International Journal of Neuropsychopharmacology (2010), **13**, 293–303. Copyright © CINP 2009
doi:10.1017/S1461145709990770

ARTICLE

CINP

Autoantibodies from schizophrenia patients induce cerebral cox-1/iNOS mRNA expression with NO/PGE_2/MMP-3 production

Sabrina Ganzinelli, Enri Borda and Leonor Sterin-Borda

Pharmacology Unit, School of Dentistry, University of Buenos Aires and Argentine National Research Council (CONICET), Buenos Aires, Argentina

Abstract

We demonstrated that circulating antibodies from schizophrenia patients, which interact with cerebral M_1 muscarinic acetylcholine receptors (M_1 mAChRs), trigger production of nitric oxide (NO), prostaglandin E_2 (PGE_2) and matrix metalloproteinase-3 (MMP-3), and act as inducers of cyclooxygenase-1 (cox-1) and inducible nitric oxide synthase (iNOS) mRNA expression in the rat frontal cortex. The corresponding affinity-purified anti-M_1 peptide IgG from schizophrenia patients, while stimulating cerebral M_1 mAChRs, increases NOS activity, PGE_2 and MMP-3 production associated with iNOS over-activity and mRNA expression. Moreover, PGE_2 and MMP-3 production is the result of cox-1 expression and activity. All these effects were inhibited by pirenzepine or haloperidol and mimicked the action of the authentic mAChR agonist. Concurrent analysis of the effects of iNOS, phospholipase C, protein kinase C and calcium/calmodulin inhibition showed that antibody up-regulation of NOS activity, PGE_2 and MMP-3 production is under the control of the endogenous NO signalling system. These results provide evidence of the role that cholinergic receptor antibodies play in the development of cerebral inflammation, which shows that an antibody that interacts with cerebral mAChRs can induce expression of pro-inflammatory mediators, and support the participation of an autoimmune process in a particular group of chronic schizophrenia patients.

Received 27 April 2009; Reviewed 7 July 2009; Revised 3 August 2009; Accepted 8 September 2009; First published online 19 October 2009

Key words: Autoantibodies, cerebral frontal cortex, cholinergic receptors, pro-inflammatory mediators, schizophrenia.

Introduction

Schizophrenia is a neuropsychiatric disease in which an autoimmune reaction occurs in the brain (Henneberg *et al*. 1993). The disturbance in self-recognition of brain antigens by the immune system is the primary phenomenon, while the signs and symptoms of schizophrenia are the secondary manifestations of the immune attack on the brain (Noy *et al*. 1994). The course of exacerbations and remissions, genetic vulnerability and the early onset in a great number of cases, hint at parallels between schizophrenia and autoimmune diseases, especially those with overt central nervous system (CNS) manifestations (Colburn *et al*. 2001).

Cellular and humoral immune responses both seem to be involved in the immune dysfunction of schizophrenia (Müller *et al*. 2000). Antibodies against different brain regions, especially the frontal cortex have been described in sera of schizophrenia patients (Heath *et al*. 1990).

Previously, autoantibodies against cerebral M_1 and astrocyte M_1 and M_2 muscarinic acetylcholine receptors (mAChRs) have been described, which account for the disease (Borda *et al*. 2002, 2004; Ganzinelli *et al*. 2006). A strong association ($\alpha = 0.05$) between the presence of serum mAChR autoantibodies and disease stage has been demonstrated. As the serum samples were collected during a psychotic exacerbation, we hypothesize that the paranoid or negative symptoms and the acute stage of the illness may influence the muscarinic cholinergic activity

Address for correspondence: L. Sterin-Borda, M.D., Pharmacology Unit, School of Dentistry, University of Buenos Aires, Marcelo T. de Alvear 2142, 4° 'B', 1122AAH, Buenos Aires, Argentina.
Tel.: (5411) 4964 1276 *Fax*: (5411) 4963 2767
Email: leo@farmaco.odon.uba.ar

induced by antibody–mAChR specific interaction. Fixation of autoantibodies to M_1 mAChRs has important functional implications, since they are directed against the agonist binding site on the cell surface and behave as partial agonists, activating intracellular messengers and altering the rate of transcription of M_1 mAChR genes in response to receptor activation (Ganzinelli *et al*. 2006). These facts raise the question of how greatly neurotransmitter autoantibodies are implicated in the pathogenesis of schizophrenia.

Several lines of research have suggested that muscarinic receptor dysfunction is associated with negative schizophrenia symptoms, and an increase in positive symptoms results from decreasing muscarinic activity (Carlsson, 1978; Tandon, 1999). Deficits in attention, memory, motor coordination and executive functions primarily implicate the frontal cortex (Andreasen *et al*. 1992; Grafman *et al*. 1995), and involve cholinergic neuron activation distributed in the associated cortex region (Yeomans, 1995). Therefore, alterations to different mAChR subtypes may mediate differentially positive, negative and cognitive symptoms of schizophrenia (Birdsall *et al*. 2001). Thus, M_1 receptor knockout mice have exhibited decreased performance in a hippocampus-based test of spatial learning and memory (Gerber, 2001).

There is active surveillance and an inflammatory response in the brain of schizophrenia patients, which can occur in response to different injuries (Henneberg *et al*. 1993). This leads us to consider that neuronal tissue damage and inflammation described in schizophrenia (Noy *et al*. 1994) might be a consequence of production of pro-inflammatory mediators induced by antibody–mAChR interaction. In particular, molecules that classically have been thought to exclusively mediate immune function have been found to actively modulate synaptic memory processes (Di Filippo *et al*. 2008).

Among the signalling events involved in mAChR activation in the cerebral frontal cortex, stimulation of nitric oxide synthase (NOS) activity (Sterin-Borda *et al*. 2003) and release of prostaglandin E_2 (PGE_2) (Orman *et al*. 2005) have been demonstrated. Prostaglandins (PGs) and NO represent some of the most relevant local mediators that participate in the modulation of many neuronal functions, including learning and memory (Mollace *et al*. 2005). NO plays a key role in the pathophysiology of chronic inflammation and neurodegenerative processes (Liberatore *et al*. 1999). Moreover, altered biosynthesis of prostanoids is clearly involved in neuroinflammatory processes (Cao *et al*. 1996).

Two major isoforms of NOS and cyclooxygenase (COX) enzymes have been identified: constitutive and inducible. The constitutive isoforms are found in virtually all normal conditions. On the other hand, in inflammatory settings, the inducible isoforms of these enzymes (iNOS and COX-2) are detected. In the brain, COX-2 is expressed constitutively and is localized in neurons and COX-1 is localized in microglia (Choi *et al*. 2009). COX-1 is prevalent in the forebrain, where PGs may be involved in complex integrative functions, such as modulation of the autonomic nervous system (Breder & Saper, 1996). Human brain tissues contain equal amounts of mRNA for cox-1 and cox-2 (Shuttleworth & Thompson, 1998). Recent evidence has indicated that there is a constant cross-talk between NO and PG biosynthesis pathways involved in the pathophysiological mechanisms underlying some inflammatory disorders (Mollace *et al*. 2005).

