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Central nervous system interaction and crosstalk between nAChRs and other ionotropic and metabotropic neurotransmitter receptors☆

Federica Bono [a,1], Chiara Fiorentini [a,1], Veronica Mutti [a], Zaira Tomasoni [a], Giulia Sbrini [a], Hanna Trebesova [b], Mario Marchi [b], Massimo Grilli [b,*,2], Cristina Missale [a,2]

[a] Division of Pharmacology, Department of Molecular and Translational Medicine, University of Brescia, 25123 Brescia, Italy
[b] Department of Pharmacy, University of Genova, 16148 Genoa, Italy

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ABSTRACT

Neuronal nicotinic acetylcholine receptors (nAChRs) are widely distributed in both the peripheral and the central nervous systems. nAChRs exert a crucial modulatory influence on several brain biological processes; they are involved in a variety of neuronal diseases including Parkinson's disease, Alzheimer's disease, epilepsy, and nicotine addiction. The influence of nAChRs on brain function depends on the activity of other neurotransmitter receptors that co-exist with nAChRs on neurons. In fact, the crosstalk between receptors is an important mechanism of neurotransmission modulation and plasticity. This may be due to converging intracellular pathways but also occurs at the membrane level, because of direct physical interactions between receptors. In this line, this review is dedicated to summarizing how nAChRs and other ionotropic and metabotropic receptors interact and the relevance of nAChRs cross-talks in modulating various neuronal processes ranging from the classical modulation of neurotransmitter release to neuron plasticity and neuroprotection.

1. Introduction

The concept of neurotransmission has been enriched by a much broader interpretation than in the past, even postulating the existence of an "active milieu" [1,2]. The presence of an active environment determines the complexity of each phenomenon. Different modulators (neuronal, glial, vascular etc) interact with the classic neurotransmitters to produce adaptations that are also due to the different targets involved. Neuronal Nicotinic Acetylcholine Receptors (nAChRs) are pharmacological targets implicated in the main brain functions and in several pathologies [3,4]. Therefore, the opportunity of modulating these proteins is an intriguing medical strategy. The nAChRs are expressed both as autonomous entities and as an integral part of coexisting receptor networks. In the latter case, the nAChRs exist both as a physical part of heteromers (receptor-receptor interaction) and/or as a unit of functional interactive proteins (receptor crosstalk) responding to integrative stimuli. The possibility of modulating one ring of a chain of receptors may be a way to overcome the limits of undruggable target therapy. Understanding the mechanisms of heteromodulation is also fundamental to prevent and address any adverse reactions. This review describes the heterodimerization of G protein-coupled receptors (GPCRs) with nAChRs and receptor crosstalk between nAChRs and other coexisting receptors.

Abbreviations: nAChRs, Neuronal Nicotinic Acetylcholine Receptors; BiFC, fluorescence complementation; FCS, fluorescence correlation spectroscopy; GPCRs, G protein-coupled receptors; ER, endoplasmic reticulum; TM, transmembrane helices; ICL3, third intracellular loops; BRET, bioluminescence resonance energy transfer; FRET, fluorescence resonance energy transfer; PLA, proximity ligation assays; BiFC, bimolecular luminescence or fluorescence complementation; PD, Parkinson's Disease; P2XR, purinergic P2X receptors; DA, dopamine; NE, norepinephrine; iPSCs, pluripotent stem cells; alpha-syn, alpha-synuclein; ACh, acetylcholine; NAc, nucleus accumbens; RLuc, Renilla Luciferase; GFP, green fluorescent protein; PI3K, phosphoinositide-3-kinase–protein kinase; p70S6K, cytosolic p70 ribosomal S6 kinase; NMDARs, N-methyl-D-aspartate receptors; GluRs, Glutamate receptors; AMPARs, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors.

☆ The multifaceted activities of nervous and non-nervous neuronal nicotinic acetylcholine receptors in physiology and pathology. Eds: Dr Cecilia Gotti, Prof Francesco Clementi, Prof Michele ZOli.

* Correspondence to: University of Genova, 16148 Genoa, Italy.
E-mail address: massimo.grilli@unige.it (M. Grilli).
[1] First co-authors
[2] Senior co-authors

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2. The concept of GPCR oligomerization and the nAChR role

While it is well established that many receptors exist as oligomeric species, consisting of two or more subunits that together form a functional receptor unit (homomeric/heteromeric receptors), the concept of receptor heteromers emerged from indirect evidence of the formation of oligomers among β-adrenergic receptors [5]. In the early 1980 s the group of Agnati and Fuxe provided further evidence, based on biochemical and functional data, that GPCRs could interact with other functional receptors at the membrane level to form new receptor entities [6–8]. These heteromers, resulting from interactions between GPCRs or between a GPCR and divergent classes of receptors, represent novel functional units. They exhibit unique pharmacology, signaling and trafficking properties that differ from those of their molecular constituents [9,10]. Interacting receptors, together with their effector and regulatory proteins, are highly organized structures, compartmentalized in membrane micro-domains [11]. Allosteric interactions between the physically coupling receptors represent the fundamental molecular mechanism underlying the activity of these complexes [12].

