Nicotinic acetylcholine receptor-mediated calcium signaling in the nervous system

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Based on the composition of the five subunits forming functional neuronal nicotinic acetylcholine receptors (nAChRs), they are grouped into either heteromeric (comprising both α and β subunits) or homomeric (comprising only α subunits) receptors. The nAChRs are known to be differentially permeable to calcium ions, with the α7 nAChR subtype having one of the highest permeabilities to calcium. Calcium influx through nAChRs, particularly through the α-bungarotoxin-sensitive α7-containing nAChRs, is a very efficient way to raise cytoplasmic calcium levels. The activation of nAChRs can mediate three types of cytoplasmic calcium signals: (1) direct calcium influx through the nAChRs, (2) indirect calcium influx through voltage-dependent calcium channels (VDCCs) which are activated by the nAChR-mediated depolarization, and (3) calcium-induced calcium release (CICR) (triggered by the first two sources) from the endoplasmic reticulum (ER) through the ryanodine receptors and inositol (1,4,5)-triphosphate receptors (IP3Rs). Downstream signaling events mediated by nAChR-mediated calcium responses can be grouped into instantaneous effects (such as neurotransmitter release, which can occur in milliseconds after nAChR activation), short-term effects (such as the recovery of nAChR desensitization through cellular signaling cascades), and long-term effects (such as neuroprotection via gene expression). In addition, nAChR activity can be regulated by cytoplasmic calcium levels, suggesting a complex reciprocal relationship. Further advances in imaging techniques, animal models, and more potent and subtype-selective ligands for neuronal nAChRs would help in understanding the neuronal nAChR-mediated calcium signaling, and lead to the development of improved therapeutic treatments.

Keywords: nicotinic acetylcholine receptors; calcium; cellular signaling; nervous system

Acta Pharmacologica Sinica (2009) 30: 673–680; doi: 10.1038/aps.2009.64; published online 18 May 2009

Introduction

Neuronal nicotinic acetylcholine (ACh) receptors (nAChRs) are in the cys-loop ligand-gated ion channel superfamily, and are activated by nicotine in addition to the endogenous neurotransmitter ACh. The nAChRs are widely expressed in the brain, located both at the synapse (presynaptically and postsynaptically) as well as extrasynaptically. Presynaptic and preterminal nAChRs can enhance neurotransmitter release, postsynaptic nAChRs can contribute to fast excitatory transmission, and extrasynaptic nAChRs can modulate many neurotransmitter systems by influencing neuronal excitability and/or intracellular processes. The nAChRs play important modulatory roles in neuronal development and synaptic plasticity, participating in cognitive functions such as learning, memory, and attention. In addition, decrease, disruption or alteration in the function of neuronal nAChRs contributes to dysfunctions associated with various neurodegenerative diseases and disorders, including (but not limited to) epilepsy, schizophrenia, Parkinson’s disease, autism, Alzheimer’s disease, and addiction.

Neuronal nAChRs are pentameric transmembrane proteins, consisting of five subunits from a portfolio of nine α (α2–α10) and three β (β2–β4) subunits. Some are homomeric nAChRs, such as α7 receptors which contain five α7 subunits, and others are heteromeric nAChRs, which comprise α and β subunits. Different subtypes of neuronal nAChRs are known to be differentially permeable to calcium ions (Ca2+). The α7 nAChR subtype has one of the
highest permeabilities to calcium\cite{7}, the activation of which can raise cytoplasmic calcium levels and trigger a series of calcium-dependent intracellular processes. Calcium ions are one of the most versatile intracellular messengers known, and impacts almost every aspect of cellular life, including excitability, exocytosis, motility, apoptosis, and transcription; this is achieved by interacting with thousands of proteins and their downstream effectors\cite{9,10}. Calcium influx through nAChRs, particularly through the α-bungarotoxin-sensitive α7-containing nAChRs, is a very efficient way to raise cytoplasmic calcium levels. Here we will present a brief summary of the calcium signals initiated by the activation of neuronal nAChRs, and its possible physiological relevance.

