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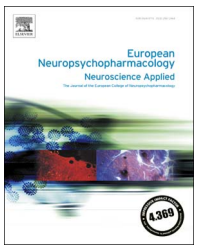

Dopamine D3 and acetylcholine nicotinic receptor heteromerization in midbrain dopamine neurons: Relevance for neuroplasticity

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Abstract
Activation of nicotinic acetylcholine receptors (nAChR) promotes the morphological remodeling of cultured dopamine (DA) neurons, an effect requiring functional DA D3 receptors (D3R). The aim of this study was to investigate the mechanisms mediating D3R-nAChR cross-talk in the modulation of DA neuron structural plasticity. By using bioluminescence resonance energy transfer^2 (BRET^2) and proximity ligation assay (PLA), evidence for the existence of D3R-nAChR heteromers has been obtained. In particular, BRET^2 showed that the D3R directly and specifically interacts with the β2 subunit of the nAChR. The D3R-nAChR complex was also identified in cultured DA neurons and in mouse Substantia Nigra/Ventral Tegmental Area by PLA. Cell permeable interfering peptides, containing highly charged amino acid sequences from the third intracellular loop of D3R (TAT-D3R) or the second intracellular loop of the β2 subunit (TAT-β2), were developed. Both peptides, but not their scrambled counterparts, significantly reduced the BRET^2 signal generated by D3R-GFP^2 and β2-Rluc. Similarly, the PLA signal was undetectable in DA neurons exposed to the interfering peptides. Moreover, interfering peptides abolished the neurotrophic effects of nicotine on DA neurons. Taken together these data first demonstrate that a D3R-nAChR heteromer is present in DA neurons and represents the functional unit mediating the neurotrophic effects of nicotine.

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1. Introduction

Dopamine (DA) plays a fundamental role in the regulation of different physiological functions, including motor activity, cognition, attention, affective behavior and positive reinforcement. In particular, DA neurons in the ventral tegmental area (VTA) modulate reward processing, impulsivity and motivation (Ikemoto and Bonci, 2014) and those in the Substantia Nigra (SN) regulate locomotor activity (Graybiel et al., 1994). Alteration of DA function in different brain areas and specific neuronal populations are related to the development of various neurological and psychiatric disorders, including Parkinson's disease (PD) and schizophrenia and underlie drug addiction (Kalia and Lang., 2015; Winton-Brown et al., 2014; Koob and Volkow, 2010).

Beside the DA transporter (DAT), that regulates spatial and temporal functions of DA (Giros et al., 1996), a crucial role in the control of DA neuron activity is exerted by DA itself through activation of D2-like receptors (D2R and D3R) and by acetylcholine (ACh) through nicotinic receptors (nAChR). The D2R is localized in both DA nerve terminals and soma and plays a major function in the control of neuronal firing and DA release (De Mei et al., 2009; Mercuri et al., 1997). The D3R is preferentially expressed in somatodendritic compartments (Diaz et al., 2000) and provides neurotrophic and neuroprotective support to DA neurons (Bellucci et al., 2008; Collo et al., 2008; Du et al., 2005; Joyce and Millan, 2007; Van Kampen and Eckman, 2006). nAChRs are a heterogeneous family of ligand-gated ion channels, composed by various α (α2-α7) and β (β2-β4) subunits, which are activated by nicotine. Different nAChR subtypes are co-localized with D2-like receptors in DA nerve terminals (Exley and Cragg 2008; Zoli et al., 2015). The α4β2 subtype, on the other hand, represents the majority of functional nAChR in DA neuron somatodendritic area (Champtiaux et al., 2003). It is well known that stimulation of nAChR increases DA neuron firing and DA release (De Kloet et al., 2015) and an antagonistic cross-talk between nAChR and D2R in the regulation of DA release has been clearly demonstrated (Quarta et al., 2007). Nicotine also promotes the morphological remodeling of DA neurons and regulates various genes controlling neuronal morphogenesis (Doura et al., 2010; Quik et al., 2006; Collo et al., 2013), an effect that may induce alterations of synaptic connections among neurons of the mesolimbic pathway leading to addiction (Luscher and Malenka, 2011). Moreover, these processes are also crucially involved in the mechanisms supporting nigro-striatal DA neuron survival. An inverse correlation has been, in fact, established between cigarette smoking and PD development (Fratiglioni and Wang, 2000; Picciotto and Zoli 2008).