Other players involved in neuroinflammation that directly or indirectly have an effect on neuronal function are the matrix metalloproteinases (MMPs) (Hendriks *et al*. 2005). Much has been learned recently about the role of MMPs in neurological diseases (Rosenberg, 2002). In the active form, these enzymes have a number of important roles but they are highly destructive in inflammation of the CNS (Rosenberg, 2002).

Taking these observations together, we focused our research on the possibility that mAChR autoantibodies contribute to the neuroinflammatory reaction in schizophrenia. We investigated whether the antibodies against mAChRs can induce cholinergic NOS activation and PGE_2 and MMP generation, and suggest a novel insight into the mechanisms involved in the ability of anti-mAChR antibodies to act as an early inducer of cox and iNOS gene expression. Thus, the possibility of a CNS-specific antigen–antibody system in schizophrenia that displays an agonistic-like activity may result in deregulation of cerebral mAChR functional activity and immune-mediated cerebral inflammation.

Material and methods

Subjects

The paranoid schizophrenia groups comprised: 37 inpatients (25 men, 12 women) that were positive for autoantibodies against M_1 mAChRs; and 10 patients that were negative for M_1 mAChR autoantibodies (used as negative control). The mean age was 43 yr (range 23–57 yr) with mean duration of illness 27.8 ± 5 yr as defined by DSM-IV criteria (APA, 1994). Patients did not have any associated organic disease and more specifically, no immune disorders. All

patients were receiving maintenance doses of neuroleptic medication. Diagnoses were made on the basis of clinical records, interview data collected with the Structured Clinical Interview for DSM-IV (SCID-P; Spitzer *et al*. 1991), and the Brief Psychiatric Rating Scale (BPRS; Overall & Goham, 1962) (schizophrenia score was 46.5, S.D. = 15.1, predominance of negative symptoms). Sera were collected during a psychotic exacerbation. The normal groups consisted of 30 age-matched healthy subjects (15 men, 15 women; mean age 45 yr). Serum immunoglobulin G (IgG) concentrations (mean ± S.E.) were: schizophrenia patients 6.4 ± 0.6 mg/ml and normal controls 7.1 ± 0.5 mg/ml. Patients and volunteers gave their written consent to participate in this study. All clinical investigations were conducted according to the principles in the Declaration of Helsinki.

Purification of human IgG

The serum IgG fraction was isolated by protein G affinity chromatography as previously described (Borda *et al*. 2002). Briefly, sera were loaded on the protein G (Sigma Chemical Co., USA) affinity column and IgG fractions were eluted with 100 mM glycine–HCl (pH 3.0), and immediately neutralized. Both IgG concentration and purification were determined by radial immunodiffusion assay.

Purification of M_1 antipeptide antibodies (anti-M_1p IgG) by affinity chromatography

The IgG fraction of four pools from nine schizophrenia patients positive for antibodies against M_1 mAChRs, two pools from five schizophrenia patients negative for antibodies against M_1 mAChRs and three pools from 10 normal subjects were independently subjected to affinity chromatography on the synthesized peptide covalently linked to AffiGel 15 gel (Bio-Rad, USA) as previously described (Borda *et al*. 2002). The IgG fraction was loaded on the affinity column equilibrated with PBS and the non-peptide fraction was first eluted with the same buffer. Their immunological reactivity against the muscarinic receptor peptide was evaluated by enzyme immunoassay. The concentration of the anti-M_1p IgG (5×10^{-7} M) that maximally increased optical density (OD 2.9 ± 0.3) values corresponds to 5×10^{-6} M total IgG concentration (OD 2.6 ± 0.2). The non-anti-M_1 peptide IgG fraction eluted from the column showed OD values (0.27 ± 0.06) similar to normal IgG (OD 0.22 ± 0.04) and to IgG fraction from negative controls (OD 0.25 ± 0.05).These results are in accordance with previous reports obtained with fewer patients (Borda *et al*. 2002).

Peptides

A 24-mer peptide ERTMLAGQCYIQFLSQPIITFGT-AM corresponding to the amino-acid sequence of the second extracellular loop of the human M_1 mAChR was synthesized by F-moc amino acids activated using HOBt/DCC (1-hydroxybenzotriazole/dicyclohexyl carbodiimide) strategy with an automatic peptide synthesizer (Applied Biosystems model 431A, USA) (Borda *et al*. 2002).

Rat cerebral frontal cortex preparations

Female Wistar rats (obtained from the Pharmacology Unit, School of Dentistry, University of Buenos Aires) were housed at our colony in small groups and kept under automatically controlled lighting (lights on 08:00–19:00 hours) and uniform temperature (25 °C) conditions. All animals were used at 3–4 months of age. The animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care. Cerebral frontal cortex slices (10 mg) were incubated in Krebs-Ringer bicarbonate (KRB) gassed with 5% CO_2 in oxygen at 37 °C throughout the different assays (Borda *et al*. 1998).

Determination of NOS activity

NOS activity was measured in cerebral frontal cortex slices by production of [U-^{14}C]citrulline from [U-^{14}C]arginine as previously described (Sterin-Borda *et al*. 2003). Briefly, tissue was incubated in KRB for 30 min in the presence of [U-^{14}C]arginine (0.5 mCi). Immunoglobulins were then added for the last 20 min, followed by centrifugation at 20 000 *g* for 10 min at 4 °C; supernatants were applied to 2-ml columns of Dowex AG 50 WX-8 (sodium form); [^{14}C]citrulline was eluted with 3 ml of water and quantified by liquid scintillation counting.

PGE_2 and MMP-3 assays

Cerebral frontal cortex slices were incubated for 60 min in 0.50 ml KRB. Immunoglobulins or pilocarpine were added for the last 20 min. Blockers were added at the beginning of the incubation. Tissues were then homogenized and thereafter, all procedures employed were those indicated in the protocol of the Prostaglandin E_2 Biotrak enzyme immunoassay (ELISA) system (Amersham Biosciences, USA). For extraction of extracellular matrix components samples were mixed with extraction buffer at 4 °C and homogenized in a glass/glass conical homogenizer. The homogenate was then subjected to three freeze–thaw cycles of 5 min each and centrifuged at 13 000 *g* for

Table 1. Oligonucleotides of primers for PCR

Gene product	Sense	Antisense	Predicted size (bp)
iNOS	5′ GAT CAA TAACCT GAA GCC CG 3′	5′ GCC CTT TTT TGC TCC ATA GG 3′	578
cox-1	5′ TAA GTA CCA GGT GCT GGA TGG 3′	5′ AGA TCG TCG AGA AGA GCA TCA 3′	160
cox-2	5′ TCC AAT CGC TGT ACA AGC AG 3′	5′ TCC CCA AAG ATA GCA TCT GG 3′	242
G3PDH	5′ ACCAC AGTCCA TGCCAT CAC 3′	5′ TCCAC CACCC TGTTG CTGTA 3′	452

iNOS, Inducible nitric oxide synthase isoform; G3PDH, glyceraldehyde-3-phosphate dehydrogenase isoform; cox, cyclooxygenase isoform.

