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Review

Expression and function of nicotinic acetylcholine receptors in stem cells

Carlos M. Carballosa [1], Jordan M. Greenberg [1], and Herman S. Cheung [1,2,*]

[1] Department of Biomedical Engineering, College of Engineering, University of Miami; Coral Gables, FL, USA
[2] Geriatric Research, Education and Clinical Center (GRECC); Miami Veterans Affairs Medical Center, Miami, FL, USA

*** Correspondence:** Email: hcheung@med.miami.edu; Tel: +1-305-575-3388; Fax: +1-305-575-3365.

Abstract: Nicotinic acetylcholine receptors are prototypical ligand gated ion channels typically found in muscular and neuronal tissues. Functional nicotinic acetylcholine receptors, however, have also recently been identified on other cell types, including stem cells. Activation of these receptors by the binding of agonists like choline, acetylcholine, or nicotine has been implicated in many cellular changes. In regards to stem cell function, nicotinic acetylcholine receptor activation leads to changes in stem cell proliferation, migration and differentiation potential. In this review we summarize the expression and function of known nicotinic acetylcholine receptors in different classes of stem cells including: pluripotent stem cells, mesenchymal stem cells, periodontal ligament derived stem cells, and neural progenitor cells and discuss the potential downstream effects of receptor activation on stem cell function.

Keywords: nicotine; nicotinic acetylcholine receptor; nAChR; stem cells; pluripotent stem cells; multipotent stem cells; regeneration potential; differentiation potential

1. Nicotinic Acetylcholine Receptors (nAChRs)

Nicotinic acetylcholine receptors (nAChRs) belong to the cholinergic family of receptors and are a class of ligand-gated ion channels that respond to the neurotransmitter acetylcholine as well as other ligands such as choline and nicotine. Functional nAChRs are composed of 5 transmembrane subunits that are arranged together to form a transmembrane pore. These pentameric receptors are created from various combinations of the 16 different nAChR subunit types. All subunits share the same molecular structure: an extracellular domain, four transmembrane subunits (TM1-TM4), and a cytoplasmic domain; however, each subunit has differences in its amino acid sequence [1]. Accordingly, subunits are classified as either α or non-α type based on the presence of a cysteine-cysteine residue within the N-terminal domain near the entrance to TM1 [1]. The Cys-Cys pair is only found on α subunits and is required for agonist binding. In total, there are 9 α subunits: α1-7, α9, α10, (α8 is identified in avian libraries but has not been observed in mammalian species) and 7 non-α subunits: β1-4, γ, δ, and ε [1,2].

The affinity for ligands and the ion gating properties of the nAChR depends on the specific subunit composition of each receptor [1]. For instance, the binding affinity for nicotine, a typical nAChR agonist, is higher in nAChRs that contain the α4 subunit versus those that contain the α7 [3]. This specificity is also observed for binding of receptor antagonists, which can competitively bind to nAChRs causing desensitization and inhibit activation. α-bungatrotoxin (α-BTX), for example, binds specifically to α7 nAChRs [4], whereas dihydro-β-erythroidine (DHβE) binds specifically to α4 containing nAChRs [5]. On the other hand, mecamylamine (MECA) is a non-specific antagonist and therefore it binds to all nAChRs, regardless of subunit composition [6]. In addition to ligand affinity these receptors also vary in their ion gating properties. For example, nAChRs composed of α4 subunits are much less permeable to Ca^{2+} ions than the α7 homomeric nAChRs, which are considered to be the most permeable nAChR to Ca^{2+} [7]. The calcium permeability of the nAChR can, however, be modified. The addition of the α5 subunit to α3 nAChRs, for instance, greatly increases the permeability to calcium [8].

nAChRs are highly conserved among all species and are widely expressed throughout the body; however, they are predominantly found in muscle (muscular nAChRs) and neuronal (neuronal nAChRs) tissues. Musclar nAChRs are found in skeletal muscles where they mediate neuromuscular transmission at the neuromuscular junction. Neuronal types, on the other hand, are mainly found in the peripheral and central nervous systems, but have been located in non-neuronal tissues including stem cells [9,10]. Muscle nAChRs are heteropentameric (i.e. 5-diferent subunits) and consist of α1, β1, γ, and either δ or ε subunits in the 2:1:1:1 stoichiometric ratio [1]. Neuronal nAChRs, however, can exist as either hetero- or homopentamers (i.e. 5-identical subunits). Heteropentameric nAChRs are the more predominately found form of neuronal nAChR as they can exist in several different combinations. For example, studies show that α2-4 and/or α6 nAChR subunits typically assemble with β2 and/or β4 subunits, but they can also assemble with α5 or β3 subunits to make functional nAChRs [2]. Homopentameric nAChRs, on the other hand, are less diverse and can only be formed from α7, α9, and α10 subunits (Figure 1).

