediamine-containing substrate solution, and the optical density determined at 490 nm. To control for the α7 nAChR content in the preparations, the plates were coated with an α7(1-208)-specific antibody and the bound antigen was revealed with an α7(179-190)-specific antibody as previously described [41, 81]. The optical density values obtained with lectins were normalized to the signal for α7 nAChR in each sample. The data of representative experiments are shown in Figure 1. It is evident that mitochondrial α7 nAChRs did not differ from the plasma membrane one by the number of N-acetylglucosamine (WGA), galactose (PNA) or O-linked glycans (Jacalin) but contained more sialic acid (SNA) and, possibly, fucose (LABA). The averaged data of three independent experiments revealed a significant (p<0.05) increase of sialic acid (33.6%) and fucose (17%) and a slight decrease of galactose (10%) in mitochondrial α7 nAChRs compared to the plasma membrane species. These data allowed us to make an important conclusion: that mitochondrial nAChRs, similar to plasma membrane nAChRs, have post-translational modifications in the Golgi (trans-Golgi network), where O-linked glycans and sialic acid are attached to the protein backbone [82]. The data also suggest that the carbohydrate content (extra sialiation and fucosylation) might be a signal targeting the newly synthesized nAChR molecule to mitochondria instead to the plasma membrane.

Conclusions

The data obtained so far indicate that nAChRs are expressed in the outer membrane of mitochondria and control the early events of mitochondria-driven apoptosis. The nAChRs are located close to VDAC, which is engaged in Ca²⁺ entry to and cyt c release from mitochondria. The nAChRs prevent VDAC oligomerization and interaction with other mitochondrial pore components that decreases Ca²⁺ influx and attenuates cyt c release. These effects are produced through mitochondrial kinases located in the intermembrane space. Our data demonstrate that mitochondria possess their own PI3K/Akt, CaMKII- and Src-dependent signaling cascades, which can be regulated by mitochondrial nAChRs (Figure 2). The main target of α7β2 nAChRs is the PI3K/Akt pathway, which was also shown to be regulated by plasma membrane α7 nAChRs in the cytosole [17, 53-54]. The cytosolic PI3K/Akt cascade results in phosphorylation of anti-apoptotic Bcl-2 and Bcl-x, while its mitochondrial counterpart might additionally phosphorylate Bcl-xS (Bcl-2-like protein located in mitochondria outer membrane) to prevent the mPTP formation [83]. Other nAChR subtypes present in mitochondria (α3β2 and α4β2)
efficiently inhibit CaKMII and Src kinases, which are assumed to be up-stream of PI3K and are also attributed to the signaling of plasma membrane nAChRs [18, 61]. Therefore, it appears that mitochondrial and plasma membrane-expressed nAChRs engage similar established signaling pathways. Taking into account that various apoptotic agents (e.g., reactive oxygen species or Ca\(^{2+}\)) affect mitochondria in non-identical ways, the presence of multiple nAChR subtypes in mitochondria is important to protect the cell from different types of stress influence.

Revealing functional nAChRs in intracellular organelles naturally puts a question about the way of their targeting to mitochondria and their physiological ligands. Our data allow suggesting that one of the targeting signals might be the carbohydrate component attached to the protein backbone that is different in the plasma membrane and mitochondrial \(\alpha 7\) nAChR subunit. We also suggest that mitochondrial \(\alpha 7\)-containing nAChRs can be triggered by choline constantly present in the cytosole and being transferred to mitochondria to be metabolized into betaine [84]. This suggestion is supported by literature data showing that mitochondria of mice maintained on choline-deficient diet were more susceptible to apoptosis induction than mitochondria of control mice [85]. Activation of \(\alpha 3\beta 2\) and \(\alpha 4\beta 2\) nAChRs requires ACh. Therefore, their involvement depends on the activity of mitochondrial choline acetyltransferase [86] and can be additionally regulated. Alternatively, it was shown that extracellular ACh can be translocated into the cytoplasm of immune cells during inflammation to prevent mitochondrial DNA release and inflammasome formation by affecting mitochondrial \(\alpha 7\) nAChRs [87].

In whole, the discovery of mitochondrial nAChRs is the first example of these receptors functioning in intracellular organelles. It reveals a novel, previously non-recognized, way of regulating cellular death or survival, opening new perspectives in the therapy of cancer and neurodegenerative diseases.
Conflicting interests

The authors have declared that no conflict of interests exist.

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Author contributions

M.S. planned experiments and analyzed results, wrote the review; G.G made experiments with live mitochondria; O.L. made immunochemical experiments with mitochondria and their supernatants; O.K. made experiments with glioblastoma cells; L.K. collected mitochondria of knockout mice; K.U. made lectin-ELISA experiments with the brain mitochondria or plasma membranes.

Abbreviations

Aβ: amyloid-beta; Ach: acetylcholine; cyt c: cytochrome c; MLA: methyllycaconitine; Miptp: mitochondrial permeability transition pore; nAChR: nicotinic acetylcholine receptor; PAM: positive allosteric modulator; TOM22: translocase of outer membrane; VDAC: voltage-dependent anion channel.

References

18. Thomsen MS, Mikkelsen JD. The α7 nicotinic acetylcholine receptor ligands methyllycaconitine, NS6740 and GTS-21 reduce lipopolysaccharide-induced TNF-α release from microglia. J Neuroimmunol 2012; 251:65-72.


48. Murray TA, Bertrand D, Papke RL, George AA, Pantoja R, Srinivasan R et al. α7β2 nicotinic acetylcholine receptors assemble, function, and are activated primarily via their α7-α7 interfaces. Mol Pharmacol 2012; 81:175-188.


of the agonist-binding domain of the α7 nicotinic acetylcholine receptor. Proc Natl Acad Sci USA 2015; 112:E2543-2552.