**Cytoplasmic calcium signals initiated by neuronal nAChR activation**

There are three types of cytoplasmic calcium signals initiated by neuronal nAChR activation; (1) direct calcium influx through the nAChR\cite{6,11}, (2) indirect calcium influx through voltage-dependent calcium channels (VDCCs) which are activated by the nAChR-mediated depolarization\cite{6,11}, and (3) calcium-induced calcium release (CICR) (triggered by the two sources listed above) from the endoplasmic reticulum (ER) through the ryanodine receptors\cite{12–14} and inositol (1,4,5)-triphosphate receptors (IP3R)\cite{13–15}.

**Direct calcium influx through nAChRs** Two primary methods have been developed for estimating the calcium permeability of nAChRs. The first utilizes the shift in the reversal potential of nAChR-mediated currents due to changes in the extracellular calcium concentration. The permeability ratio PCA/PNa, which is the relative permeability of calcium to sodium ions, was then estimated using the Goldmann-Hodgkin-Katz constant field equation\cite{16,17}. The PCA/PNa ratio estimated in this way was ~2 for heteromeric neuronal non-α7 nAChRs, and ~10 for homomeric α7 or the heteromeric α9/α10 nAChRs\cite{2,3,7,8}. However, this relative approach has serious limitations.

The second method for estimating the calcium permeability of nAChRs is based on fluorescent calcium indicators\cite{7,18–20}. This approach relies on the simultaneous recording of fluorescence signals and transmembrane currents, and requires perfect voltage control of the cell and the absence of CICR. In this way, the percentage of the total current flowing through a given ion channel that is carried by calcium ions, the so-called “fractional calcium current” (usually indicated as Pf), can be measured. Heteromeric neuronal nAChRs have a Pf of 2%–5%, whereas homomeric α7 nAChRs (which have the highest Pf) range from 6%–12% (depending on the species)\cite{7,21}.

Interestingly, for the α3 subunit-containing human nAChRs, incorporation of the α5 subunit significantly increases calcium permeability\cite{22}. Populations of nAChRs composed of α4 and β2 subunits with different stoichiometries can be expressed in oocytes with different functional properties; eg the (α4)/(β2) stoichiometry has been demonstrated to have a much greater Ca\textsuperscript{2+} permeability than does the (α4)/(β2) stoichiometry\cite{23}.

**Indirect calcium influx through VDCCs** Activation of nAChRs can depolarize neurons, inducing the activation of the VDCCs and subsequent calcium influx. The nAChRs that contain α3 and/or β2 subunits in brain and ganglionic neuronal preparations are associated predominantly with calcium signals that are mediated by depolarization and the activation of VDCCs\cite{12,14,24}, as well as the α7 nAChRs\cite{25,26}. Calcium influx through VDCCs augments the primary calcium signals generated by the direct influx through nAChRs\cite{11,14}. These two mechanisms may be physiologically complementary; calcium entry through inwardly rectifying nAChRs will be robust under either resting or hyperpolarized potentials, whereas calcium influx through VDCCs will occur mainly at more depolarized potentials (~40 mV)\cite{27}.

**Intracellular calcium release from internal stores** In addition to the entry of extracellular calcium through channels in the plasma membrane, the cytoplasmic concentration of calcium reflects a complex interplay between buffering and mobilization capacities. In particular, calcium release from intracellular stores (via CICR) can have a crucial role in defining calcium responses\cite{28}. The activation of α7 nAChRs can generate calcium transients via entry through the channel pore itself (independently of VDCCs), which can then activate CICR from ryanodine-dependent stores\cite{12–14}. In neurons of the substantia nigra pars compacta, depletion of internal calcium stores inhibits the increase in cytoplasmic calcium levels induced by nicotine and the α7 nAChR-selective agonist choline\cite{12}. Blockade of ryanodine receptors in neuroblastoma cells also significantly reduces the increase in cytoplasmic calcium induced by activation of β2 and α7 subunit-containing nAChRs\cite{14}. Functional coupling between α7 nAChRs and ryanodine receptors has also been observed in cultured hippocampal astrocytes, where α7 nAChR-mediated calcium signals arise primarily from CICR through ryanodine receptors\cite{13}.