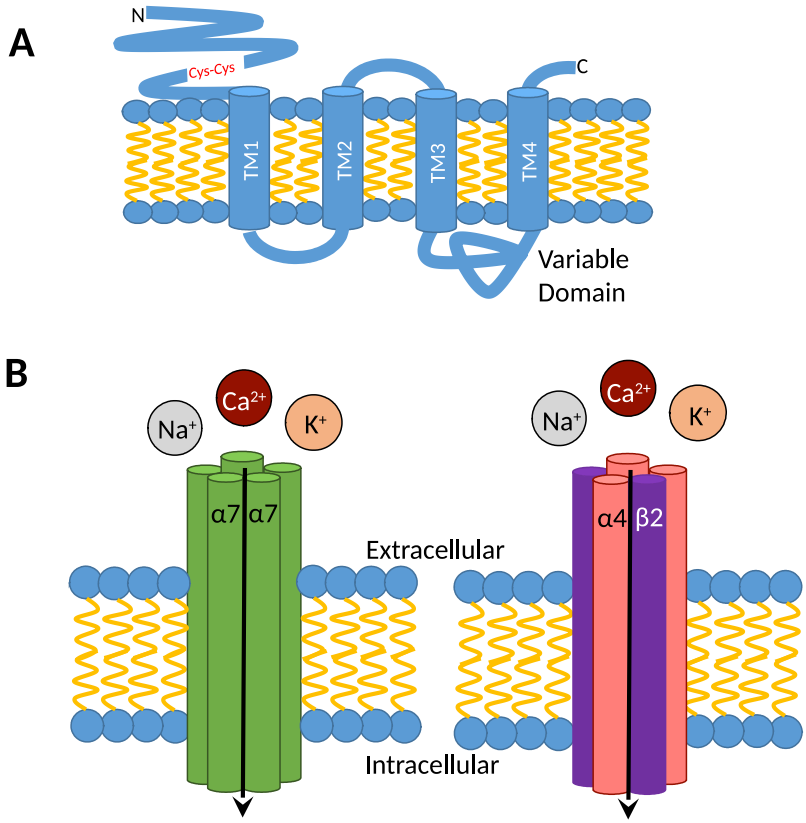

Figure 1. A: Subunit structure of nAChR showing the cys-cys pair that defines α-subtype nAChRs. **B:** A prototypical α7 homopentameric nAChR (left) and heteropentameric α4β2 nAChR (right).

The following review will focus on the expression and function of nAChRs in stem cell populations that currently exhibit potential for use in therapeutic applications for tissue regeneration. Specifically, this review will cover embryonic, induced-pluripotent, mesenchymal and neuronal stem cell types. As stem cell-based therapies become more common in the clinic, understanding the effects mediated by the activation of these receptors will be crucial due to the continued use of tobacco products, which contain nicotine. Effects of activation include, among others, changes to stem cell DNA synthesis, proliferation, and differentiation mediated either through direct action of ion flux or as a result of activation of secondary messenger pathways [11].

2. Pluripotent Stem Cells

Pluripotent stem cells are defined by their ability to undergo unlimited self-renewal and differentiate into any of the three germ layers. There are two classes of pluripotent stem cells, embryonic stem cells (ESCs) and induced-pluripotent stem cells (iPSCs). ESCs are isolated from the inner cells mass of blastocyst stage embryos and are the only naturally occurring pluripotent stem cell source. These cells where first identified in 1998 by the laboratory of James Thomson [12]. These cells have been widely used for studying developmental biology, drug discovery, and regenerative medicine since their discovery, but have also been marred by ethical dilemmas

associated with their isolation. iPSCs were first reported in 2006 by Yamanaka et al. [13]. In contrast to ESCs, iPSCs are not naturally occurring but are instead derived from somatic cells that have been reprogrammed to express pluripotency markers such as OCT4 and Sox2 [14]. iPS technology has allowed the use of pluripotent stem with no associated ethical dilemmas as well as the ability to isolate patient specific pluripotent stem cells. iPS technology, however, is still in its infancy and has risks associated with reprograming such as epigenetic memory [15].

3. Expression of Subunits in Pluripotent Stem Cells

Multiple groups have reported the presence of nAChRs in pluripotent stem cells. In regards to human ESC's all subunits have been identified at the gene level, whereas only α3 and α7 have been identified on the protein level [16]. Confirmation of RT-PCR results were carried out for α3, α4, α7, β2 and β4 using immunohistochemistry which showed that of those studied only α3 and α7 were present [16]. Meanwhile, non-human primate ESC's (nhpESC) show expression of α1, α5, α7, β1, and β2 subunits at the genetic level [17]. This data however is incomplete as these were the only subunits that were investigated in these studies. These subunits were selected as they represent the most likely subunits found in the downstream application investigated, nhpESC derived lung epithelial cells [17]. Murine ESC's show genetic expression of α3, α4, α7 and β2 subunits [18], but, similar to the previous studies this study only investigated these specific subunits. Murine iPSCs have been shown, via western blot, to express α4 and α7 and lower expression of α1 and α3 relative to α4 [5]. Once again, however, only a limited number of subunits were investigated: α1, α3, α4, α7 and α9 [5]. In another study, the same group verified detection of the α4 and α7 subunits through immunofluorescence and western blot [19]. All of these results have been compiled by investigating small subsets of possible subunits, and a complete study of all genes and proteins of possible subunits has yet to be completed. In addition to this, none of these studies performed immunostaining or western blot using knock out controls. It has been previously shown that the antibodies for α3, α4, α7, β2, and β4 subunits are not highly specific and that data utilizing either immunoblotting or immunostaining for these subunits should be coupled with additional technical data to support these findings [20]. Where appropriate we have included details on the specific subunits antagonists used to alleviate any concern.

