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The α7 nicotinic acetylcholine receptor ligands methyllycaconitine, NS6740 and GTS-21 reduce lipopolysaccharide-induced TNF-α release from microglia

Morten S. Thomsen *, Jens D. Mikkelsen

Neurobiology Research Unit, University Hospital Copenhagen, Rigshospitalet, Copenhagen, Denmark

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ABSTRACT

The anti-inflammatory properties of, particularly the α7, nicotinic acetylcholine receptors (nAChRs) in the peripheral immune system are well documented. There are also reports of anti-inflammatory actions of nicotine in the CNS, but it is unclear, whether this is due to activation or inhibition of nAChRs.
Here we investigate the mechanisms behind α7 nAChR-mediated modulation of TNF-α release. We show that α7 nAChR agonists or positive allosteric modulators do not affect LPS-induced release of the pro-inflammatory cytokine TNF-α from cultured microglia. This suggests that classical activation of, *i.e.* ion-flux through, the α7 nAChR does not reduce TNF-α release from activated microglia. Contrarily, the α7 nAChR antagonist methyllycaconitine and the weak (<10%) agonist NS6740 reduced LPS-induced TNF-α release, indicating that α7 nAChR antagonism conveys anti-inflammatory properties on microglia. The effect of methyllycaconitine or NS6740 was not due to changes in MAPK signaling. These results suggest that the anti-inflammatory effects of nicotine seen *in vivo* are not due to classical activation of the α7 nAChR, and further suggest that antagonism of α7 nAChRs may reduce neuroinflammation.

1. Introduction

Neuroinflammation is evident in diseases, such as stroke and epilepsy, but more subtle, long-lasting forms of neuroinflammation may also underlie the pathophysiological changes in schizophrenia, Alzheimer's disease and Parkinson's disease, and may contribute to the underlying pathological process of these diseases (reviewed in Wyss-Coray, 2006; Monji et al., 2009; Panaro, 2012).

Nicotine has been shown to have anti-inflammatory and neuroprotective properties in animal models of neurodegenerative disease (Park et al., 2007a; Takeuchi et al., 2009; Toulorge et al., 2011). Several nicotinic acetylcholine receptors (nAChRs) are involved in these effects of nicotine, although the α7 nAChR may have the most prominent role (reviewed in Buckingham et al., 2009; Cui and Li, 2010; Bencherif et al., 2011). It has thus been shown that nicotine acts on the α7 nAChR to reduce cytokine expression in the rat brain after intracerebral injections of lipopolysaccharide (LPS) (Tyagi et al., 2010) and in a murine experimental autoimmune encephalomyelitis model (Nizri et al., 2009).

The protective effects of nicotine *in vivo* may be related to α7 nAChRs located on neurons or glia. However, the α7 nAChR is also critical for vagal nerve-mediated reduction of TNF-α release from peripheral macrophages, the so-called cholinergic anti-inflammatory pathway (Wang et al., 2003; Tracey, 2007), inviting the possibility that peripheral immune cells invading the brain in response to an insult is the basis of the protective effects of nicotine *in vivo*.

Direct neuroprotective effects of nicotine in cultured neurons have been shown against a variety of insults, such as glutamate excitotoxicity, oxygen and glucose deprivation, endotoxins and amyloid toxicity (Kaneko et al., 1997; Kihara et al., 2001; Egea et al., 2007; Park et al., 2007a). In addition, it has been shown that nicotine can reduce LPS-induced release of the pro-inflammatory cytokine TNF-α from cultured rodent microglia, and that this effect is sensitive to the α7 nAChR antagonists methyllycaconitine (MLA) and bungarotoxin (Shytle et al., 2004; De Simone et al., 2005; Suzuki et al., 2006), suggesting that α7 nAChRs on microglia are important for the anti-inflammatory actions of nicotine *in vivo*. In addition, the selective α7 nAChR agonist PNU-282987 can reduce TNF-α release from an NIH3T3 cell line engineered to over-express the α7 nAChR (Li et al., 2009).