To date, it is ascertained that receptor-receptor assembly likely occurs early, during protein synthesis, at the endoplasmic reticulum (ER) level, thus implying that GPCRs heteromers reach the plasma membrane as a pre-formed unit. For most GPCRs heteromers, the domains involved in receptor-receptor interaction have been identified by means of both bioinformatic and experimental studies. These include crystallization and atomic force microscopy techniques, mass spectrometry, as well as super-resolution imaging-based approaches [13].

In general, GPCR oligomers interact via multiple interfaces involving both the highly conserved transmembrane (TM) helices, mainly TM4, TM5 and TM6, as well as the carboxyl tail and the third intracellular loop (ICL3), while interactions between the extracellular domains are less frequent [14]. Moreover, electrostatic interactions between positive- and negative-charged intracellular domains (e.g. arginine-rich and serine-phosphate-rich motifs) are likely responsible for most interactions between receptors [15–17], including GPCR heteromerization with non-GPCRs [16,18].

To date, a wide range of GPCRs heteromers has been reported [19, 20], besides the acquisition of novel techniques specifically developed to detect a receptor-receptor interaction. Pharmacological studies and biochemical techniques, such as cross-linking and co-immunoprecipitation, have represented the first approaches used to demonstrate the oligomerization of GPCRs. Biophysical techniques, such as bioluminescence and fluorescence resonance energy transfer (BRET and FRET, respectively), are now the preferred system to monitor receptor-receptor interaction. However, these techniques, allowing the detection of energy transfer between fluorescent donors and acceptors (FRET) or between luminescent donors and fluorescent acceptors (BRET) when they are in proximity (less than 10 nm) [21,22], require the transfection of opportunely tagged receptors into artificial cell systems. More recently, the proximity ligation assay (PLA) has achieved the opportunity to detect GPCRs oligomers in their physiological environment. This technique is based on specific antibodies against the two interacting receptors, linked to complementary DNA probes. Only when the two interacting receptors are in close proximity (less than 17 nm) it result in DNA polymerization and amplification with fluorogenic oligonucleotides [23,24]. To date, many other techniques are available for investigating GPCR heteromers, including the bimolecular luminescence or fluorescence complementation (BiFC), based on two non-luminescent/fluorescent fragments that can re-associate upon the interaction of the two tagged receptors [25] and the fluorescence correlation spectroscopy (FCS), able to quantitatively assess the interaction between proteins both in in vitro and in vivo models [26].

Given the high propensity of GPCRs to form heteromers and with the aim of preventing the study of GPCR heteromers without physiological relevance, several different standards have been proposed for the identification and characterization of GPCR heteromers [27]. In particular, the group of Gomes and collaborators [19] indicated as the “Criterion 1” the need to prove that in native tissues, the interacting receptors co-localize and physically interact. In some cases, transgenic animals expressing fluorescent-tagged receptors have been developed, thus allowing the isolation and visualization of GPCR heteromers, using co-immunoprecipitation and immunofluorescence techniques, the latter also in living animals [28].

Once identified as a physiological GPCRs heteromer, the unique pharmacological, signalling, and trafficking properties, should be described using a combination of appropriate methods as required by “Criterion 2”. These include radioligand-binding assays, intracellular signalling assays, and techniques able to monitor receptor-receptor localization and trafficking from intracellular sites to membranes and vice-versa, such as immunocytochemistry and live imaging microscopy. All these issues should be demonstrated first in a heterologous expression system, which allows to define the properties of the individually expressed receptors and to compare them with those of the corresponding heteromeric complexes, and then in native cells/tissues using knock-out mice lacking one of the interacting receptors [19].

The last criterion (“Criterion 3) states that the expression of a GPCRs heteromer, as well as its properties, should be lost by disrupting the receptor-receptor interaction. To this end, different experimental strategies have been developed, including transgenic mice expressing receptors with mutations affecting GPCRs heteromers formation [29–31], and interfering peptides designed to target and disrupt the predicted interacting interfaces. Furthermore, the generation of specific ligands that selectively recognise GPCRs heteromers in native tissues, such as selective antibodies [32] or bifunctional/bivalent or dual-acting compounds, represents a breakthrough in this field. This opens up new opportunities for the development of selective and effective drugs with fewer side effects. Given that GPCR heteromers represent a new mechanism to explain how neurotransmitter systems interact, it follows that they should be hypothesised to play a role in the pathogenesis of most diseases. To date, the relevance of GPCRs heteromers in the pathogenesis of various diseases has been described [19,20,33], including pre-eclampsia [34,35], asthma [36], acquired immune deficiency syndrome [37], heart failure [38], liver fibrosis [39], schizophrenia [40,41] and Parkinson’s Disease (PD).