Cytoplasmic calcium signals can also be enhanced by activation of the IP\textsubscript{3} receptor (IP\textsubscript{3}R) second-messenger system and the subsequent release of calcium from intracellular stores. The involvement of IP\textsubscript{3}R-dependent calcium stores
in neuronal nAChR signaling was shown when nAChR-induced calcium responses were reduced with IP$_3$-selective antagonists$^{[13-15]}$. The functional interaction between IP$_3$ and ryanodine receptor-dependent calcium signals is considered to be a key signaling mechanism$^{[28]}$. Reports that nAChR-induced release of calcium from IP$_3$Rs is secondary to that from ryanodine receptors are consistent with their sequential activation$^{[13, 14]}$. Although it is unclear how nAChR stimulation activates IP$_3$Rs, possible mediators are a calcium-dependent phospholipase C (PLC)$^{[29]}$, and/or calcium-sensor proteins$^{[30]}$ activated following nAChR activation. The ability to activate different sources of calcium confers a further spatial and temporal dimension to the calcium signals evoked by nAChR activation. By converting acute nAChR stimulation into sustained cellular events, calcium signals may be a crucial link between nAChRs and the downstream processes that impinge on many neuronal functions.

Another source of intracellular Ca$^{2+}$ stores includes the mitochondria$^{[31]}$. The ER-mitochondrial interactions are important for the continued filling of the ER$^{[32, 33]}$. The efflux of Ca$^{2+}$ from the mitochondria through the mitochondrial Na$^+$/Ca$^{2+}$ exchanger can contribute to the overfilling of some intracellular Ca$^{2+}$ stores, particularly those that are in extremely close proximity to mitochondria$^{[34, 35]}$. The repeated activation of nAChRs can enhance calcium release from mitochondria$^{[36, 37]}$.

**Downstream signaling events mediated by nAChR-mediated calcium responses**

Calcium signals initiated by the activation of nAChRs can initiate responses via a variety of different mechanisms. According to the duration and timing, we have grouped these into instantaneous effects, short-term effects, and long-term effects.

**Instantaneous effects**

*Regulation of cytoplasmic calcium levels* The immediate impact of nAChR activation is the direct influx of cations (including calcium ions) through the channel pore. This instantaneous membrane depolarization can then activate the VDCCs, thereby increasing cytoplasmic calcium levels. Both the direct calcium influx through nAChRs, and indirect calcium influx through VDCCs, can then trigger calcium release from intracellular calcium stores as noted above$^{[6]}$. In chick ciliary ganglion neurons, a major portion of the nAChR-induced changes in cytoplasmic calcium levels is due to influx through VDCCs$^{[11, 26]}$. In cultured hippocampal neurons, about 85% of the nAChR-mediated calcium level changes were blocked by cadmium, a VDCC blocker, suggesting that most of the calcium influx was through VDCCs$^{[25]}$. In cultured hippocampal astrocytes$^{[13]}$, activation of nAChRs leads to a rapid and large calcium transient. This rise in cytoplasmic calcium level is entirely dependent on direct calcium influx, without any contribution from VDCC activation.

