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Subsynaptic localization of nicotinic acetylcholine receptor subunits: A comparative study in the mouse and rat striatum

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HIGHLIGHTS

- nAChRs subunits (α7, α4, α6, β2) display different subsynaptic localizations.
- All nAChRs subunits (α7, α4, α6, β2) studied are present presynaptically.
- The α6 subunit is mostly postsynaptically located.

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ABSTRACT

The striatum is the primary input station of the basal ganglia network, playing an essential role in sensorimotor, cognitive and motivational functions. Nicotinic acetylcholine receptors (nAChRs) were identified in nerve terminals of the striatum, where they are known to modulate neurotransmitter release, therefore critically regulating striatal functions. However, the subsynaptic (i.e. pre-, post- and extra-synaptic) localization of the different nAChRs subtypes present in the striatal synapses is still unclear, which might be associated with different roles in the control of synaptic transmission. In the present study we analyzed the subsynaptic distribution of particularly relevant nAChRs subunits, namely α7, α6, α4 and β2, in rat and mice striatal synapses (synaptosomes). In the rodent striatum we found that the α7 subunit, which predominantly forms homomeric nAChRs, was mainly present at the presynaptic active zone. The α4 and β2 subunits displayed a similar distribution, being primarily present at the presynaptic and/or extrasynaptic zones (mice and rats, respectively), which was expected since these two subunits together form heteropentameric nAChRs. In contrast, the α6 subunit was mainly present in the postsynaptic fraction, albeit being also present in pre- and extra-synaptic fractions. Altogether, this work details the striatal subsynaptic distribution of some of the main nAChRs subunits, underlining the possible relevance of striatal nAChRs in controlling neurotransmission, with potential relevance for Parkinson's disease, nicotine addiction and other dopaminergic disorders.

1. Introduction

The striatum is the main gateway of the basal ganglia, a brain network essential in sensorimotor, cognitive and motivational functions [2,29]. Ionotropic nicotinic acetylcholine receptors (nAChRs) are present and control all known striatal functions [13,15,19,36] and the main neurochemical impact of nAChRs seems to be the modulation of neurotransmitters release [12,30,34]. The nAChRs consist of five subunits assembled at the plasma membrane to form ligand-gated channel permeable to ions. The many possible combinations in which the different nAChR subunits can potentially co-assemble lead to a diversity of distinct nAChR subtypes with specific pharmacological and functional properties [1,12]. In fact, the activation of presynaptic nAChRs induces the release of GABA [14,15,19], glutamate [4,20], and dopamine [10,28,33] in the rat or mouse striatum to control the key functioning of GABAergic medium spiny neurons [6,8,15] as well as their extrinsic cortical and mesencephalic control [9,19]. However, it is still unclear if particular nAChRs subtypes display a different relative distribution at different subsynaptic sites, such as pre-, post- and extra-synaptic zones.

Abbreviations: FELASA, Federation for Laboratory Animal Science Associations; nAChR, nicotinic acetylcholine receptor; SDS, sodium dodecyl-sulphate.

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The present study aims to detail the localization and density at the subsynaptic level of some relevant subunits of nAChRs, such as α7, α4, α6 and β2. To address these objectives we carried out a subsynaptic fractionation of purified striatal synapses (synaptosomes) obtained from rats and mice and quantified the density of nAChRs subunits by immunoblot, using commercially available antibodies against these proteins. It was also our goal to assess if the distribution of the nAChR subunits is similar in Wistar rats and C57BL/6 mice, two animal models extensively used in neuroscience research [3,31]. This effort to map the localization of the nAChRs subunits within striatal synapses may provide insights about the involvement of the different nAChRs subtypes in disorders affecting striatal circuits, such as Parkinson's disease and addiction.

2. Methods

2.1. Animals

All experimental procedures were conducted in accordance with the principles outlined in the EU guidelines (86/609/EEC) and by FELASA, and were approved by the Animal Welfare Committee of the Center for Neuroscience and Cell Biology of Coimbra.

The animals used in this work were male Wistar rats (10–14 weeks old) and male C57BL/6 mice (10–12 weeks old) obtained from Charles River (Barcelona, Spain). The animals were deeply anesthetized with halothane (no reaction to handling or tail pinch, while still breathing) before decapitation with a guillotine, and their brains rapidly removed to ice-cold sucrose solution.