Among the various receptor partners involved in the formation of GPCR heteromers, the nAChR is particularly relevant. nAChR is in fact a partner receptor in various GPCRs heteromers, including those with purinergic P2X receptors (P2XR); on this line, a receptor complex formed by the α4β2 nAChRs and the P2X2R has been identified by using FRET microscopy both in transfected Hek293 cells and in hippocampal neuron membranes [42]. Moreover, using co-immunoprecipitation, an interaction between the α3β2 nAChRs and the P2X1–3 receptors has been reported and functionally linked to the control of evoked norepinephrine (NE) release from rat hippocampal synaptosomes [43]. In addition, clear evidence of the ability of nAChR to physically interact with dopamine (DA) and glutamate receptors (GLURs) has been clearly demonstrated [44].

3. Interaction between nAChR and DA receptors in dopaminergic neurons

As observed for many neurotransmitter systems, the release of DA from DAergic neurons of the ventral tegmental area (VTA) and substantia nigra (SN) is controlled by the activation of nAChRs. These receptors are expressed at high levels both in DA nerve terminals and in somatodendritic compartments. In particular, the α4β2 and the α6β3 are the main nAChR subtypes expressed in these neurons, with the α4β2 nAChR as the predominant form. Their activation, by modifying presynaptic membrane excitability, increases DA release [45–49]. DA neurons also express receptors for DA, belonging to the D2R family. DA receptors are GPCR classified into two families, the D1-like and D2-like receptors, based on their structural, pharmacological, and functional

characteristics [50]. The D1-like receptors, D1 and D5 (D1R and D5R) are post-synaptic receptors coupled to stimulatory G (Gs) proteins that activate adenylyl-cyclase (AC) leading to cyclic AMP (cAMP) accumulation and protein kinase A (PKA) activation. On the contrary, D2-like receptors (D2R, D3R and D4R) are both pre- and post-synaptic and, by coupling to inhibitory G (Gi) proteins, inhibit AC activity decreasing cAMP levels and PKA activity [50]. Two spliced isoforms of the D2R have been identified, the long ($D2_LR$) and short ($D2_SR$) isoforms, which differ by 29-amino acids in the third cytoplasmic loop [51]. $D2_SR$ represents the subtype mainly localized at presynaptic sites [52,53].

Several properties are associated with each DA receptor, in terms of distribution in both CNS and non-CNS tissues, affinity for DA and ability to activate many other signalling pathways, both in a G protein-dependent or independent way [50,54]. Interestingly, DA receptors have been reported to form heteromers with other DA receptors, such as the D1R-D2R [55], the D1R-D3R [56,57] the D2R-D3R heteromers [58], with other GPCRs such as adenosine or histamine receptors [59] and with non-GPCRs such as the NMDA receptors [59–61] and the nAChRs.

Presynaptic D2R and D3R, localized both at the somatodendritic level and at synaptic terminals of midbrain DA neurons, act as autoreceptors, reducing DA synthesis and release [62–67]. Whether D2R and D3R possess peculiar activities is still unclear and difficult to define since the high sequence homology, common intracellular signalling and distribution, combined with the almost complete lack of selective D2R or D3R ligands [50,54,68].

In DA neurons, D2R/D3R and nAChR are co-localized and functionally interrelated in modulating DA release; interestingly, a large body of evidence has shown that both receptors also support DA neuron trophism and protection. On this line, D3R ability in exerting neurotrophic and neuroprotective effects on DA neurons has been reported [69–72]. Besides observing an inverse correlation between cigarette smoking and the development Parkinson's Disease (PD) [73,74], the capacity of nicotine to regulate various genes involved in morphology and survival of DA neurons [75] and to induce neuroprotection by stimulating both α7 and α4/α6β2 nAChRs have been provided in both in vitro and in vivo experiments [76–78]. More recently, the ability of both D3R and nAChR in protecting DA neurons from injury has been reported by using two different in vitro models, primary cultures of mouse DA neurons and human DA neurons derived from healthy induced pluripotent stem cells (iPSCs). Both D3R agonists and nicotine significantly impaired the pathological accumulation of alpha-synuclein (alpha-syn) induced by glucose deprivation [79,80]. Intriguingly, nicotine-induced neuroprotection specifically required D3R [80]. The ability of nicotine to promote structural plasticity of DA neurons through the activation of α4 and α6-containing nAChR has also been reported using both cell models [81,82]. In primary mouse DA neurons, in fact, nicotine significantly increased DA neuron dendritic arborization and soma size, an effect prevented by the α4β2 nAChR antagonist dihydro-β-erythroidine and lost in transgenic mice lacking the nAChR α4 subunit (α4-KO) [82] or the nAChR α6 subunit (α6-KO) [81]. Interestingly, as shown for neuroprotection, the activity of D3R, but not of D2R, was required for nicotine-induced neurotrophic effects since this effect was blocked by D3R-preferential antagonists and totally lost in DA neurons derived from D3R knock-out mice (D3R-KO) [82].