However, MLA has also been shown to reduce ibotenate-induced cortical lesions *in vivo* (Laudenbach et al., 2002) as well as protecting against NMDA excitotoxicity in hippocampal slices (Ferchmin et al., 2003). These data, combined with the fact that high concentrations of nicotine (10–100 μM), which are known to desensitize nAChRs, produce anti-inflammatory or neuroprotective effects (Kaneko et al.,

Abbreviations: LPS, lipopolysaccharide; MLA, methyllycaconitine; MAPK, mitogen-activated protein kinase; nAChR, nicotinic acetylcholine receptor; PAM, positive allosteric modulator; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol.

* Corresponding author at: Neurobiology Research Unit, University Hospital Copenhagen, Juliane Maries Vej 24, DK-2100 Copenhagen, Denmark. Tel.: +45 35456736; fax: +45 35456713.
E-mail address: morten.s.thomsen@nru.dk (M.S. Thomsen).

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1997; Egea et al., 2007; Park et al., 2007a), has led to the hypothesis that desensitization rather than activation of α7 nAChRs underlies these effects of nicotine (Buckingham et al., 2009).

In addition to its potential anti-inflammatory actions, the α7 nAChR has been shown to improve cognitive function in animals and humans, and it is therefore a potential drug target for diseases where cognition is impaired, such as schizophrenia and Alzheimer's disease (reviewed in Thomsen et al., 2010). Since the pathophysiology of these disorders also involve neuroinflammation, it is particularly pertinent to study the effects of the α7 nAChR on neuroinflammation. However, although the α7 nAChR is an interesting target to reduce inflammation and neurodegeneration, the underlying mechanisms are elusive. It is thus not known whether activation or inactivation of the α7 nAChR is responsible for these effects, and it is not known whether the target cells are primarily neurons, glia or invading peripheral immune cells. In this study we address the first issue by examining whether activation or inactivation of the α7 nAChR is able to reduce LPS- or $A\beta_{1-42}$-induced TNF-α release from rat microglia cultures by studying the effects of selective α7 nAChR agonists and positive allosteric modulators (PAMs) as well as MLA.

2. Materials and methods

2.1. Compounds and materials

A list of the small-molecule compounds used is presented in Table 1. 1,4-diazabicyclo[3.2.2]nonan-4-yl(5-(3-(trifluoromethyl)phenyl)furan-2-yl)methanone (NS6740) (Briggs et al., 2009), (2,4)-dimethoxybenzylidene anabaseine dihydrochloride (GTS-21) (Briggs et al., 1997), 1,4-diazabicyclo[3.2.2]nonane-4-carboxylic acid, 4-bromophenyl ester (SSR180711) (Biton et al., 2007), 2-methyl-5-(6-phenyl-pyridazin-3-yl)-octahydro-pyrrolo[3,4-c]pyrrole (A-582941) (Bitner et al., 2007), 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea (PNU-120596) (Hurst et al., 2005), and 1-(5-chloro-2-hydroxy-phenyl)-3-(2-chloro-5-trifluoromethyl-phenyl)-urea (NS1738) (Timmermann et al., 2007) were kindly provided by the Department of Medicinal Chemistry at NeuroSearch A/S (Ballerup, Denmark). N-(4-chlorophenyl)-α-[[(4-chloro-phenyl)amino]methylene]-3-methyl-5-isoxazoleacetamide (AVL-3288, also known as XY4083 and CCMI) (Ng et al., 2007) was a kind gift from Kelvin W. Gee, University of California, Irvine. Methyllycaconitine citrate was purchased from Ascent Scientific (Bristol, UK). Lipopolysaccharide, choline chloride and dexamethasone were purchased from Sigma-Aldrich. $A\beta_{1-42}$ was synthesized by Caslo Laboratory Aps. $A\beta_{1-42}$ oligomers were obtained following the protocol of Chromy et al. (2003). Briefly, the peptide was dissolved to 1 mM in cold 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and incubated for 1 h at 20–22 °C, after which the HFIP was evaporated, and the peptide was stored at −80 °C until use. Before use, the peptide was resuspended to 5 mM in anhydrous DMSO, diluted to 200 μM in culture medium and incubated for 24 h at 4 °C. The resulting suspension was cleared by spinning it at 14,000 *g* for 10 min and discarding the pellet.