4. Function of nAChR in Pluripotent Stem Cells

Activation of pluripotent stem cell nAChRs has been demonstrated to lead to effects in cell proliferation, viability, apoptosis, and even differentiation. It should be noted, before a discussion of these effects, that the majority of the following studies investigated the effects due to nAChR agonist nicotine, whose effects are known to be dose-dependent [21,22]. For example, Yu, et. al. show that concentrations on the order of 10^{-2} to 10^{-4} M nicotine lead to cell death of human H9 ESCs whereas 10^{-6} to 10^{-8} M lead to enhanced cell survival and proliferation [21]. With regards to proliferation and viability, Ishiszuka et al. reports that application of 300 nM nicotine leads to increases in DNA synthesis and proliferation of the 20D17 murine iPSC line [19]. Landgraf et. al. meanwhile reports that presence of 10^{-5} to 10^{-7} M nicotine leads to decreased cell proliferation of murine CGR8 ESC,

but interestingly show that nicotine increases the viability of these cells [18]. Several groups also report that exposure to nicotine may lead to an increase in apoptosis [23,24]. H7 and H9 human ESC's exposed to 1.8 μM nicotine showed an increase in apoptotic cells measured by the TUNEL assay. This effect was abolished with the use of the antagonist D-tubocurarine, indicating that this increase in apoptosis is receptor mediated. [24] Proliferation (and by association, viability and apoptosis) and differentiation are the defining characteristic of stem cells and therefore critical to overall stem cell functionality, and thus studies on the effects of nicotine on these traits is of utmost importance.

It appears that these observed changes may be the result of changes in cell cycling. Ben-Yehuda et. al. report that non-human primate ESCs that were exposed to 100 nM nicotine during differentiation to a fibroblast lineage showed clustering in genetic changes related to cell cycling [17]. In differentiating cells, cells exposed to nicotine did not cluster separately from unexposed samples [17]. These results suggest that overall gene expression patterns between the two groups are similar. However, of the less than 100 genes that were statistically significantly different in the two groups, cell-cycle genes were over-represented in the number of genes identified [17]. Interestingly, adult fibroblasts (not stem cell derived) exposed to nicotine continuously for up to three passages did not exhibit this downregulation of cell cycling related genes [17]. These results indicate that the observed changes are a result of the presence of nicotine specifically during differentiation and not a result of overall exposure. This report also demonstrated similar results in the expression of N-myc, which showed a significant decrease in nhp-ESCs exposed to 100 nM nicotine during differentiation to a fibroblast lineage [17]. N-myc is known to stimulate proliferation via ribosome biogenesis and negative regulation of cell cycle proteins. In addition to its role in cell cycling, N-myc overexpression can also lead to apoptosis of cells [25]. Ishizuka et. al. also commented on the effects of nicotine on cell cycling in murine iPSCs. In this report they show that exposure of 300 nM nicotine to iPSCs lead to increase in cells found the S and G_2 phases and a decrease to cells in the G_1 phase [19]. As the S phase is the phase in which DNA replication occurs, this augmentation of cell cycling may be responsible for the observed increases in DNA synthesis. Additionally, the G_2 phase immediately precedes mitosis, so the reduction in G_1 phase may indicate that activation of nAChR pushes cells through the G1 phase more rapidly than non-treated groups [26].

In addition to changes in cell cycling, activation of nAChRs may also lead to changes such as increased DNA synthesis and proliferation in pluripotent stem cells as a result of the influx of Ca^{2+} and activation of secondary messenger pathways [11]. Ishizuka et. al. showed that many of the previously described effects are a result of the increase in intracellular calcium ($[Ca^{2+}]_i$) mediated through the α4 and α7 subunits. When antagonists to either of these specific subunits were applied (DHβE for α4 and α-BTX for α7), observed changes as a result of nicotine exposure decreased. Interestingly, these effects were not observed in the absence of extracellular calcium, suggesting that the effects were mediated by calcium gating receptors on the stem cell surface. One potential downstream effect due to increases in $[Ca^{2+}]_i$ is the activation of the Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) via phosphorylation. When this pathway was inhibited through KN93, a CaMKII inhibitor, DNA synthesis was significantly inhibited [19]. Zhao and Reece showed that murine embryos treated with 3–6 μM nicotine were smaller and exhibited severe birth defects, however these effects were not observed in groups receiving inhibition of increased $[Ca^{2+}]_i$ using

BAPTA-AM, a Ca^{2+} chelator [23]. Although specific subunits of nAChR were not investigated in this study, the results imply the presence and importance of Ca^{2+} gating nAChRs in embryological development.

nAChR activation has also been shown to play a role in the differentiation potential of pluripotent stem cells. Concentrations between 2.5–3.7 μM of nicotine showed a decreased in the amount of Oct3/4+ positive nuclei in human ESCs [24]. Oct 3/4 expression is tightly controlled to the potency of pluripotent stem cells [27]. This decrease could be inhibited by the use of the nAChR antagonist D-tubocurarine, which suggests that this result is receptor mediated [24]. This result is contradicted by Ishizuka et. al., however, who report that treatment with 300 nM nicotine has no effect on the Oct3/4 expression at the gene level [19]. The difference in reported changes to Oct 3/4 expression may partially be due to the dose dependence relationship of nicotine and 10x nicotine concentration used in the Zdrakovic et. al. experiments. Yu et. al, report that the onset of differentiation of ESCs to endothelial cells leads to significantly lower genetic expression of α5, α7, and α9, but higher expression of α1. This data hints at the potential role that nAChRs have in the differentiation of these cells [21]. A change in differentiation potential as a consequence of nAChR activation, however, has yet to be observed in iPSCs.