These observations led to the idea that D2R/D3R and nAChR could work together to modulate DA release and support DA neuron well-being by also forming receptor heteromers. Along this line, evidence was provided for the ability of the nAChR β2 subunit to physically interact with both $D2_SR$ and D3R [83,84] resulting in the formation of two distinct heteromers, the D2R-nAChR [83,85] and D3R-nAChR [84, 86,87]. These complexes are involved in critical aspects of DA transmission such as plasticity and protection, respectively.

3.1. The D2R-nAChR heteromer: role in the regulation of DA release

The idea that DA and acetylcholine (ACh) modulate DA release from nerve terminals by acting through a receptor heteromer composed by D2R and nAChR has been demonstrated using both transfected cells and rat striatal membranes for identification (Criterion 1) [83], and in vivo microdialysis, to study the physiological role (Criterion 2) [83,85]. Co-immunoprecipitation experiments, performed in Hek293 cells transfected with the nAChR α4 and β2 subunits and the short isoform of the D2R have shown that $D2_SR$ interacts with the β2 but not with the α4 subunit. The same results were obtained by performing co-immunoprecipitation in soluble extracts from rat striatal membranes [83]; whether D3R is involved in this oligomer has not been investigated.

The relevance of the D2R-nAChR heteromer was then defined measuring extracellular DA levels in the shell of the rat nucleus accumbens (NAc) by microdialysis in response to local infusion of different combinations of nicotine, D2R/D3R agonist quinpirole and receptor antagonists [83]. Similarly, DA release in response to a combination of receptor agonists and antagonist was determined in vitro in rat and mouse synaptosomes [85]. Using these approaches, a D2R/D3R-mediated modulation of nAChRs-induced DA release was described, suggesting that one mechanism by which D2R reduces DA release is through the D2R-nAChR heteromer; moreover, evidence has also been provided that nAChRs activation is required for the D2R/D3R to become operative [85].

These data suggest that the two receptors work together in controlling DA release from nerve terminals and that the formation of the D2R-nAChR heteromer may explain the crosstalk between these receptors [88] (Fig. 1); accordingly, targeting the D2R-nAChR heteromer could represent a novel strategy for treating diseases related to DA neuron dysfunctions, such as PD and addiction. On this line, a series of putative bifunctional derivatives were synthetized by connecting the β2-containing nAChR antagonist N-n-alkyl nicotinium salts with spacers of different length to the D2R agonist 2-(alkylaminomethyl)chromanes. These compounds were fully characterized using a combination of functional and pharmacological assays, showing a reduction in DA release [89]. Among these, the compound named "Compound 2" showed high affinity for both nAChR and D2R and combined a nAChR antagonist/D2R partial agonist profile. This drug can be used for both the study of the properties of heteromer and the development of nicotine addiction drugs [89].

3.2. The D3R-nAChR heteromer: role in DA neuron plasticity and protection

Evidence for the formation of the D3R-nAChR heteromers, their properties and physiological role have been progressively collected by a combination of biochemical, biophysical and immunofluorescence studies [84,86,87], providing a clear application of all three criteria defined by Gomes et al. [19]. According to Criterion 1, a direct and specific interaction between D3R and the nAChR β2, but not α4 subunit, was first demonstrated by BRET in Hek293 cells. The researchers co-transfected the cells with the β2-subunit fused to the donor molecule Renilla Luciferase (RLuc) and D3R tagged with the green fluorescent protein (GFP), as the acceptor molecule [84]. Moreover, the D3R-nAChR heteromer was visualized in PLA experiments. In particular, a PLA signal was detected in both mouse DA neurons and mesencephalic brain sections from wild-type mice but not from D3R-KO and α4-KO mice [84]. PLA experiments were also performed in human DA neurons derived from iPSCs, where expression of the D3R-nAChR heteromer was detected in both soma and dendrites [86].

Interestingly, two different cell-permeable interfering peptides, that represent one of the most useful tools to study GPCRs heteromers (Criterion 3), were designed and characterized after identifying in the sequence of D3R and nAChR β2 subunit the most plausible interfaces involved in the formation of stable non-covalent interactions [14]. These peptides contain the positively charged 215–225 arginine-rich regions of the intracellular loop 3 (ICL3) of the D3R (TAT-D3R) and the negatively