2.2. Mixed glia culture

Pregnant Sprague Dawley rats were obtained from Charles River (Germany). Four adult male rats were obtained from Taconic Europe (Denmark). One- to five day old Sprague Dawley rat pups were decapitated, and their brains dissected under aseptical conditions. Using microdissection tools under a microscope, the cortex was dissected while taking care to avoid meninges or blood vessels. The dissected tissue was briefly minced using tweezers and transferred to ice-cold dissection medium (2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 μg/ml streptomycin in high glucose DMEM). The tissue was then spun at 200 *g* for 2 min at 20–22 °C, resuspended in 0.05% Trypsin-EDTA (Invitrogen) containing 0.0015 kU DNase I (Sigma-Aldrich) and passed through a 10 ml pipette several times before being incubated at 37 °C for 15 min while shaking. Culture medium (dissection medium containing 10% fetal bovine serum) was then added to stop the trypsinization and the cells were spun at 200 *g* for 2 min at 20–22 °C, resuspended in a small volume of culture medium and triturated several times through a 10 ml pipette, and then through a 10 ml pipette fitted with a 200 μl pipette tip. The cell suspension was then left for 5 min at 37 °C, and the supernatant transferred to a different tube. The number of viable cells was counted using a hemocytometer and trypan blue, and the cells were seeded at $0.5–1\times10^5$ cells/ml in poly-L-lysine (Sigma-Aldrich, Saint Louis, MO) coated culture flasks. Cultures were maintained in a humidified incubator at 37 °C with 5% CO_2. The culture medium was changed every 3–4 days. The mixed glia cultures were confluent after 12–14 days.

2.3. Microglia-enriched culture

Confluent mixed glia cultures were shaken on a shaking table at ~180 rpm for 3–5 h at 37 °C. The medium was then removed, centrifuged at 200 *g* for 8 min at 20–22 °C, and resuspended in a smaller volume of culture medium. The number of viable cells was counted using a hemocytometer and trypan blue, and the cells were seeded at $0.5–1\times10^5$ cells/well in poly-L-lysine coated 96-well plates. The purity of the microglia-enriched cultures using this method was confirmed by fluorescent immunocytochemical staining for the microglia marker OX-42 and the astrocyte marker GFAP, which showed that the cultures contained >99% microglia (data not shown).

2.4. TNF-α ELISA

For TNF-α release studies involving LPS, the resulting microglia-enriched cultures, 18–24 h after plating, were exposed to a combination of compounds as indicated in the figure labels for 30 min after which they were exposed to LPS for 4 h. For TNF-α release studies involving $A\beta_{1-42}$, the resulting microglia-enriched cultures, 2 h after plating, were exposed to a combination of compounds as indicated in the figure label for 30 min after which they were exposed to $A\beta_{1-42}$ for 24 h. After

Table 1
Compounds used.

Compound	Type	EC_{50} (μM)	E_{max} (% of Ach)	Comments	References
SSR180711	Partial agonist	0.9–4.4	36–51%		Biton et al. (2007)
A-582941	Partial agonist	4.3–7.9	52–59%		Bitner et al. (2007)
Choline	Full agonist	1600	91%	Metabolite of ACh	Alkondon et al. (1997)
NS6740	Weak agonist	$IC_{50}=0.003$	8.3%		Briggs et al. (2009)
GTS-21	Partial agonist	6.0	23%	Also α4β2* nAChR antagonist	Briggs et al. (1997), Kem et al. (2004)
MLA	Antagonist	$IC_{50}=0.0001$	n/a		Alkondon et al. (1992)
PNU-120596	PAM II	0.2	n/a		Hurst et al. (2005)
NS1738	PAM I	3.9	n/a		Timmermann et al. (2007)
AVL-3288	PAM I	0.7	n/a		Ng et al. (2007)

EC_{50} and E_{max} refer to effects on the human α7 nAChR receptor. n/a = not applicable.