Exposure to 300 nM nicotine did not effect the expression of Flk-1, a marker of vascular progenitor cells [28], in murine iPSCs differentiating towards mesodermal progenitor cells [5]. The same study also looked at the effect of nicotine on the differentiation of murine iPSCs to neural progenitors, but once again did not observe any changes to differentiation potential toward the neural progenitor lineage [5]. Serobyan et. al., meanwhile reports that triggering of nAChRs in embryoid body leads towards a shift in spontaneous differentiation toward the hemangioblast lineage [16].

5. Multipotent Stem Cells

Functional nAChRs have also recently been identified on the surface of mesenchymal stem cells (MSCs), a multipotent adult stem cell population capable of differentiating into various mesodermal lineages like osteogenic, chondrogenic, and adipogenic lineages [29]. MSCs reside in stem cell niches and have been identified within various adult tissues like bone marrow [30], adipose tissue [31], and umbilical cord blood [32]. In addition to being readily available, MSCs exhibit immunosuppressive and homing properties [33] and are therefore an ideal therapeutic stem cell source [34].

6. nAChR Subunit Expression in MSC

Both α and β nAChR subunits have been identified in human MSCs (hMSCs). In 2009, Hoogduijn et al. were the first to identify the expression of α3, α5, and α7 nAChR subunits in bone marrow-derived human MSCs (BM-MSCs) via reverse transcription-polymerase chain reaction (RT-PCR) and flow cytometry [35]. Although the authors did not observe expression in the other investigated subunits (i.e. α9, α10, β2 and β4), it is important to mention that this study was limited in scope as only a partial nAChR subunit panel was evaluated (mRNA expression for α2, α4, α6,

and β3 was not conducted). A definitive conclusion on the full expression of nAChR subunits from this adult stem cell population therefore could not be made at the time.

A full nAChR subunit expression panel on hMSCs was, however, conducted by Schraufstatter et al. shortly thereafter. Initial analysis using RT-PCR indicated the expression of α1, α2, α3, α4, α5, α7, α9, β2, β3 and β4 subunits in hMSCs [36], but only the presence of α7, β2, and β4 subunits could be confirmed after further analysis with DNA sequencing, western blotting, and immunofluorescence [36]. Although these results were compared side-by-side to the previous Hoogduijn study, the precise origin (i.e., sex, age, location) of the Schraufstatter et al. hMSCs was never disclosed. Instead, cells were described as human MSCs from the Tulane Center for Gene Therapy. It is assumed that these cells were derived from the bone marrow; however, as previously mentioned, hMSCs can be isolated from various sources therefore a comparison between current and previous BM-MSC data should be made with caution.

Most recently, Zablotni et al. confirmed the presence of several nAChR α-subunits on hMSCs derived from the long bone diaphyseal fractures of both men and women [37]. Interestingly, subunit expression was dependent on the sex and osteoporotic health of the donor [37]. For instance, healthy male-derived hMSCs expressed the α3, α5, α7 and α9 nAChR subunits, whereas healthy female-derived hMSCs expressed α2, α5, α6, α7, α9, and α10 subunits [37]. Osteoporotic-derived hMSCs, on the other hand, expressed the same subunits as healthy females with the additional expression of the α3 nAChR subunit [37]. In addition to the variation in expression among patients of different sex and osteoporotic health, Zablotini et al. also observed that the expression of the specific subunits within each group was often not consistent for all patients. While the α7 subunit was expressed in all (4/4) healthy female patients, the α2 subunit was only expressed in half (2/4) of the healthy female patients [37]. The authors suggest that these inconsistencies in expression are likely attributed to patient-specific conditions such as hormone levels, nutrition, and/or drug use. In a separate study, the expression of nAChR α-subunits was investigated after hMSCs were differentiated into osteogenic, chondrogenic, and adipogenic lineages (according to the protocols outlined). Interestingly, variations for some subunits were again observed between differentiated groups and within differentiated subjects. For example, while the α7 subunit remained mostly unchanged throughout all groups, the α3 subunit did not. In pre-differentiated male hMSCs, the α3 subunit was expressed in 3/4 patients; however, after differentiation the subunit expression was increased in osteoblasts (4/4), maintained in chondrocytes (3/4), and abolished in adipocytes (0/4) [37]. These differences in expression suggest that nAChRs might be involved in the stem cell differentiation process. This suggestion is further strengthen by the widespread and maintained expression of the α7 subunit in all groups and differentiated states. Although Zablotni et al.'s results confirm previous observations of α-subunit expression in hMSCs, a western blot or other protein expression analysis is needed to confirm the actual translation of these genes into proteins that can then be assembled into functional nAChRs.