*Regulation of neurotransmitter release* At presynaptic terminals, the activation of nAChRs can initiate neurotransmitter release directly by raising intraterminal calcium levels due to calcium influx through the channel pore, as well as indirectly by calcium influx through VDCC activation due to membrane depolarization. For example, in hippocampal synaptosomes, the activation of $\alpha_3\beta_4$ nAChRs induced the release of noradrenaline without the involvement of VDCCs$^{[38]}$, whereas in striatal dopamine synaptosomes with $\beta_2$ subunit-containing nAChRs$^{[39]}$, the nAChR-induced release of dopamine was mediated by VDCCs$^{[38, 40]}$. In addition, presynaptic $\alpha_7$ nAChRs at excitatory synapses can increase the probability of glutamate release in the presence of tetrodotoxin and cadmium$^{[41]}$. Lastly in hippocampal mossy fiber terminals, calcium entry through $\alpha_7$ nAChRs can initiate CICR from presynaptic stores, and elicit bursts of miniature excitatory postsynaptic currents$^{[42]}$. All of these examples of calcium-dependent neurotransmitter release facilitated by presynaptic nAChRs are consistent with the activation of exocytotic mechanisms$^{[43]}$.

**Short-term effects** Although electrical signaling and neurotransmitter release related to nAChR activation can occur within milliseconds, downstream events and regulatory feedback mechanisms operate over longer time periods, often requiring seconds to minutes$^{[4]}$. These events usually depend on cellular signaling without gene expression, and we will refer to these as 'short-term effects'.

*Regulation of neurotransmitter release* In addition to inducing neurotransmitter release as noted above, presynaptic nAChRs may also modulate transmitter release through calcium-mediated signal transduction cascades. For example, protein kinase C (PKC) has been proposed to modulate striatal dopamine release by nAChR activation$^{[44]}$. Furthermore, nAChR and PKC-mediated stimulation of extracellular signal-regulated mitogen-activated protein kinase (ERK/MAPK)$^{[45]}$ and annexin phosphorylation$^{[46]}$ have been reported to contribute to the regulation of exocytosis in adrenomedullary cells.

*Regulation of nAChR desensitization* The nAChRs can undergo desensitization, a reversible reduction in response during sustained agonist application, which has been proposed to be important in controlling synaptic efficacy, responses to cholinergic agents, and certain nAChR-related
disease states\cite{47}. For α7-containing nAChRs in rat hippocampal interneurons\cite{48}, and for native nAChRs in chromaffin cells\cite{49,50}, the recovery from desensitization is delayed by high cytoplasmic calcium levels. This is probably because calcium catalyzes the activity of enzymes such as protein kinase C (PKC) and/or calcineurin, whose dynamic balance controls the recovery process\cite{47}. In addition, α7 nAChRs on chick ciliary ganglion neurons can undergo substantial activity-dependent inactivation\cite{4,51}; this inactivation or rundown depends on receptor activation, calcium influx, calcium release from internal stores, calmodulin, and CaMKII activity.

**Long-term effects** A critical element of the long-term consequence of the regulation of synaptic signaling at the cellular level is probably that of transcriptional regulation. In addition to influencing neurotransmitter release, a role for nAChRs in the regulation of cell signaling and gene expression has been reported\cite{52}. In neuroblastoma cells, exposure to nicotine influences the expression of a diverse set of genes, including transcription and protein-processing factors, and proteins associated with RNA binding and the plasma membrane\cite{53}. For nAChRs, such transcriptional control is usually triggered by calcium influx directly through the receptors, indirectly through VDCCs, and through release from internal stores\cite{4,54–58}.

**Regulation of neurotransmitter release** The activation of nAChRs can influence gene expression for immediate early genes and genes involved in transmitter synthesis\cite{15,59,60}. In the chick ciliary ganglion\cite{61}, the nAChR-mediated control of transcription relies on calcium influx and calcium release from internal stores to activate first CaMKII/IV, and then ERK/MAPK. These enzymes activate the transcription factor CREB (the cAMP response element-binding protein), which can alter gene expression.