30 min at 4 °C. The detergent-soluble supernatant was recovered and stored at −70 °C. The enzymatic activity of MMP-3 was determined according to the manufacturer's instructions (Amersham Matrix Metalloproteinase-3, Biotrak activity assay system; GE Healthcare, USA). The PGE_2 and MMP-3 results were expressed as ng/ml.

mRNA isolation and cDNA synthesis

Total RNA was extracted from rat cerebral frontal cortex slices by homogenization as previously reported (Ganzinelli *et al*. 2006). Quantization of iNOS and cox mRNA levels was performed by a method that involves simultaneous co-amplification of both the target cDNA and a reference template (MIMIC) with a single set of primers (Sterin-Borda *et al*. 2003). MIMIC for iNOS and cox mRNA and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were constructed using a PCR MIMIC construction kit (Clontech Laboratories, USA). The sequence of oligonucleotide primer pairs used is listed in Table 1. The relative mRNA expression of iNOS and cox in each group was compared to those from the respective normal group and reported as a percentage of normal.

Drugs

Pirenzepine, pilocarpine, trifluoperazine (TFP), aminoguanidine, staurosporine, and haloperidol were purchased from Sigma Chemical Co., USA (4-octadecylphenyl)-4-oxobutenoic acid (OBAA), 1-[4,5-bis (4-methoxyphenyl)-2-thiazolyl] carbonyl-4-methylpiperazine hydrochloride (FR 122047) and 5-bromo-2-(4-flurophenyl)-3-[4-(methylsulfonyl)phenyl]-tiophene (DuP 697) were purchased from Tocris Cookson Inc., USA; 1-6-17β-3-methoxgestra-1,3,5-(10)-trien-17yl-aminohexyl-1-H-pyrrole-2,5-dione (U-73122) from ICN Pharmaceuticals Inc., USA. Stock solutions were freshly prepared in the corresponding buffers.

Statistical analysis

Analysis was performed using the computer program Graph Prism (Graph Pad, USA). Student's *t* test for unpaired values was used to determine the level of significance. Pearson's analysis was applied to establish correlation. Differences between means were considered significant if p was $\leqslant 0.05$.

Results

Previously, we have demonstrated the existence of circulating antibodies against the cerebral frontal cortex membrane in schizophrenia patients, which are able to interact molecularly with human M_1 mAChR synthetic peptide (Borda *et al*. 2002). Knowing that the amino acid sequences of the second extracellular loop M_1 mAChR of rat and humans have strong homology (92%), we studied the mAChR-mediated effect of autoantibodies from schizophrenia patients on the rat cerebral frontal cortex. For this purpose, we used the affinity purified anti-M_1 peptide IgG (M_1p IgG) from schizophrenia patients.

Autoantibody stimulation resulted in activation of a number of enzymatic pathways associated with M_1 mAChR signalling. Figures 1*a*, 2*a* and 3*a* show the ability of M_1p IgG to stimulate NOS activity, and PGE_2 and MMP-3 production, respectively, in a concentration-dependent manner, reaching the maximal capacity of stimulation at 10^{-7} M. The corresponding IgG fraction eluted from the column with M_1 peptide (non-peptide fraction) gave negative results. A reduction in M_1p IgG-increased NOS, PGE_2 and MMP-3 production was observed in the presence of 5×10^{-6} M

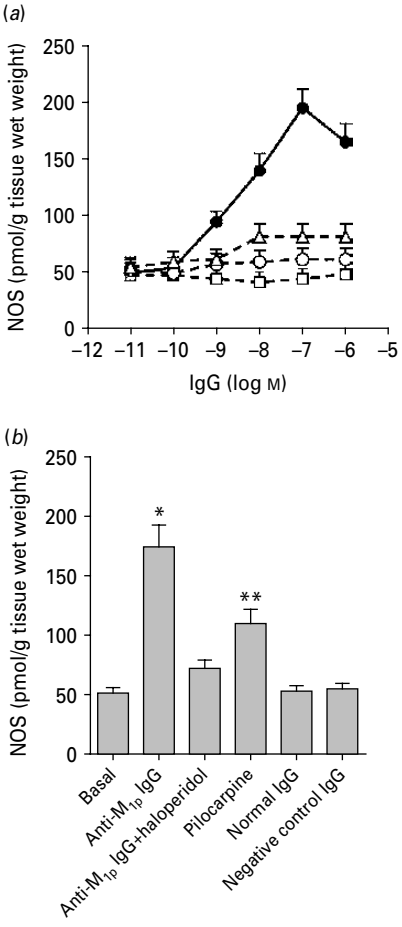

Fig. 1. (*a*) Stimulation of NOS activity. Dose–response curve of anti-M_1 peptide IgG from schizophrenia patients alone (●) or in combination with 10^{-7} M pirenzepine (○), pre-incubated with 10^{-5} M M_1 peptide (□) or schizophrenic IgG eluted from M_1 peptide (non-peptide fraction) (△). (*b*) Effect of 10^{-7} M anti-M_1 peptide IgG from schizophrenia patients (anti-M_1p IgG) alone or in the presence of 10^{-6} M haloperidol, 10^{-7} M pilocarpine, 10^{-7} M normal IgG or 10^{-7} M negative control IgG. Values are mean ± S.E.M. of four pools from nine schizophrenia patients positive for M_1 mAChRs, two pools from five schizophrenia patients negative for M_1 mAChRs and three pools from 10 normal subjects, performed in duplicate in each group. * $p<0.0001$ *vs.* baseline. ** $p<0.0001$ *vs.* anti-M_1 peptide IgG from schizophrenia patients.