7. Functionality of hMSC nAChRs

Functional nAChRs, specifically those gating for the calcium ion (i.e. α7), have been identified on hMSCs [35,36]. The functionality of these receptors can be determined via measurements of $[Ca^{2+}]_i$, which should increase upon proper activation of α7 nAChR. In an *in vitro* study, Hoogduijn

et al. investigated the functionality of hMSC nAChRs by measuring changes in $[Ca^{2+}]_i$ via Fluo-3-AM imaging. Briefly, cultured hMSCs were loaded with Fluo-3-AM, a calcium-specific fluorescent probe, exposed to 1 μM nicotine, and immediately imaged under confocal microscopy. Increases in $[Ca^{2+}]_i$ were observed for almost half (22/50) of the hMSCs imaged, suggesting the presence of calcium-gating nAChRs like the homopentameric α7 receptor [35]. Additional studies by Hoogduijn et al. were conducted to determine whether receptor activation with nicotine affected the cAMP production or phosphorylation of extracellular signal-regulated protein kinases (ERK). After exposing hMSCs to 0.1 – 10 μM nicotine, no significant changes in the production of cAMP when compared to non-nicotine treated cells was observed [35]. Nicotine (1 μM) exposure did, however, increase the phosphorylation of ERK in hMSCs. When phosphorylated, ERKs are activated and capable of regulating cell proliferation and differentiation [38]. Along with migration, the processes of stem cell proliferation and differentiation are critical for effective stem cell-based regeneration [39]. By mediating the effects that interfere with these processes, nAChRs are undoubtedly capable of influencing stem cell health and differentiation and regeneration potentials. Altogether, this data suggests that the observed nicotinic effects are predominantly regulated by the homopentameric α7 nAChRs. The other nAChR subunits identified in this study, α3 and α5, are incapable of forming homopentameric receptors with themselves [40] or heteropentameric receptors with each other (none have yet to be identified.) Moreover, since no β–subunit proteins were identified to form complexes with the identified α-subunit proteins it is very unlikely that the α3 and α5 subunits are involved in the formation of functional nAChRs in hMSCs.

The presence of functional homopentameric α7 nAChRs in hMSCs was also confirmed in separate *in vitro* studies conducted by Schraufstatter et al. [36]. Briefly, cultured hMSCs were loaded with Fluo 4-AM, exposed to 2 μM nicotine, and immediately imaged using a fluorescent plate reader. hMSCs exposed to nicotine experienced a significant increase in $[Ca^{2+}]_i$ compared to non-nicotine-treated controls and calcium-chelated groups, thus confirming the presence of functional α7 nAChRs in hMSCs [36]. The researches acknowledged that one of the consequences of increased $[Ca^{2+}]_i$ is the increased vulnerability to cellular apoptosis. In further investigations, Schraufstatter et al. demonstrated that hMSCs exposed to 10 mM - 0.1 μM nicotine experienced a dose-dependent effect on cell viability, with doses above 1 μM resulting in significant trypan blue uptake and Annexin V staining [36]. The toxic threshold dose of 1 μM nicotine was further investigated in order to determine its effect on the inherent homing characteristics of hMSCs. After 16 hours of exposure to nicotine, hMSCs exhibited significantly reduced growth factor-mediated migration potential [36]. Interestingly, this inhibition was ameliorated by 100 nM α-BTX pretreatments, thus providing further evidence that α7 nAChRs mediate these nicotine-induced effects. Our group has also previously shown that 1 μM nicotine is capable of inhibiting hMSC proliferation and migration potentials *in vitro* [41].

8. Periodontal Ligament Derived Stem Cells

Periodontal ligament derived stem cells (PDLSCs) are a subset of hMSCs that reside in the periodontal ligament (PDL). Similar to hMSCs, PDLSCs are capable of mesodermal lineage differentiation [42]. Moreover, these cells are capable of differentiating in various oral tissues, like

cementum, alveolar bone, and periodontal ligament, [43,44] and are therefore critical to maintaining the overall health within the oral cavity. PDLSCs are also easily accessible after routine dental procedures (e.g., orthodontic extractions) and are thus an ideal source for therapeutic stem cells [45].

9. nAChR Subunit Expression in PDLSC

Both nAChR subunits and functional nAChRs have been identified on PDLSCs. Kim et al. were the first to confirm the expression of both α7 and β4 nAChR subunits and functional α7 nAChRs in PDLSCs through RT-PCR and nAChR antagonist studies, respectively [6]. The researchers' analyzed all nAChRs with the exception of the α9 and α10 subunits, which were not investigated at any level (rationale for exclusion not provided). Consequently, the presence or absence of these subunits could not be confirmed in PDLSCs. In a separate study, Zhou et al. confirmed the presence of functional α7 nAChRs on PDLSCs through RT-PCR, western blot, and nAChR-specific antagonist studies using α-BTX [46]; however, it should be noted that only the α7 nAChR subunit was examined in these studies. These results also confirmed the findings of his collaborators' previous study demonstrating the expression of α7 and β4 nAChR subunits in PDL tissues [4].