As the rate-limiting step in catecholamine biosynthesis, tyrosine hydroxylase (TH) is a major control point in neurotransmitter release from catecholamine-containing neurons, and is subject to diverse regulatory mechanisms\cite{62}. Long-term treatment with nicotine increases the concentration of TH-mRNA and, consequently, TH-activity, both in vivo and in chromaffin cells\cite{62}. This effect is calcium-dependent and mediated by protein kinase A (PKA)\cite{63}. The nicotine-induced activation of expression of the gene encoding TH requires a prolonged increase in calcium concentration, and the activation of store-dependent calcium channels\cite{63} and ERK/MAPK\cite{64}.

**Involvement in synaptic plasticity and memory mechanisms** Agonists and antagonists of nAChRs can improve and impair performance in cognitive tasks, respectively\cite{65}. However, elucidating the cellular mechanisms that underlie the contribution of nAChRs to cognitive function is a daunting task. CREB and ERK/MAPK signaling cascades have attracted particular attention because their activities are central to long-term plasticity in the nervous system\cite{66}. This might have physiological relevance to many functions, including (but not limited to) addiction, learning and memory\cite{67}. The nAChRs mediate the calcium-dependent activation of ERK/MAPK and CREB in several neuronal models\cite{61,68,69}. The hippocampus has received particular attention as a key area for memory processing, and nAChR-mediated intracellular calcium increases promote activation of CaMKII/IV and ERK/MAPK, and the sustained phosphorylation of CREB\cite{69,70}. In addition, in rat brain slices that contain both the ventral tegmental area (VTA) and the nucleus accumbens (NAc), it has been demonstrated\cite{71} that activation of presynaptic α7 nAChRs induces long-term potentiation (LTP; a putative cellular model for learning and memory) of the excitatory input to the VTA if nicotine application is paired with postsynaptic stimulation. In hippocampal preparations, presynaptic α7 nAChRs are found to enhance the probability of LTP\cite{72}. These actions might also contribute to the mechanisms that underlie the effects of nicotine on cognition. Activation of the hippocampal ERK/MAPK pathway is required for the formation of contextual and spatial memories in mammals\cite{66}. Thus, factors that interfere with the activation of this pathway by α7 nAChRs might contribute to cognitive decline.

**Involvement in reward and dependence** Drug dependence is thought to involve plastic changes in neuronal circuits that are associated with ‘rewarding’ behaviours. Nicotine dependence, which is mediated by interaction with nAChRs, is likely to involve the modification of signaling cascades that modulate synaptic plasticity and gene expression, as proposed for other drugs of abuse\cite{62,73,74}. Like other addictive substances and rewarding behaviours, nicotine increases the release of dopamine from the mesolimbic projections to the NAc\cite{6,75}. Although somatodendritic nAChRs on dopamine-containing neurons of the VTA can excite these neurons directly, which results in transient responses that are terminated by desensitization of nAChRs\cite{70}, the stimulation and subsequent desensitization of GABA-containing neurons in the VTA also contributes to an excitatory effect through removal of the inhibitory influence of GABA\cite{77}.

For nicotine addiction, studies show that in rats, nicotine withdrawal (but not chronic treatment with nicotine itself) significantly reduced the levels of CREB and phosphorylated CREB in rat cortex and the amygdala\cite{78}. Phosphorylated CREB also decreased in the NAc in mice following chronic
consumption of nicotine in their drinking water\textsuperscript{[79]}. Changes in phosphorylated CREB in the NAc are consistent with previous reports that decreased CREB activity in this region contributes to drug reinforcement\textsuperscript{[80]}.