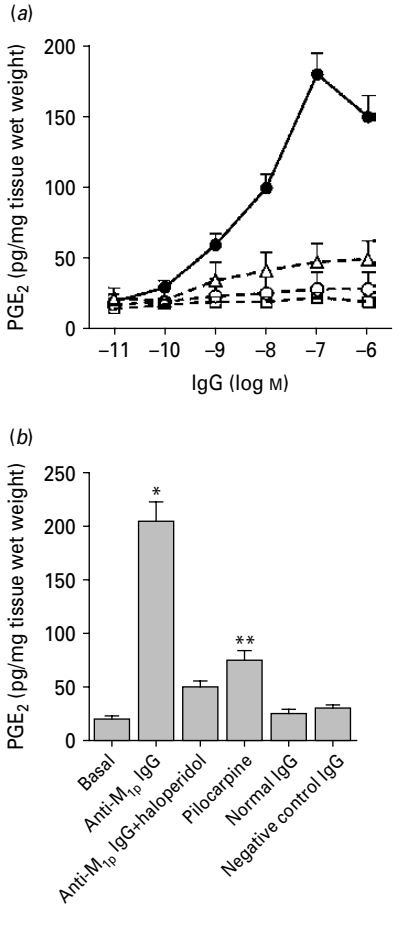

Fig. 2. (*a*) PGE_2 production. Dose–response curve of anti-M_1 peptide IgG from schizophrenia patients alone (●) or in combination with 10^{-7} M pirenzepine (○), pre-incubated with 10^{-5} M M_1 peptide (□) or schizophrenic IgG eluted from M_1 peptide (non-peptide fraction) (△). (*b*) Effect of 10^{-7} M anti-M_1 peptide IgG from schizophrenia patients (anti-M_1p IgG) alone or in the presence of 10^{-6} M haloperidol, 10^{-7} M pilocarpine, 10^{-7} M normal IgG or 10^{-7} M negative control IgG. Values are mean ± S.E.M. of four pools from nine schizophrenia patients positive for M_1 mAChRs, two pools from five schizophrenia patients negative for M_1 mAChRs and three pools from 10 normal subjects, performed in duplicate in each group. * $p<0.0001$ *vs.* baseline. ** $p<0.0001$ *vs.* anti-M_1 peptide IgG from schizophrenia patients.

pirenzepine or after pre-incubating the IgG with 10^{-5} M synthetic peptide. Moreover, *in-vitro* experiments with 10^{-6} m haloperidol inhibited the M_1p IgG-increased NOS, PGE_2 and MMP-3 production (Figs 1*b*, 2*b*, 3*b*). As can be seen in Figs 1*b*, 2*b* and 3*b*, the maximum effect of M_1p IgG on NOS activity, and PGE_2 and MMP-3 production, respectively, was significantly higher than the maximum effect of the authentic agonist pilocarpine (10^{-7} M). As a control, the IgG fraction from normal subjects and those from negative controls (schizophrenia patients without M_1p IgG), gave negative results. Figure 4 demonstrates that, under identical experimental conditions, there was a significant correlation between M_1p IgG-stimulated NOS activity, and PGE_2 and MMP-3 production (PGE_2: Pearson $r=0.9902$, $p<0.0001$, $R^2=0.9806$; MMP-3: Pearson $r=0.9889$, $p<0.0002$, $R^2=0.9779$). These results indicated that the activation of M_1

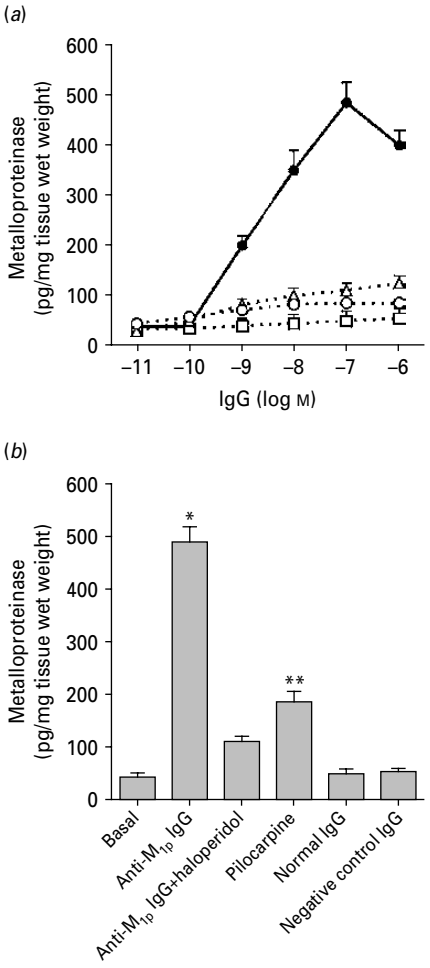

Fig. 3. (*a*) Metalloproteinase-3 production. Dose–response curve of anti-M_1 peptide IgG from schizophrenia patients alone (●) or in combination with 10^{-7} M pirenzepine (○), or pre-incubated with 10^{-5} M M_1 peptide (□) or schizophrenic IgG eluted from M_1 peptide (non- peptide fraction) (△). (*b*) Effect of 10^{-7} M anti-M_1 peptide IgG from schizophrenia patients (anti-M_1p IgG) alone or in the presence of 10^{-6} M haloperidol, 10^{-7} M pilocarpine, 10^{-7} M normal IgG or 10^{-7} M negative control IgG. Values are mean ± S.E.M. of four pools from nine schizophrenia patients positive for M_1 mAChRs, two pools from five schizophrenia patients negative for M_1 mAChRs and three pools from 10 normal subjects, performed in duplicate in each group. * $p < 0.0001$ *vs*. baseline. ** $p < 0.0001$ *vs*. anti-M_1 peptide IgG from schizophrenia patients.

mAChRs by IgG from schizophrenia patients increased the production of PGE_2 and MMP-3, perhaps as a result of stimulation of NOS activity.

To investigate if the endogenous NO signalling system participates in the increase of NOS activity, and PGE_2, and MMP-3 production by M_1p IgG, we studied the effect of selective inhibitors of enzymatic

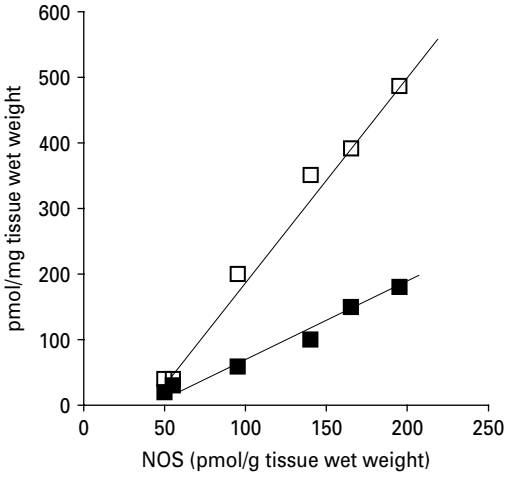

Fig. 4. Correlation in the stimulatory effect of anti-M_1 peptide IgG from schizophrenia patients (10^{-10} to 10^{-6} M) on prostaglandin E_2 (■) and metalloproteinase-3 (□), plotted as a function of NOS.

pathways commonly associated with M_1 mAChR activation. Figure 5(*a–c*) shows that the inhibition of phospholipase C (PLC) by U-73122 (5×10^{-6} M), inhibition of protein kinase C (PKC) by staurosporine (5×10^{-9} M) and inhibition of calcium-calmodulin by TFP (5×10^{-6} M), and iNOS inhibition by aminoguanidine (10^{-5} M) attenuated the increase in NOS activity, and PGE_2 and MMP-3 production by schizophrenic IgG.