10. Functionality of PDLSC nAChRs

To date, only the α7 and β4 nAChR subunit proteins have been confirmed in PDLSCs [6,46]. Out of all the possible arrangements using these two subunits, only the homopentameric α7 and heteropentameric α7β4 conformations form functional nAChRs. The homopentameric α7 nAChR occurs naturally and has been previously identified in PDLSCs [6,46]. The functional α7β4 heteropentameric nAChR, on the other hand, does not occur naturally (or has yet to be identified) and can only be created through forced experimental means [47]. Therefore, although both α7 and β4 subunits have been identified in PDLSCs, only the homopentameric α7 nAChR is believed to be present and functional in these cells.

PDLSC homopentameric α7 nAChRs have been shown to mediate nicotine-induced effects on cell viability [6]. In an *in vitro* study conducted by Kim et al., PDLSCs were exposed to a range of nicotine concentrations, between 0 and 10 mM, for 24 or 48 hours before being evaluated by MTT analysis for changes in cell viability. The results showed that nicotine caused a significant decrease in PDLSC viability after 24 and 48 hours of culture in concentrations greater than or equal to 0.1 mM. The reduction in cell viability could be explained by the observed increases in DNA fragmentation and by the increase in cells in subG_1 [6], the cell phase associated with apoptosis [48]. Moreover, PDLSCs treated with 10^{-2} M nicotine experienced an increase in the expression of p53, a pro-apoptotic tumor suppressor protein [49], after only 30 min. [6]. In order to confirm if these effects were regulated by nAChRs, Kim et al. measured the expression of several apoptotic proteins in the presence or absence of α-BTX, the aforementioned α7-specific nAChR antagonist, and MECA, the non-specific nAChR antagonist (concentrations not provided) [6]. PDLSCs treated with nicotine (1 μM - 10 mM) for 48h experienced a decrease in the expression of Bcl-2, [6] a prosurvival, anti-apoptotic protein from the mitochondrial pathway [48], and an increase in the expression of cleaved caspase-3 [6], an activated pro-apoptotic enzyme [50]. These effects, however, were significantly

reversed, to a similar extent, in PDLSCs pretreated with α-BTX and MECA [6]. The similarity in data between α-BTX and MECA pretreatments further suggests that α7 is the predominant nAChR in PDLSCs.

In a separate *in vitro* study Zhou et al. demonstrated that homopentameric α7 nAChRs mediated the detrimental effects of nicotine on PDLSC osteogenic differentiation potential [46]. PDLSCs were pre-treated with 0 or 100 μM α-BTX and cultured in an osteo-inductive media (PDLSC culture media supplemented with 100 nM dexamethasone, 50 μg/mL ascorbic acid, and 5 mM β-glycerophosphate) containing 0 or 0.1 mM nicotine before being evaluated for evidence of osteogenic differentiation. After 3-weeks, PDLSCs cultured in osteo-inductive medias showed significant increases in osteogenic gene and protein expression (i.e., alkaline phosphatase, osteocalcin, bone sialoprotein, and runt-related transcription factor 2) and also showed evidence of mineralization through positive alizarin red S and alkaline phosphatase staining [46]. We recently observed similar results, but at lower nicotine concentrations (1 μM) and shorter differentiation periods (i.e. 2-weeks) [41]. PDLSCs treated with nicotine-containing osteo-media managed to show evidence of osteogenic differentiation, albeit at significantly lower levels than non-nicotine treated and α-BTX pre-treated groups [46]. These results suggest that the homopentameric α7 nAChRs regulate, to an extent, the osteogenic differentiation potential of PDLSCs. In an additional study, Zhou et al. also confirmed that these receptors could regulate the wnt/β–catenin pathway [46], which was previously shown in a separate study to be involved in PDLSC osteogenic differentiation [51]. PDLSCs exposed to 0.1 mM of nicotine showed an increase in the expression of active-β-catenin protein and a decrease in wnt-related transcriptional factors DKK-1 and GSK-3β; however, all of these effects were reversed with α-BTX pretreatments [46]. Altogether, this data provides further evidence the role of homopentameric a7 nAChRs in PDLSC regeneration potential.

11. Neural Progenitor Cells

In the mature adult, the number of neuronal stem cell (NSCs) sources is extremely limited. NSCs reside in the few remaining regions of neurogenesis (i.e., subventricular zone of the lateral ventrical and subgranular zone of hippocampus) [52,53] and are capable of differentiating into neurons [54–56], astrocytes [57–59], and oligodendrocytes [60,61]. Given their neuronal differentiation potential, NSCs are capable of providing therapeutic options for neurodegenerative disorders like Parkinson's [62] and Alzheimer's [63]. As previously mentioned, nAChRs are predominantly found on neuronal tissues [64–66]. Not surprisingly neural progenitor cells (NPCs) also express nAChRs [55,67–69].