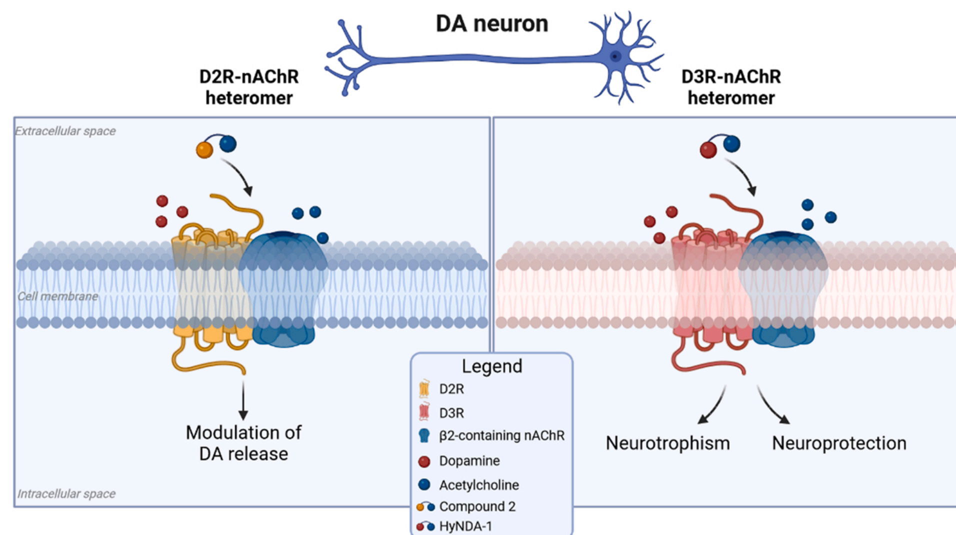

Fig. 1. Schematic representation of the interaction and dimerization between nAChRs and DA receptors in dopaminergic neurons. Created by Biorender.com.

charged 439–449 aspartate-rich region of the ICL2 of the β2 subunit (TAT-β2) [84]. When used in BRET experiments, both interfering peptides, but not their scrambled counterparts, used as controls, specifically affected the ability of the β2 subunit to interact with D3R [84]. Similarly, treating mouse cultures of DA neurons or human iPSCs-derived DA neurons with the TAT-D3R interfering peptides specifically impaired the D3R-nAChR heteromer assembly in PLA experiments [84,86]. Thus, the IC3 loop of the D3R and the ICL2 of the nAChR β2 subunit crucially contribute to the direct interaction of D3R with the nAChR to form the heteromer.

The physiological significance of this heteromer was deeply investigated in both mouse and human DA neurons. D3R-nAChR heteromer represents the functional unit supporting plasticity and protection of DA neurons induced by both nAChR and D2R/D3R agonists [71,84,86,87]. In fact, both chronic treatments with nicotine and quinpirole, significantly increased the arborization and the soma size of DA neurons. This growth is an effect totally lost when the D3R-nAChR heteromer was disrupted by interfering peptides or when treatments were performed in DA neurons derived from D3R-KO mice [84,87]. Moreover, in mouse and human DA neurons, both nicotine and D3R agonists prevented the accumulation of alpha-syn induced by glucose-deprivation and the subsequent morphological damages. It is reasonable that this neuroprotective effect depends on the stimulation of the D3R-nAChR heteromer since its disruption impaired nicotine-induced neuroprotection [86].

Therefore, both nicotine and D3R agonists exert neurotrophic and neuroprotective effects on DA neurons only through the D3R-nAChR heteromer, while the two receptors, if individually expressed, are not sufficient for inducing these effects. Furthermore, data from D3R-KO mice exclude D2R involvement. Interestingly, the combination of low doses of nicotine and quinpirole, usually ineffective in exerting neurotrophic effects when singularly administered, was sufficient for inducing structural plasticity. Hence it is probable a synergy of the two agonists in ensuring D3R-nAChR heteromer activation, even under conditions of low neurotransmitter levels [87]; on this line, D3R is the DA receptor with the highest affinity for DA [90].

The signalling pathway associated with D3R-nAChR heteromer stimulation by nicotine or D2R/D3R agonists was also investigated first in transfected cells, and then in mouse DA neurons. (Criterion 2). In particular, the phosphoinositide-3-kinase–protein kinase (PI3K)-ERK1/2/Akt signaling cascade, deeply involved in neurotrophic and neuroprotective effects was analysed [70,82,91]. By comparing Hek293 cells expressing the D3R or the α4β2-nAChR, or co-expressing both receptors, a long-lasting activation of the ERK1/2/Akt cascades, that requires PI3K activity, was defined as the signalling pathway specifically activated by the D3R-nAChR heteromer [87]. On the contrary, when the two receptors were expressed individually, this pathway was transiently activated and was independent of PI3K [87]. Similarly, in mouse DA neurons, the activation of the D3R-nAChR heteromer by nicotine or by quinpirole resulted in the persistent PI3K-dependent activation of the ERK1/2/Akt pathway. The result is the expression of the immediate-early gene c-Fos and the sustained phosphorylation of cytosolic p70 ribosomal S6 kinase (p70S6K) [87], crucial effectors for sustaining dendritic remodelling and neuronal protection [91].