A functional interplay between nAChR and D3R has been reported. D3R antagonists block, in fact, nicotine-induced conditioned place preference and drug seeking behavior and nicotine increases D3R, but not D2R, mRNA with strong implication in nicotine sensitization (Khaled et al., 2010; Le Foll et al., 2005; Pak et al., 2013; Smith et al., 2015). Moreover, we reported that nicotine increases the dendritic arborization of cultured DA neurons and that this effect was prevented by preferential D3R antagonists and was lost in D3R knock out (D3R-KO) mice (Collo et al., 2013).

Interaction between nAChR and D3R might involve either the convergence of their signaling pathways or transactivation mechanisms or the formation of heteromeric complexes. In particular, one of the properties of G protein-coupled receptors (GPCR) is their propensity to physically associate with both closely-related and structurally-divergent receptors to generate heteromers with novel pharmacological and transductional properties, suggesting that heteromerization represents a key integrative mechanism at the synaptic level. In particular, DA receptors have been reported to form heteromers not only with other GPCR, but also with ion channel receptors including the glutamate NMDAR (Fiorentini et al., 2003; Lee et al., 2002; Cahill et al., 2014) and the GABA-AR (Liu et al., 2000).

In this paper we investigated the mechanisms mediating D3R-nAChR cross-talk in the modulation of DA neuron structural plasticity. We report the existence of a D3R-nAChR heteroreceptor complex resulting from the direct interaction of the D3R with the β2 nAChR subunit. We identified the amino-acid sequences involved in the interaction and, by using specific interfering peptides, we demonstrated that this heteromer is the functional receptor unit mediating nicotine-induced morphological remodeling of DA neurons.

2. Experimental procedures

2.1. Animals

CD1 mice were obtained from Charles River Laboratory (Calco, Italy). D3R knock out mice (D3R-KO) were from Jackson Laboratory (Bar Harbor, ME) (B6.129S4-Drd3Tm1Dac/J). α4 knock out mice (α4-KO) were kindly provided by Dr Uwe Maskos (Paris). C57BL6/J singenic mice were used as control. Animals were bred and housed in the animal-house facility of the University of Brescia with water and food ad libitum and a 12-h light-dark cycle. Animal use was in accordance with the Directive 2010/63/EU. All procedures were conformed to the National Research Guide for the Care and Use of Laboratory Animals and were approved by the Animal Research Ethical Committee of the University of Brescia. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Antibodies

Polyclonal anti-D3R (S-16) was from Santa Cruz Biotechnologies (Heidelberg, Germany). Monoclonal anti-α4 nAChR subunit was from Sigma-Aldrich (Milano, Italy). Monoclonal anti-tyrosine hydroxylase (TH) was from Millipore (Milano, Italy). The horseradish peroxidase-conjugated anti-rat and anti-goat secondary antibodies were from Santa Cruz Biotechnologies.

2.3. Generation of TAT-peptides

Cell-permeable peptides were obtained by linking a 11 amino acid sequence of cell-penetrating human immunodeficiency virus trans-activator of transcription (TAT) to either the 215–225 arginine-rich region of D3R (TAT-D3R; NH_2-YGRKKRRQRRR**LKQRRRKRIL**-COOH) or the 439–449 aspartate-rich region of β2 nAChR subunit (TAT-β2; NH_2-YGRKKRRQRRR**HMRSEDDDQSVS**-COOH) (GenScript, Piscataway, USA). Cell-permeable peptides whose sequence has been scrambled maintained the same overall positive or negative charge of TAT-D3R or TAT-β2 respectively (TAT-D3R-Sc, NH_2-YGRKKRRQRRR**IRKLRLRQRK**-

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