In order to discern which arachidonic acid (AA) cascade enzymes are implicated in the induction of PGE_2 and MMP-3 production by schizophrenic IgG, we used several inhibitors of this enzymatic cascade. It can be seen in Fig. 5(*b*, *c*) that the inhibition of phospholipase A_2 (PLA_2) by OBAA (5×10^{-6} M) or COX-1 inhibition by FR 122047 (5×10^{-8} M) prevented the stimulatory action of M_1p IgG-induced PGE_2 and MMP-3 production. As control FR 122047 did not modify M_1p IgG-induced NOS activity (data not shown). COX-2 inhibition by DuP 697 (5×10^{-8} M) had a minor effect. These results indicate that schizophrenic IgG stimulation of mAChRs may trigger production of PGE_2 and MMP-3, as a result of an increase in iNOS pathways with PLA_2 and COX-1 activation, with little COX-2 participation.

To resolve the role of iNOS and COX isoforms in the action of IgG from schizophrenia patients on cerebral M_1 mAChR activation, we used specific primers for iNOS, cox-1 and cox-2 mRNA. Figure 6 shows the results of RT–PCR and quantitative RT–PCR analysis, which demonstrate that M_1p IgG (10^{-8} M) stimulation for 1 h increased iNOS and cox-1 mRNA levels, with

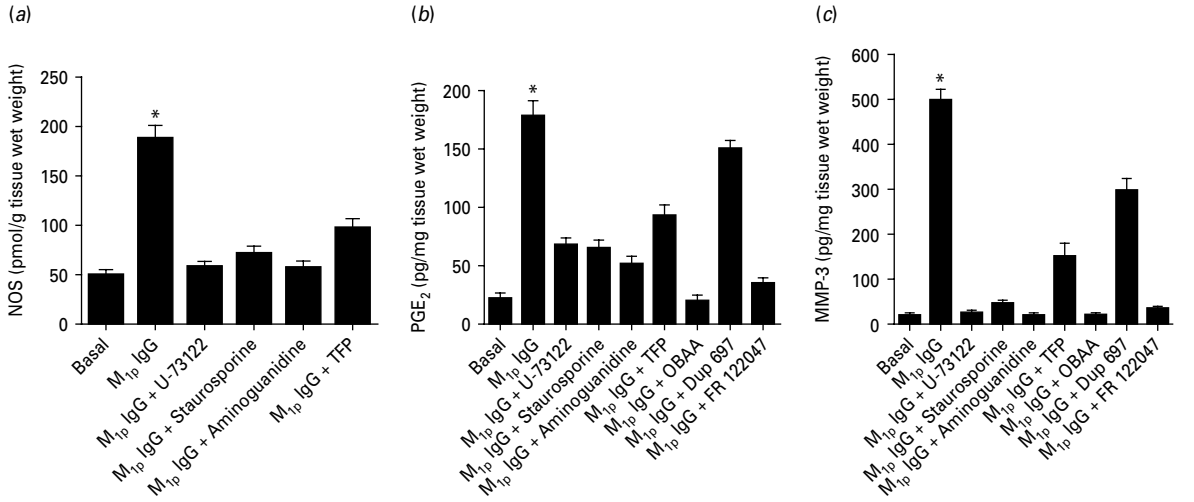

Fig. 5. Anti-M_1 peptide IgG from schizophrenia patients (M_1p IgG) increased NOS activity (*a*), induced prostaglandin E_2 (PGE_2) accumulation (*b*), or increased metalloproteinase-3 (MMP-3) (*c*), in the absence or presence of 5×10^{-6} M U-73122, 10^{-9} M staurosporine, 10^{-5} M aminoguanidine, 5×10^{-6} M TFP, 5×10^{-6} M OBAA, 5×10^{-8} M DuP 697 or 5×10^{-6} M FR 122047. Values are mean ± S.E.M. of four pools from nine schizophrenia patients positive for M_1 mAChRs performed in duplicate in each group. * $p < 0.0001$ *vs*. baseline.

no modification of cox-2 mRNA levels. The depleted fraction of anti-peptide IgG had no effect (data no shown). Figure 6 also shows that the authentic agonist pilocarpine (10^{-8} M) mimicked the antibody action. Moreover, when M_1p IgG (10^{-8} M) and pilocarpine (10^{-8} M) were assayed together, a synergic stimulatory effect on iNOS and cox-1 mRNA levels was observed. These results demonstrate that IgG from schizophrenia patients acts as an inducer of iNOS and cox-1 mRNA, with a synergic effect between the antibody and the mAChR agonist.

Discussion

Sera from schizophrenia patients contain serum autoantibodies. These autoantibodies are able to interact with the second extracellular loop of the human M_1 mAChR. The molecular interaction of schizophrenia antibodies with cerebral frontal cortex M_1 mAChR has the capacity to generate the pro-inflammatory substances NO, PGE_2 and MMP-3, and induces cox-1 and iNOS mRNA.

Immune-mediated neurological manifestation in schizophrenic syndrome has been described (Henneberg *et al*. 1993) but the precise immune mechanism remains unclear. Here, we demonstrated the possible role of autoantibodies against cerebral mAChRs on CNS alterations in schizophrenia patients.

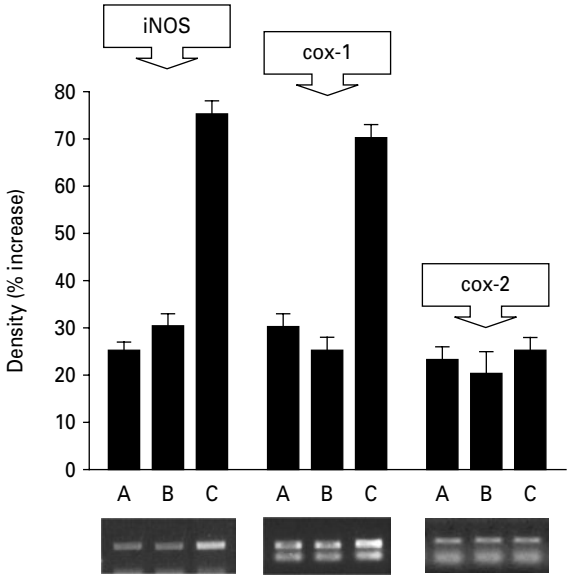

Fig. 6. Effect of anti-M_1 peptide IgG from schizophrenia patients on quantitative RT–PCR analysis of iNOS, cox-1 and cox-2 mRNA expression. Cerebral frontal cortex slices were incubated for 1 h in the presence of 10^{-8} M anti-M_1-peptide IgG from schizophrenia patients alone (A), 10^{-8} M pilocarpine alone (B), 10^{-9} M anti-M_1-peptide IgG plus 10^{-8} M pilocarpine (C). Values are mean ± S.E.M. of four pools from nine schizophrenia patients positive for M_1 mAChRs in each group. RT–PCR products obtained from the analysis are shown.