Since the D3R-nAChR heteromer represents a strategic unit supporting DA neuron wellness [71,84,86,87] (Fig. 1), an attempt to develop heteromer-selective compounds led to the generation of a dual-acting compound named HyNDA-1. This bifunctional compound is composed by A-84543, a selective α4β2 nAChR agonist connected by a partially rigidified spacer to ropinirole, a D3R preferential agonist [92]. HyNDA-1 displays a high affinity for both β2-subunit-containing nAChR and D3R, significantly exerts neurotrophic effects in both mouse and human iPSCs-derived DA neurons. Interestingly, a peculiar characteristic of the HyNDA-1 compound is its ability to bring the two interacting receptors together, as shown by the increase in the affinity of interaction between D3R and nAChR in BRET experiments [92]. Abnormalities in D3R-nAChR heteromer expression, by impairing the neurotrophic and neuroprotective support for DA neurons, may contribute to DA neuron vulnerability, predisposing to the development of degenerative diseases such as PD [93]. Accordingly, in addition to many other abnormalities that characterize DA neurons bearing the G2019S mutation in LRKK2 [94], a remarkable reduction in the D3R-nAChR heteromer expression was demonstrated [95]. This mutation is present in more than 10 % of familial PD and in approximately 2 % of sporadic PD [96]. In fact it affects the function of LRRK2, which is involved in many cellular activities, including the trafficking of receptors from intracellular sites to the membrane [94,97]. Interestingly, normalizing the pathological activity of LRRK2 was sufficient to rescue the expression of the D3R-nAChR heteromer membrane, ensuring their support to DA neuron activity [95].

4. The concept of receptor crosstalk and the nAChRs role

Receptor-receptor crosstalk differs from receptor-receptor interaction because the proteins involved are usually close but not physically linked [98]. More specifically, all interactions that do not satisfy the

above criteria [19] (or lack information) could be defined as receptor crosstalk. This aspect allows for many combinations and identifies functional synaptic units composed of multiple dynamic receptors [99–105]. Ionotropic, metabotropic, and nuclear receptors are involved as well as channels, exchangers, and transporters [106,107]. Many concepts converge in the analysis of phenomena related to receptor crosstalk: metamodulation, co-transmission, and plasticity [108–114]. Moreover in 2005, Zhang and Oppenheim proposed that the crosstalk between chemokine receptors and neuropeptide membrane receptors can be the bridge between the immune and nervous systems [115]. The common denominator of these crucial aspects is complexity and fine-tuning signals. Receptor-receptor crosstalk is a dynamic mechanism regulated by development, chronic exposures, and any phenomena affecting each individual receptors involved [88,111,112,116]. The difficulty of studying how this phenomenon affects brain function has increased dramatically with the discovery of "receptor sharing" [117]. The authors described that A2A receptors, which can recognize and decode extracellular signals, can be safely transmitted from source to target via extracellular vesicles. Whether and to what extent this may affect functional crosstalk is now an intriguing hypothesis [118].

To understand the receptor crosstalk, it is necessary to evaluate the possible reasons for the interplay between different membrane structures. For example, we know that under basal conditions N-methyl-D-aspartate receptors (NMDARs) have a steric block that prevents ionic conduction. Therefore, collaborative actions can represent the primer for their activation, unblocking the channel and allowing the ligand action (Fig. 2). Functional crosstalk can generate the synergic activation of intracellular ion stores that sustains the external ion flux, producing an increase of neurotransmitter release or second messenger activation. Likewise, literature has demonstrated that dynamic expression in membranes (trafficking) can be affected by the cooperative action of nearby receptors. Accordingly, an alteration of trafficking increases (or reduces) the final effects of ligands, modifies the desensitization state of receptors and induces a transient modification of the subunit composition of receptors. Over the past few decades, the scientific community has extensively studied the functional crosstalk between proteins expressed on the same membranes [98,106,107,110–112]. The main reason is to understand if there is the possibility to modulate undruggable targets by selective compounds acting on ancillary partners [112]. Different technics can describe this phenomenon. One of the most important protocols is the release of neurotransmitters from synaptosomes in superfusion [112,119,120]. This technique combines two fundamental aspects to detect a clear interaction. First, it is often performed with purified synaptosomes, which are a simple model to analyse only the synaptic compartments without connection to the axon or neuronal body. Second, superfusion removes the biophase and emphasizes only direct modulations. It also has other interesting features such as the possibility to integrate the information gradually [121]. Indeed, we can compare synaptosomes with gliosomes in superfusion from the same tissue preparation to analyse the contribution of glial cells [122–125]. All particles have a native set of receptors with the same properties as those found in the brain of a living animal. In addition, we can identify receptor-receptor interactions using the same terminals to perform co-immunoprecipitations analysis [113]. Synaptosomes can also be paired with synaptoneurosomes allowing to discuss the role of post synaptic entities [126].

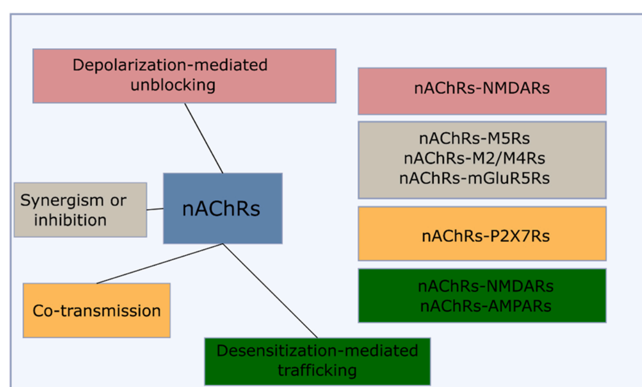

Fig. 2. Schematic representation of receptor crosstalk involving nAChRs.