2.2. Subsynaptic fractionation of nerve terminals

The fractions from rodent striatal synaptosomes were separated as initially described by Phillips et al. [23], with some modifications introduced by our group [26]. Briefly, striata (caudate-putamen) from 5 to 8 rats or 10 to 12 mice were homogenized at 4 °C in isolation solution [0.32 M sucrose, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM phenylmethylsulfonylfluoride (PMSF)] and centrifuged at 100,000 × *g* for 3 h at 4 °C. Synaptosomes were collected and pelleted at 15,000 × *g* for 30 min at 4 °C. Pellets were resuspended in 1 ml of isolation solution, diluted in cold 0.1 mM $CaCl_2$ and a 2× concentrated solubilization buffer (2% Triton X-100, 40 mM Tris, pH 6.0) was added. The synaptosomal membranes were then incubated for 30 min on ice with mild agitation and the insoluble material (synaptic junctions, comprising the presynaptic active zone and the postsynaptic density) was pelleted (40,000 × *g* for 30 min at 4 °C). The supernatant (extra-synaptic fraction, corresponding to membranes in the vicinity, but outside the active zone) was decanted and proteins precipitated with 6 volumes of acetone at −20 °C and recovered by centrifugation (18,000 × *g* for 30 min at −15 °C). The synaptic junctions pellet was washed twice in pH 6.0 solubilization buffer, resuspended in 5 ml of 1% Triton X-100 and 20 mM Tris (pH 8.0), incubated for 30 min on ice with mild agitation, centrifuged (40,000 × *g* for 30 min at 4 °C) and the supernatant (presynaptic fraction, corresponding to the presynaptic active zone) processed as above. The increase of pH from 6.0 to 8.0 leads to the dissociation of the extracellular web filaments that strongly connect the presynaptic active zone to the postsynaptic density; this allows the solubilization of the active zone while the postsynaptic density is mostly maintained, because the amount of detergent is not sufficient to achieve its solubilization [23]. PMSF (1 mM) was added to the suspension in all extraction steps to minimize proteolysis. The pellets from the supernatants and the final insoluble pellet (postsynaptic density fraction) were solubilized in 5% SDS (W/V in water).

2.3. Western blot

The experiments were performed as previously described [11]. The antibodies used were as follows: rat anti-nicotinic α7 receptor (1:3000), rabbit anti-nicotinic α4 receptor (1:3000) and rabbit anti-nicotinic α6 receptor (1:600) (Abcam Biochemicals, Cambridge, UK), rabbit anti-nicotinic α6 receptor (1:3000) and rabbit anti-nicotinic β2 receptor (1:15,000) (Merck Millipore, MA, USA), mouse anti-SNAP-25 (1:60,000), rabbit anti-synaptophysin (1:60,000), mouse anti-syntaxin (1:60,000) and mouse anti-PSD-95 (1:100,000) (Sigma, Sintra, Portugal), alkaline phosphatase-labeled (AP) goat anti-rabbit (1:20,000) or anti-mouse (1:20,000) antibodies (GE Healthcare, Lisbon, Portugal), AP chicken anti-rat (1:3000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.4. Data presentation

All data are expressed as means ± SEM of the indicated number of independent experiments (*n*).

3. Results

The efficiency of the subsynaptic fractionation procedures was assessed by quantifying the immunoreactivity of proteins that are specific markers of different synaptic regions: (i) SNAP-25 and syntaxin in the presynaptic active zone, (ii) PSD-95 in the postsynaptic density and (iii) synaptophysin (a synaptic vesicle protein) outside the active zone, i.e. in extra-synaptic regions. As shown in Fig. 1A and B, the presynaptic active zone fraction displayed higher levels of SNAP-25 and syntaxin as compared with the post- or extra-synaptic fractions, whereas the postsynaptic fraction exhibited higher levels of PSD-95, being this protein almost residual in the other subsynaptic fractions. The density of synaptophysin was higher in extra-synaptic than in presynaptic fractions, and it was almost inexistent in the postsynaptic density fraction. The labeling of synaptophysin observed in the presynaptic fraction is possibly due, at least partially, to the presence of docked synaptic vesicles [24] (Fig. 2).

The subsynaptic distribution of the immunoreactivity of nAChR subunits in the subsynaptic fractions obtained from rat and mouse striatum is detailed in Fig. 1A and B, respectively, where the tested antibodies against the different nAChR subunits recognized bands of identical molecular weight in rats and mice. The results showed that the α7 subunit was localized mainly at the presynaptic active zone (51.5 ± 4.4% of total immunoreactivity, $n = 3$) and was also present extra-synaptically outside the active zone (36.7 ± 3.3%, $n = 3$), but it had a much smaller presence at the postsynaptic density (11.8 ± 2.7%, $n = 3$). In mice, the α7 subunit was also mainly present at the presynaptic active zone (63.6 ± 6.1% of total immunoreactivity, $n = 4$), similarly to what was observed in rat striatum. However, the relative levels of the α7 subunit at the postsynaptic density (24.5 ± 5.6% of total immunoreactivity, $n = 4$) and outside the active zone (11.9 ± 1.6%, $n = 4$) were different from these detected in the rat striatum.

The striatal subsynaptic localization of the β2 subunit was found to be different in rats and mice: thus, the β2 subunit was predominantly located at extra-synaptic sites (86.1 ± 2.5% of total immunoreactivity, $n = 3$) in rats, whereas in mice it was expressed in similar relative amounts both at pre- and extra-synaptic regions (about 40%, $n = 3$). Likewise, the subsynaptic distribution of the α4 subunit in the striatum of rats and mice was dissimilar: thus, the α4 subunit was predominantly localized at extra-synaptic sites (88.8 ± 9.5% of total immunoreactivity, $n = 3$), having a lower density at the presynaptic active zone (about 10%, $n = 3$) in rats, whereas in mice the α4 subunit was mainly present at the presynaptic active