The most common CNS manifestations described in schizophrenia patients are alterations in cognitive function with subtle abnormalities in the frontal lobes (Goldberg *et al*. 1991), and many of the cognitive aspects of the disease are linked to cholinergic dysfunction of brain tissue (Yeomans, 1995). Therefore, the nature of the cholinergic CNS dysfunction may be related to the effect of autoantibodies on the cerebral muscarinic cholinergic system.

The autoantibodies were not only able to interact molecularly with the human M_1 mAChR peptide, but also displayed agonistic activity triggering NO, PGE_2 and MMP-3 production. Studies on signalling events involved in mAChR activation in rat cerebral frontal cortex have implicated stimulation of NOS and PGE_2 (Borda *et al*. 1998; Orman *et al*. 2005; Sterin-Borda *et al*. 2003). The increased production of PGE_2 and MMP-3 and over-activity of NOS are dependent on iNOS, as they are blocked by aminoguanidine at concentrations that are known to inhibit iNOS activity. Moreover, we observed a significant correlation between the increase in NOS activity and PGE_2/MMP-3 production triggered by the autoantibodies. Regarding the effect of haloperidol inhibiting M_1p IgG-increased NOS, PGE_2 and MMP-3, we can interpret this as the antipsychotic drug behaving like an inhibitor on M_1 mAChRs. Indeed, we have previously demonstrated that haloperidol is able to inhibit the binding of specific mAChR radiolabelled antagonists in a concentration-dependent manner on rat cerebral frontal cortex membrane (Borda & Cremaschi, 1997). Concurrent analysis of the effects of PLC, PKC and calcium-calmodulin inhibition on the increased generation of NO and PGE_2/MMP-3 triggered by schizophrenia antibodies suggest that the mechanism by which IgG up-regulates pro-inflammatory mediators seems to involve stimulation of phosphoinositide (PI) hydrolysis through PLC activation, whose second messenger stimulates iNOS activity with over-production of NO. Thus, the anti-M_1p IgG mimics the effect of the authentic agonist (Sterin-Borda *et al*. 2003). However, the autoantibodies' effect was higher than that obtained with the full agonist pilocarpine, which was used to compare their actions. This difference could be due to the greater selectivity of anti-M_1p IgG for the M_1 mAChR subtypes while pilocarpine would bind to other cerebral mAChR subtypes (M_1–M_5); as reported for positive allosteric modulators of mAChRs used for the treatment of schizophrenia (Conn *et al*. 2009).

In inflammatory disease of the CNS, iNOS and NO products are present mainly in macrophages and microglia of active lesions (Bagasra *et al*. 1995) that are associated with the induction of neuronal apoptosis (Estévez *et al*. 1998). The idea that iNOS is detrimental during CNS inflammation is supported by the finding of decreased axon necrosis after treatment with aminoguanidine (Rose *et al*. 1998). NO produced during inflammation is very reactive and forms products such as peroxynitrite, which directly induces neurodegeneration (Redford *et al*. 1997), but it can also induce the production of pro-inflammatory substances such as PGE_2 (Orman *et al*. 2005).

Our data indicate that the endogenous NO signalling system is a key factor in the generation of PGE_2 induced by IgG from schizophrenia patients, and is able to increase the rate of transcription of cox-1 in response to mAChR activation. It has been reported that NO is able to dramatically alter the pattern of early gene expression in hippocampal granule cells (Morris, 1995). The major new finding of the present study is that anti-M_1 mAChR antibodies behaving as cholinergic agonists have the capacity to alter the rate of transcription of specific pro-inflammatory target genes, which triggers the production of PGE_2 in response to receptor-mediated signalling events at the cell membrane. The transcription is rapidly induced following receptor activation, and therefore, the target genes can be classified as early genes (Sterin-Borda *et al*. 2003). In this way, we demonstrated positive regulation of cox-1 and iNOS mRNA in the early stages after activation of cerebral M_1 mAChRs by autoantibodies. The expression of cox-1 and iNOS early genes may play an important role in coupling receptor stimulation to long-term tissue responses (Ganzinelli *et al*. 2006). Therefore, the anti-cerebral mAChR autoantibodies might play a role in the pathophysiological mechanisms that underlie the inflammatory process described in schizophrenia.

The positive PGE_2/MMP-3 regulation by IgG from schizophrenia patients observed in the present study was mediated mostly by PLA_2 and COX-1, as it was prevented by specific blockade of these two enzymes. Therefore, the activation of rat cerebral frontal cortex M_1 mAChRs by the antibody that triggers PGE_2/MMP-3 production is preceded by NOS activation, which in turn catalyses PLA_2/AA release and induces immediate early cox-1 mRNA, without affecting cox-2 mRNA levels. The schizophrenia autoantibody has been shown to effectively increase the rate of transcription of cox-1 and iNOS mRNA in response to mAChR activation. COX-1 is prevalent in the forebrain where PGs may be involved in complex integrative functions, such as modulation of the autonomic nervous system (Breder & Saper, 1996). Moreover, COX-1 localized in microglia plays a major role in the neuroinflammatory process. By contrast, COX-2 which is