5. Functional interplay between nAChRs and glutamate receptors

GluRs and nAChRs are often expressed on the same membranes [127–129]. The reason for this partnership is still debated and it could be the key to overcoming the limitations of direct therapy [130]. Recently, Stone summarized the data on the relationships and interactions between ionotropic GluRs and nAChRs in the CNS [44]. In the CNS, this interplay characterizes multiple brain areas such as the cortex, striatum, hippocampus, and several neurotransmitter systems including glutamate, DA, and NE. Both NMDARs and AMPARs are co-expressed with nAChRs and interact functionally. The nature of the interaction seems to depend on the subtypes involved, and the activity of nAChRs always anticipates that of GluRs. Interestingly, metabotropic GluRs also cooperate with nAChRs [131], suggesting a variety of mechanisms promoting cholinergic and glutamatergic cross-coupling. The nAChRs-GluRs crosstalk is a part of a broader reciprocal modulation. Indeed, nAChRs expressed on presynaptic nerve terminals induce glutamate release through exocytosis of glutamate-containing vesicles as well as aspartate-containing vesicles [132]. These amino acids are endogenous ligands of GluRs and can simultaneously activate both metabotropic and ionotropic signals. In addition, chronic activation of nAChRs modifies GluRs, glutamate transporters and cystine-glutamate exchangers, leading to an alteration in glutamate homeostasis [133–136].

5.1. *The GluRs and nAChRs crosstalk: role in neuronal DA release*

Presynaptic NMDARs efficiently modulate DA release from dopaminergic terminals and their composition is a combination of GluN1 and GluN2 subunits. Within the latter, GluN2B appears to be the most characteristic and data on the presence of GluN3 subunits are still inconsistent. These receptors are co-expressed with nAChRs subtypes in adult rodent nerve endings. In a pioneering paper, Glowinski and collaborators demonstrated that nicotine removes the Mg^{2+} block on nearby NMDARs by altering the membrane potential, allowing the subsequent activation of these receptors [137]. The result is a synergic modulation of DA release that increase the amount of DA efflux.

In 2014 we demonstrated that a lower concentration of nicotine maintained for 10 min reduces the NMDA-induced DA release from NAc synaptosomes [138]. This effect is due to the fine regulation of GluRs inputs performed by prolonged nicotine treatment. Chronic exposure of nAChRs to nicotine-induced a reduction in GluN2B subunits present at the plasma membrane, that was compatible with the induction of receptor trafficking. Both α4β2 and α6β3 containing nAChR subtypes appear to be involved. Dopaminergic terminals of the NAc also contain α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), that rapidly cycle between the synaptic membrane and intracellular compartments [139]. The dynamics of these receptors are sensitive to prolonged exposure to nicotine [140]. Therefore, a nicotine-mediated and pep2-SVKI sensible AMPARs internalization takes place at the same time as that described for NMDARs. Interestingly, the synchronous downregulation of GluRs mediated by nicotine pre-treatment revealed that the desensitization of nAChRs in the dopaminergic terminal causes the concomitant reduction of glutamatergic targets. The analysis of these interactions could be interpreted as a ubiquitous process however we know that only cyclothiazide-sensitive AMPARs respond to the nicotinic influence [140].

This mechanism could be a sophisticated regulation of the synaptic firing, but it is also possible that it is a mechanism to alter the synaptic sensitivity by reassembling receptor subunits. It is interesting to note that patients with epilepsy who use tobacco products or nicotine patches show a reduction in seizure frequency. This effect could be due to the regulation of several mechanisms such as DRs balance [141], nAChRs subunit regulation [142] and GluRs expression [143,144] Accordingly, it has been reported that NMDA, AMPA, and kainate can induce seizures in animal models and glutamate receptor antagonists inhibit seizures in animals [145].

5.2. The GluRs and nAChRs crosstalk: role in neuronal NE release

Recently, Pittaluga described the pharmacological differences between NMDARs located on striatal terminals and those expressed on hippocampal noradrenergic terminals [139]. The main difference seems to be the presence of the N1 cassette in the GluN1 subunit of NMDARs, which stimulates NE release. The noradrenergic terminals concomitantly express nAChR subtypes. These heteromeric receptors differ from those described at DA nerve endings. However, they remain depolarizing receptors capable of removing the Mg^{2+} block in the NMDAR channel [146]. The nicotinic subtypes α3β4 and α3β2 also colocalize with mGluR5 receptors [131]. Glutamate activation of the metabotropic cascade targeting IP3-sensitive calcium stores, which are per se ineffective in inducing NE exocytosis, becomes capable of upregulating nAChRs agonist-induced NE efflux. Taken together, these results describe how fluctuating glutamate concentrations can sustain nicotine activity by alternatively generating a parallel influx of external ions or recruitment of internal stores.