12. nAChR Subunit Expression in NPCs

Takarada et al. identified the presence of several nAChR subunits in NPCs derived from the neocortex of embryonic Std-ddY mice (15.5d) and embryonic Wistar rats (18d). RT-PCR analysis of α2-α6, α7, α9, and β2-β4 nAChR subunits showed the mRNA expression of α2-α5, α7, α9, β2 and β4 nAChR subunits in rats and α3-α5, α7, α9, β2 and β4 nAChR subunits in murine NPCs [55]. Expression for all nAChR subunit mRNA, however, was observed in the rat and mouse whole brain,

which was used as the positive control for these studies [55]. Additional western blot and immunocytochemical analysis using α4 and β2 antibodies confirmed the expression of α4 and β2 nAChR subunit proteins in mouse NPCs [55]. Expression of α4 and β2 nAChR subunit proteins have also been confirmed in NPCs derived from the hippocampus of C57BL/6 mice via immunocytochemistry [68]. Fetal mouse cerebral cortical neural precursors have also been shown to express α3, α4, α7, β2, and β4 nAChR subunit mRNA [69]. The expression of the remaining nAChR subunits in these cells, however, remains unknown since the corresponding mRNA was never investigated.

13. Functionality of NPC nAChRs

Functional nAChRs have been identified in NPCs and have been shown to regulate several cellular processes. Functional α4β2 nAChRs have been confirmed in NPCs derived from the murine neocortex [55]. Briefly, NPCs (i.e. neurospheres cultured in epidermal growth factor for 10 days) were loaded with Fluo-3-AM and stimulated with various concentrations of nicotine (1 mM and 1 μM) and with or without α4β2-specific antagonist DHβE (1 mM and 1 μM) before being imaged for relative fluorescent intensity. The researchers noted that 1 mM nicotine induced a 20%+ increase of $[Ca^{2+}]_i$ in non-pretreated NPCs, but only a 10%+ increase in 1 mM DHβE pretreated NPCs [55]. This data confirms the presence of functional α4β2 nAChRs and suggests that these receptors mediate nicotine-induced rises of $[Ca^{2+}]_i$ in NPCs. This data also suggests that additional functional, Ca^{2+}-gating nAChRs, like the homopentameric α7 nAChR, might be present in NPCs, as a complete abolition of $[Ca^{2+}]_i$ was not observed with DHβE. These α7 receptors were investigated along with α4β2 receptors during experiments that evaluated the effect of nicotine on murine NPC viability. Murine neurospheres were cultured in 10 μM nicotine for 10 days and pretreated with or without 10 μM of α4β2-specific antagonists DHβE or 4-(5-ethoxy-3-pyridinyl)-N-methyl-(3E)-3-buten-1-amine (TC2559) or α7-specific antagonist methyllycaconitine (MLA). Neurosphere size was significantly decreased due to nicotine after only 4 days in culture, with effects persisting throughout the 10-day experiment; however, DHβE and TC2559 pretreatments prevented nicotine-induced reductions in neurosphere size, while MLA had no effect [55]. Similar trends were also observed during NPC differentiation. Briefly, NPCs were treated with EGF and with or without 1 μM nicotine for 10 days and cultured for an additional 4 days in regular culture media before undergoing immunocytochemical analysis for neuronal marker MAP2 and astroglial marker GFAP [55]. The differentiation protocol itself increased NPC expression of both MAP2 and GFAP, while nicotine (1 μM) increased the expression of MAP2, but decreased the expression of GFAP [55]. DHβE and TC2559 (1 μM) pretreatments reversed these nicotinic effects, while MLA had no effect [55].

Functional α4β2 nAChRs have also been identified on NPCs derived from the hippocampus of C57BL/6 mice [68]. Initial studies indicated that 0.1 μM – 5 μM of ZY-1, an α4β2-specific nAChR agonist, significantly increased the proliferation of C57BL/6 hippocampus-derived NPCs neurospheres [68]. 1 μM concentrations of ZY-1 were shown to significantly increase neurosphere proliferation, migration potential, and viability. Pretreatments with 0.1 μM of DHβE were shown to significantly decrease neurosphere viability only, as this was the only experiment in which pretreatments were conducted [68].

Functional nAChRs on BALB/c mice neurospheres (E16) were also identified in a separate study; however, receptor subunits were never identified through RT-PCR, western blotting, or immunocytochemical techniques [67]. Instead, nAChR functionality was determined by investigating the effects of nicotine and MECA, the non-specific nAChR antagonist, on neurosphere size. Neurospheres were cultured in 1 mM – 8 mM nicotine-containing media with or without 10 μM MECA for 5 days before being imaged to determine changes in neurosphere number and size. The results showed that nicotine significantly decreased the total number and overall size of neurospheres with MECA pretreatments reversing these effects [67]. In summary, the α4β2 nAChR appears to be the major mediator of effects observed in NPCs.

14. Conclusion

The data presented in this review confirms the widespread presence of stem cell nAChRs and demonstrates the involvement and impact of these receptors in stem cell function. The expression of these receptors is summarized in Table 1. Although not mentioned in this review, due to their lack of therapeutic potential, it is important to note that nAChRs have also been identified on cancer stem cells (CSCs), which, according to the cancer stem cell theory, give rise to cancerous tissues [70]. For instance, Hirata et al. has demonstrated the presence of α7 nAChRs and their involvement in the proliferation of breast CSCs [71]. Scherl et al., on the other hand, has shown the expression of α1, α3, α5 and α7 nAChR subunits in squamous cell carcinomas of the head and neck [72]. In addition, it was determined that as these tumors progressed, the expression of α1 and α5 subunits increased, suggesting a strong correlation between nAChRs and tumorigenesis. Tu et al. has shown that the knockdown of α7 nAChR subunits can inhibit cancer cell proliferation and improve the sensitivity of cancer cells to chemotherapeutic drugs (i.e., gastric cancer cells to taxane) [73]. Consequently, these receptors are currently being investigated as potential targets for cancer treatment.