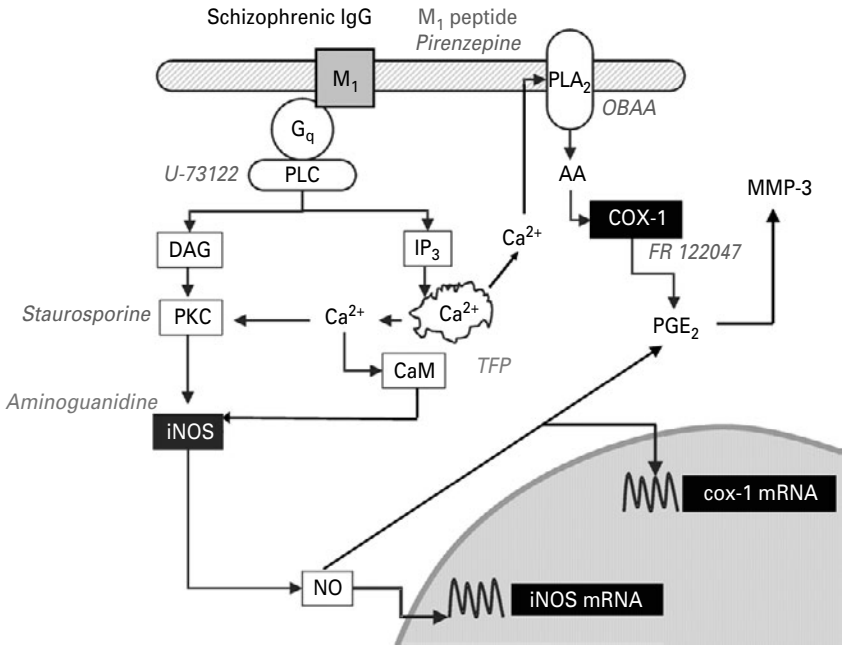

Fig. 7. Proposed model to explain the mechanism whereby schizophrenic IgG up-regulates iNOS and cox-1 mRNA levels and prostaglandin E_2/metalloproteinase-3 (PGE_2/MMP-3) in cerebral frontal cortex. Schizophrenia anti-M_1 peptide IgG (schizophrenic IgG) acting on G_q-protein activates PLC, mediating production of inositol triphosphate (IP_3) and 1-2 diacylglycerol (DAG). IP_3 triggers intracellular release of calcium stores (Ca^{2+}). Free Ca^{2+} binds to calcium-calmodulin and sensitizes PKC activation via DAG. Subsequent PKC translocates to the membrane and the Ca^{2+}-calmodulin complex (CaM) increase both iNOS activity and iNOS mRNA levels, that in turn increases production of NO. The overproduction of NO also triggers the induction of cox-1 mRNA levels. Alternatively, the rise in cytosolic Ca^{2+} activates phospholipase A_2 (PLA_2) with subsequent activation of COX-1 which induces generation of PGE_2 and MMP-3. Inhibitory agents are indicated in italics.

localized in pyramidal neurons is expected to be activated during ischaemia and excitotoxicity (Choi *et al*. 2009). The fact that M_1 mAChR antibodies are able to interact with neuronal and astrocyte M_1 mAChRs (Borda *et al*. 2002, 2004) could explain the preferential cox-1 mRNA autoantibody inflammatory effect.

An altered biosynthesis of prostanoids is clearly involved in neuroinflammatory processes (Cao *et al*. 1996). Indeed, COX-1-positive microglia accumulate significantly in the perilesional area and the developing necrotic core after injury, and the number of COX-1-positive cells is persistently elevated for up to 4 wk following injury (Schwab *et al*. 2000). However, cox-1 expression does not change in post-mortem schizophrenia brain (Maida *et al*. 2006). On the other hand, over-expression of cox-1 and subsequent abnormal release of prostanoids have been described after exposure of brain cells to different inflammatory agents (Minghetti & Levi, 1998).

An important feature of the activity of IgG is its ability to increase production of MMP-3, and its production may be mediated though the induction of cox-1 and PGE_2, as it was prevented by FR 122047, which is known to selectively inhibit COX-1 activity. Inflammatory processes in the brain lead to the induction of MMP-3, which is highly destructive and attacks several extracellular matrix molecules (Rosenberg, 2002). MMP-3 has been shown to be localized in macrophage-like cells and was mainly found in neurons and microglia (Maceda & Sobel, 1996). Pericytes around the blood vessels contain MMP-3 in the ischaemic region, and this is released into the extracellular space, where it can do more damage (Rosenberg, 2002). Brains injected with lipopolysaccharides show early elevation in MMP-3 mRNA gene expression (Mun-Bryce *et al*. 2002).

On the basis of our results, we postulated that early agonistic-promoting activation of mAChRs initiated by antibodies, bind to and persistently activate cholinergic receptors, which results in the production of large amounts of pro-inflammatory and cytotoxic NO, PGs and MMPs. The release of NO, PGE_2 and MMP-3 by the induction of iNOS and cox-1 gene expression may contribute to immune neuroinflammation. The agonistic activity displayed by these autoantibodies may induce desensitization, internalization and/or

intracellular degradation of the mAChRs, which leads to a progressive decrease in cerebral M_1 mAChR surface expression and activity. Subsequently, this leads to greater alterations in cognitive function, including synaptic plasticity and memory, in schizophrenia patients.

An illustration of bringing together the various systems studied and proposing a mechanism by which anti-M_1 peptide IgG from schizophrenia patients might induce cox-1 and iNOS mRNA, thereby triggering the production of pro-inflammatory mediators, is shown in Figure 7.

Acknowledgements

The authors thank Mrs Elvita Vannucchi and Mrs Fabiana Solari for the excellent technical assistance. We also thank W. Rasband for NIH image. UBACYT (University of Buenos Aires) and CONICET (Argentine National Research Council); Buenos Aires, Argentina funded this work.

Statement of Interest

None.

References

Andreasen NC, Rezai K, Alliger R, Swayze VW, *et al.* (1992). Hypofrontality in neuroleptic-naive patients and in patients with chronic schizophrenia. Assessment with xenon 133 single-photon emission computed tomography and the Tower of London. *Archives of General Psychiatry* **49**, 943–958.

Bagasra O, Michaels FH, Zheng YM, Bobroski LE, *et al.* (1995). Activation of the inducible form of nitric oxide synthase in the brains of patients with multiple sclerosis. *Proceedings of the National Academy of Sciences USA* **23**, 12041–12045.

Birdsall NJM, Nathanson NM, Schwarz RD (2001). Muscarinic receptors: it's a knockout. *Trends in Pharmacological Sciences* **22**, 215–219.

Borda T, Cremaschi G (1997). Multireceptor interactions of haloperidol on rat cerebral frontal cortex 'in vitro'. *Acta Physiologica et Therapeutica Latinoamericana* **173**, 173–178.

Borda T, Genaro A, Sterin-Borda L, Cremaschi G (1998). Involvement of endogenous nitric oxide signalling system in brain muscarinic acetylcholine receptor activation. *Journal of Neural Transmission* **105**, 193–204.

Borda T, Gomez R, Berria M, *et al.* (2004). Antibodies against astrocytes M_1 and M_2 muscarinic cholinoceptor from schizophrenic patients' sera. *Glia* **45**, 144–154.

Borda T, Perez Rivera R, Joensen L, Gomez RM, *et al.* (2002). Antibodies against cerebral M_1 cholinergic muscarinic receptor from schizophrenic patients molecular interaction. *Journal of Immunology* **168**, 3667–3674.

Breder CD, Saper CD (1996). Expression of inducible cyclooxigenase mRNA in mouse brain after systemic administration of bacterial lipopolysaccharide. *Brain Research* **713**, 64–69.