**Involvement in neuroprotection** Nicotine and other nAChR agonists are neuroprotective in several models of neuronal death, both in vivo and in vitro\textsuperscript{[81]}. The nAChR-mediated neuroprotection against excitotoxicity is calcium-dependent\textsuperscript{[82–84]}, and does not involve blockade of glutamate receptor function\textsuperscript{[83–85]}. Excessive activation of the N-methyl-D-aspartate (NMDA) receptor is thought to play a prominent role in a variety of acute and chronic neurological injuries\textsuperscript{[86, 87]}. In hippocampal slices, nicotine-mediated protection against acute NMDA excitotoxicity is mediated by the activation of phosphatidylinositol 3-kinase (PI3K) and the ERK/MAPK pathway\textsuperscript{[84]}. These signalling molecules could increase the expression of calcium buffering proteins such as calbindin-D28K, which have been implicated in the nAChR-dependent amelioration of excitotoxic insults\textsuperscript{[85]}. In cortical cultures, the nicotine-induced calcium-dependent activation of the phosphatase calcineurin is proposed to mediate the protection afforded by nicotine against glutamate excitotoxicity\textsuperscript{[88]}.

**Perspectives**

Compared to the rapid nAChR-mediated current, the corresponding kinetics of the intracellular calcium response are relatively slow; eg the time-to-peak for nAChR-mediated current responses in dendrites of interneurons was 39 ms, whereas the duration of the calcium response was 7 sec\textsuperscript{[53]}. In general, the prolonged duration of the evoked Ca\textsuperscript{2+} response by nAChR stimulation is due to several factors, including (but not limited to) the fact that the intracellular removal mechanisms for calcium are slower, and the secondary Ca\textsuperscript{2+} release from intracellular stores (eg, the ER and mitochondria) or from the extracellular space via VDCC activation. Thus the relatively large and prolonged Ca\textsuperscript{2+} accumulation mediated by nAChRs helps these “fast” receptors create prolonged responses at the level of Ca\textsuperscript{2+} homeostasis\textsuperscript{[3]}.

The direct and indirect calcium influx resulting from the activation of neuronal nAChRs can generate specific, complex calcium signals that mediate instantaneous (such as presynaptic neurotransmitter release), short-term (such as the modulation of desensitization through cellular signaling) and long-term effects (such as neuroprotection via gene expression). The regulation of calcium signaling is one of the most important aspects induced by the activation of neuronal nAChRs\textsuperscript{[6]}, which are cell-type, location, and receptor subtype-specific. However the spatial and temporal characteristics of nAChR-mediated calcium signaling are far from understood. In addition, nAChR activity can be regulated by cytoplasmic calcium levels\textsuperscript{[48, 89, 90]}, suggesting a complex reciprocal relationship. Consistent with this, we recently found that the activation of the M\textsubscript{1} muscarinic ACh receptor in hippocampal interneurons decreased the function of the α7 nAChRs in a calcium-dependent manner\textsuperscript{[89]}.

Physiological roles for neuronal nAChRs continue to be delineated. For example the functions of nAChRs on glia, especially on astrocytes, which occupy about half of the total volume of the brain and are critical for understanding the full picture of various brain functions\textsuperscript{[91, 92]}, have received considerably less attention to date than for receptors expressed in neurons. However evidence suggests that astrocytic nAChRs may be involved in neuroprotection. Furthermore there may be a role for nAChRs in inflammatory responses in the brain as in the periphery\textsuperscript{[93–95]}. The identification of novel gene targets regulated by neuronal nAChR function remains a big challenge, requiring the continued elucidation of the signaling pathways through which nAChRs regulate their expression. The ERK/MAPK signaling cascade is considered to lie at the center of many signaling pathways, and so a particular association with α7 nAChRs in relation to long-term behavioural changes is still waiting to be addressed\textsuperscript{[6]}.

Although the intracellular mechanism that mediate the modulation of neuronal nAChRs are not fully resolved, further advances in imaging techniques, animal models, and more potent and subtype-selective ligands for nAChRs would almost certainly help in understanding the neuronal nAChR-mediated calcium signaling and lead to better therapeutic treatments.

**Acknowledgements**

We would like to thank S DUDEK and S B SIMONS for advice in preparing the manuscript. Research was supported by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences; the National Natural Science Foundation of China (Ng 30470900), Natural Science Foundation of Guangdong Province (Ng 04020253) and SRF for ROCS, SEM.

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