The majority of these studies determined the downstream implications of nAChR activation on stem cell function through the use of the nAChR agonist nicotine. Nicotine is a tobacco plant alkaloid found in cigarettes and nicotine replacement therapies, like electronic cigarettes, and is often abused due to its addictive properties. The nicotine delivered from these products eventually absorbs into the bloodstream where it is quickly distributed throughout the whole body via the circulatory system. The distribution of nicotine across several tissues impacts the overall health of the user as well as any potential offspring as nicotine has been shown to readily accumulate in the breast milk [74–76] and placental tissues [77–80]. Consistent nicotine use can therefore lead to the vast accumulation of nicotine within many tissues, most of which harbor resident stem cell populations.

We, and others, have shown the dose-dependent effects of nicotine on stem cell function, specifically proliferation, migration, and differentiation potential. These effects are mediated, in part, by specific nAChRs present on the stem cell surface as shown by the reviewed studies herein, which observed a partial reversal of nicotinic-induced effects when stem cells were pretreated with receptor specific antagonists. The partial reversal of effects suggests that there may be other functional nAChRs present on the stem cell surface that also mediate the nicotinic effect or that the nicotinic effect is mediated by an additional pathway not involving nAChRs. It is therefore imperative to determine the full expression of nAChR subunit mRNA and proteins in experiments dealing with

nicotine and stem cell function in order to accurately define which nAChRs are responsible for the observed effects. The experiments discussed herein merit further review given the widespread expression of nAChRs, their impact on stem cell regeneration potential, and the popularity of nicotine-containing products.

Table 1. Summary of stem cell nAChR expression from studies reviewed in text. Brackets denote which subunits were actually investigated in each respective study. N/A corresponds to a lack of any subunit investigation.

Pluripotent Stem Cells					
Cell Source	**Species**	**Subunit mRNA Expression**	**Subunit Protein Expression**	**Functional nAChRs**	**Ref.**
iPSC	Murine (20D17)	N/A	**α**: α4, α7 **β**: N/A [α1, α3, α4, α7, α9]	α4, α7	[5]
iPSC	Murine (20D17)	N/A	**α**: α4, α7 **β**: N/A [α4, α7]	α4, α7	[19]
ESC	Monkey (nhpESC 4706)	**α**: α1, a5, α7 **β**: β1, β2 [α1, α5, α7, α9, β1, β2]	N/A	N/A	[17]
ESC	Murine (CGR8)	**α**: α3, α4, α7 **β**: β2 [α3, α4, α7, β2]	N/A	N/A	[18]
ESC	Human (WH09)	**α**: α1- α7, α9, α10 **β**: β1 - β4 [α1 - α7, α9, β1 - β4]	**α**: α3, α7 **β**: N/A [α3, α4, α7, β2, β4]	N/A	[16]
Multipotent Stem Cells					
Cell Source	**Species**	**Subunit mRNA Expression**	**Subunit Protein Expression**	**Functional nAChRs**	**Ref.**
BM-MSCs	Human	**α**: α3, α5, α7 **β**: N/A [α3, α5, α7, α9, β2 - β4]	N/A	α7	[35]
MSCs	Human	**α**: α1 - α5, α7, α9 **β**: β2 - β4 [α1 - α10, β1 - β4]	**α**: α7 **β**: β2, β4 [α3, α4, α7, β2, β4]	α7	[36]

BM-MSCs	Human (M)	**α**: α3, α5, α7, α9 **β**: N/A [α2 - α7, α9, α10]	N/A	N/A	[37]
	Human (F)	**α**: α2, α5 - α7, α9, α10 **β**: N/A [α2 - α7, α9, α10]	N/A	N/A	
PDLSC	Human	**α**: α7 **β:** β4 [α1 - α7, β1 - β4]	N/A	α7	[6]

Neuronal Progenitor Cells

Cell Source	Species	Subunit mRNA Expression	Subunit Protein Expression	Functional nAChRs	Ref.
Neocortex	Murine (Std-ddY)	**α**: α3 - α5, α7, α9 **β**: β2, β4 [α2 - α7, α9, β2 - β4]	**α**: α4 **β**: β2 [α4, β2]	α4β2	[55]
	Rat (Wistar)	**α**: α2 - α5, α7, α9 **β**: β2, β4 [α2 - α7, α9, β2 - β4]	N/A	N/A	[55]
Hippocampus	Murine (M) (C57BL/6J)	N/A	**α**: α4 **β**: β2 [α4, β2]	α4β2	[68]
Cerebrum	Murine (M) (C57BL/6J)	**α**: α3, α4, α7 **β**: β2, β4 [α3, α4, α7, β2, β4]	N/A	N/A	[69]

Conflict of Interest

The authors declare no conflict of interest.

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