Cao C, Matsumura K, Yamagata K, Watanabe Y (1996). Endothelial cells of the rat brain vaculature express cyclooxygenase 2 mRNA in response to systemic interleukin-1beta: a possible site of prostaglandin synthesis responsible for fiver. *Brain Research* **733**, 263–272.

Carlsson A (1978). Antipsychotic drugs, neurotransmitters and schizophrenia. *American Journal of Psychiatry* **135**, 165–169.

Choi SH, Aid S, Bosetti F (2009). The distinct roles of cyclooxygenase-1 and -2 in neuroinflammation: implications for translational research. *Trends in Pharmacological Science* **30**, 174–181.

Colburn KK, Green LM, Wong AK (2001). Circulating antibodies to guanosine in systemic lupus erythematosus: correlation with nephritis and polyserositis by acute and longitudinal analyses. *Lupus* **10**, 4–10.

Conn PJ, Jones CK, Lindsley CW (2009). Subtype-selective allosteric modulators of Muscarinic receptors for the treatment of CNS disorders. *Trends in Pharmacological Science* **30**, 148–155.

Di Filippo M, Sarchielli P, Picconi B, Calabresi P (2008). Neuroinflammation and synaptic plasticity: theoretical basis for a novel, immune-centred, therapeutic approach to neurological disorders. *Trends in Pharmacological Science* **29**, 402–412.

Estévez AG, Spear N, Manuel SM, Barbeito L, *et al.* (1998). Role of endogenous nitric oxide and peroxynitrite formation in the survival and death of motor neurons in culture. *Progress in Brain Research* **118**, 269–280.

Ganzinelli S, Borda T, Sterin-Borda L (2006). Regulation of m1 muscarinic receptors and nNOS mRNA levels by autoantibodies from schizophrenic patients. *Neuropharmacology* **50**, 362–371.

Gerber DJ (2001). Hyperactivity, elevated dopaminergic transmission and response to amphetamine in M_1 mAChR-deficient mice. *Proceedings of the National Academy of Sciences USA* **98**, 15312–15317.

Grafman J, Holyak KJ, Boller F (eds) (1995). Structure and functions of the human prefrontal cortex. *Annals of the New York Academy of Sciences* **769**, 1–411.

Heath RG, McCarron KL, O'Neil CE (1990). Antiseptal brain antibody in IgG of schizophrenic patients. *Biological Psychiatry* **27**, 681–682.

Hendriks JJ, Teunissen CE, de Vries HE, Dijkstra CD (2005). Macrophages and neurodegeneration. *Brain Research Reviews* **48**, 185–195.

Henneberg AE, Ruffert S, Henneberg HJ, *et al.* (1993). Antibodies to brain tissue in sera of schizophrenic patients. *Psychiatry and Clinical Neurosciences* **242**, 314–317.

Liberatore GT, Jackson-Lewism V, Vukosavic S, Mandir AS, *et al.* (1999). Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. *Nature Medicine* **5**, 1403–1409.

Maceda A, Sobel RA (1996). Matrix metalloproteinases in the normal human central nervous system, microglial, nodules, and multiple sclerosis lesions. *Journal of Neuropathology and Experimental Neurology* **55**, 300–309.

Maida ME, Hurley SD, Daeschner JA, Moore AH (2006). Cytosolic prostaglandin E2 synthase expression is decreased in discrete cortical regions in psychiatric disease. *Brain Research* **1103**, 164–173.

Minghetti L, Levi G (1998). Microglia as effector cells in brain damage and repair: focus on prostanoids and nitric oxide progress. *Neurobiology* **54**, 99–125.

Mollace V, Muscoli C, Masini E, Cuzzocrea S, *et al.* (2005). Modulation of prostaglandin biosynthesis by nitric oxide and nitric oxide donors. *Pharmacological Reviews* **57**, 217–52.

Morris BJ (1995). Stimulation of immediate early gene expression in striatal neurons by nitric oxide. *Journal of Biological Chemistry* **270**, 24740–24744.

Müller N, Riedel M, Gruber R, Ackenheil M, *et al.* (2000). The immune system and schizophrenia. an integrative view. In: Conti A, Maestroni G, McCann S, Sternberg E, Lipton J, Smith C (Eds). *Neuroimmunomodulation* (p. 456). New York: New York Academy of Science.

Mun-Bryce S, Lukes A, Wallace J, Lukes-Marx M, *et al.* (2002). Stromelysin-1 and gelatinase A are upregulated before TNF-alpha in LPS-stimulated neuroinflammation. *Brain Research* **933**, 42–49.

Noy S, Achiron A, Laor N (1994). Schizophrenia and autoimmunity a possible etiological mechanism? *Neuropsychobiology* **30**, 157–159.

Orman B, Sterin-Borda L, Reina S, *et al.* (2005). Signal transduction underlying carbachol-induced PGE_2 generation and cox-1 mRNA expression of rat brain. *Neuropharmacology* **48**, 757–765.

Overall JE, Gorham DR (1962). Brief psychiatric rating scale. *Psychological Reports* **10**, 799–801.

Redford EJ, Kapoor R, Smith KJ (1997). Nitric oxide donors reversibly block axonal conduction: demyelinated axons are especially susceptible. *Brain* **120**, 2149–2157.

Rose JW, Hill KE, Wada Y, Kurtz CI, *et al.* (1998). Nitric oxide synthase inhibitor, aminoguanidine, reduces inflammation and demyelination produced by Theiler's virus infection. *Journal of Neuroimmunology* **81**, 82–89.

Rosenberg GA (2002). Matrix metalloproteinases in neuroinflammation. *Glia* **39**, 279–291.

Schwab JM, Nguyen TD, Postler E, Meyermann R, *et al.* (2000). Selective accumulation of COX-1 expressing microglia cell/macrophages in lesions of human focal cerebral ischemia. *Acta Neuropathologica* **99**, 609–614.

Shuttleworth TJ, Thompson JL (1998). Muscarinic receptor activation of arachidonate-mediated Ca^{2+} entry in HEK293 cells is independent of phospholipase C. *Journal of Biological Chemistry* **273**, 32636–32643.

Spitzer RL, Williams JB, Gibbon M (1991). *Manual for the Structured Clinical Interview for DSM-II-R with Psychotic Screen*. Washington, DC: American Psychiatric Press.

Sterin-Borda L, Ganzinelli S, Berra A, Borda E (2003). Novel insight into the mechanisms involve in the regulation of the m1 muscarinic receptor, iNOS and nNOS mRNA levels. *Neuropharmacology* **45**, 260–269.

Tandon R (1999). Cholinergic aspects of schizophrenia. *British Journal of Psychiatry* **174**, 7–11.

Yeomans JS (1995). Role of tegmental cholinergic neurons in dopaminergic activation, antimuscarinic psychosis and schizophrenia. *Neuropsychopharmacology* **12**, 3–16.