5.3. The GluRs and nAChRs crosstalk: role in neuronal glutamate release

Glutamate nerve terminals express the most representative CNS nAChR subtypes [147–150]. α4β2 and α7 receptors are very different targets [3,151]: the first is a heteromeric receptor permeable to the Na^{+} ions; the second is usually a homomeric receptor with high conductance for the Ca^{2+} ions. In the last few decades, several works have increased the knowledge of these receptors and have focused on the specificities of some alternative structures. For example, it has been shown that some α7 subtypes can be enriched through the insertion of β2 subunits, revolutionizing the dogma of this receptor [152]. In addition, α7 receptors have been described as ionotropic receptors with metabotropic accessory function [153]. In 2010, Lin and colleagues showed that α7-nAChRs enhanced the presynaptic surface expression of NMDARs, leading to increased glutamate release during early synaptic development [154]. This receptor crosstalk was confirmed a few years later in adult glutamatergic synaptosomes [155]. NMDARs stimulating glutamate release are distinct from those involved in DA and NE release [139]. Pharmacological characterization indicates that glutamatergic NMDARs contain GluN2A and GluN3A subunits [156]. Nicotine pre-exposure of NAc-glutamatergic synaptosomes caused a significant functional upregulation of NMDARs. This effect was selectively mediated by crosstalk with α7 receptors but not with α4β2 receptors. Biotinylation studies showed an increased density of GluN2A subunits in synaptosomal plasma membranes after pre-treatment with nicotine and choline. Taken together, these data indicate that functional crosstalk of nAChRs and NMDARs is not a negligible mechanism based on simple colocalization; in fact, it occurs when selective subtypes are involved. A physio-pathological significance of this interaction was indicated by Snyder and colleagues who showed that β-amyloid regulated NMDAR trafficking through interaction with the α7 receptors [157].

6. Receptor crosstalk between nAChRs and other receptors

nAChRs are cooperative receptors, which in turn are sensitive to modulatory effects exerted by other nearby expressed receptors. The main scenario in which nAChRs appear to be influenced by their partner is when they coexist with muscarinic receptors. This described interaction is peculiar because it is stimulated by the same endogenous ligand. Acetylcholine produces functional crosstalk between nAChRs and muscarinic autoreceptors at cholinergic and GABAergic nerve terminals. M2/M4 activation generates negative feedback on coexisting α4β2 receptors. Unlike on DA-terminal M5 muscarinic subtypes, they cooperate with α4β2 and α6β3 nAChRs subtypes by a mechanism like that described for mGluR5-nAChRs interaction. The analysis of these regulatory mechanisms reinforces the idea that nAChRs are often part of a receptor complex that is activated in cascade after their opening. Interestingly, on the glutamatergic nerve terminals the α7 receptors became more efficient in the inducing exocytosis when the partner P2X7 receptor is simultaneously activated. This is clear case of cotransmission since it is known that ATP is often left by the same terminals that contain acetylcholine [158].

7. Conclusions

Central nervous system functions are the result of a concert of actions mediated by protein networks. nAChRs actively collaborate within these functional groups to facilitate the function of their partners. In particular, numerous interaction phenomena that regulate mesolimbic cortical dopaminergic transmission have been characterized in recent years. At this level, phenomena such as heteromerization and receptor crosstalk have occurred, which can finely modulate the intensity and duration of the dopaminergic stimulus. Nevertheless, the interdependence of the action of some receptors has been shown to be capable of transiently modifying the set of targets expressed in the membrane, from time to time favouring the expression of some subtypes over others. nAChRs have been shown to establish close functional relationships, especially with dopaminergic receptors and glutamatergic receptors. Interestingly, these receptors can in turn cooperate among themselves [159–161]. One of the next challenges will be to complete the picture of possible interactions and move to a higher level where the whole functional cluster is considered. Knowledge of all possible forms of modulation could be the key to new pharmacological interventions.

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CRediT authorship contribution statement

Federica Bono: Writing – original draft preparation, manuscript revision, Drawing and figure making; **Chiara Fiorentini**: Writing – original draft preparation, manuscript revision, **Veronica Mutti**: Writing – original draft preparation, manuscript revision, **Zaira Tomasoni Mutti**: Writing – original draft preparation, manuscript revision, **Giulia Sbrini**: Writing – original draft preparation, Drawing and figure making, manuscript revision, **Hanna Trebesova**: Writing – original draft preparation, Drawing and figure making, manuscript revision, **Mario Marchi**: Conceptualization, Funding acquisition, **Massimo Grilli** Conceptualization, Writing – review & editing, Funding acquisition, **Cristina Missale**: Conceptualization, Writing – original draft preparation, Writing – review & editing, Funding acquisition.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability

review article.

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