Autoantibodies from schizophrenia patients induce cerebral cox-1/iNOS mRNA expression with NO/PGE₂/MMP-3 production

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Abstract
We demonstrated that circulating antibodies from schizophrenia patients, which interact with cerebral M₄ muscarinic acetylcholine receptors (M₄ mAChRs), trigger production of nitric oxide (NO), prostaglandin E₂ (PGE₂) 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-M₄ peptide IgG from schizophrenia patients, while stimulating cerebral M₄ mAChRs, increases NOS activity, PGE₂ and MMP-3 production associated with iNOS over-activity and mRNA expression. Moreover, PGE₂ 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, PGE₂ 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.

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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 M₄ and astrocyte M₁ and M₂ muscarinic acetylcholine receptors (mAChRs) have been described, which account for the disease (Borda et al. 2002, 2004; Ganzinelli et al. 2006). A strong association (a = 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...
induced by antibody–mAChR specific interaction. Fixation of autoantibodies to M₁ 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 M₁mAChR 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, M₁ 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 E₂ (PGE₂) (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 (Bredner & 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 PGE₂ 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 M₁mAChRs; and 10 patients that were negative for M₁mAChR 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 M₁ antipeptide antibodies (anti-M₁ IgG) by affinity chromatography

The IgG fraction of four pools from nine schizophrenia patients positive for antibodies against M₁ mAChRs, two pools from five schizophrenia patients negative for antibodies against M₁ 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-M₁ IgG (5×10⁻⁸ 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-M₁ 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 ERTMLAGQCYIQFSPQIFTFGT-AM corresponding to the amino-acid sequence of the second extracellular loop of the human M₁ mAChR was synthesized by F-moc amino acids activated using HOBr/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% CO₂ 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-¹⁴C]citrulline from [U-¹⁴C]arginine as previously described (Sterin-Borda et al. 2003). Briefly, tissue was incubated in KRB for 30 min in the presence of [U-¹⁴C]arginine (0.5 mCi). Immunoglobulins were then added for the last 20 min, followed by centrifugation at 20000 g for 10 min at 4 °C; supernatants were applied to 2-ml columns of Dowex AG 50 WX-8 (sodium form); [¹⁴C]citrulline was eluted with 3 ml of water and quantified by liquid scintillation counting.

PGE₂ 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 E₂ 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/tissue conical homogenizer. The homogenate was then subjected to three freeze–thaw cycles of 5 min each and centrifuged at 13000 g for
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 glycer-aldehyde-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-octadecyloxybenzaldehyde)-4-oxobutenoic acid (OBAA), 1-[4,5-bis (4-methoxyphenyl)-2-thiazolyl] carbonyl-4-methylpiperazine hydrochloride (FR 122047) and 5-bromo-2-(4-fluorophenyl)-3-[4-(methylsulfonyl)phenyl]-thiophene (DuP 697) were purchased from Tocris Cookson Inc., USA; 16,17β-3-methoxygestra-1,3,5(10)-trien-17ylaminoheptyl-1H-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 < 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$_1$p IgG) from schizophrenia patients.

Autoantibody stimulation resulted in activation of a number of enzymatic pathways associated with M$_1$ mAChR signalling. Figures 1a, 2a and 3a show the ability of M$_1$p 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$^{-5}$ M. The corresponding IgG fraction eluted from the column with M$_1$ peptide (non-peptide fraction) gave negative results. A reduction in M$_1$p IgG-increased NOS, PGE$_2$ and MMP-3 production was observed in the presence of 5 × 10$^{-4}$ M.
pirenzepine or after pre-incubating the IgG with $10^{-5}$ M synthetic peptide. Moreover, in-vitro experiments with $10^{-4}$ M haloperidol inhibited the M₁ peptide (non-peptide fraction) (△). The effect of $10^{-5}$ M anti-M₁ peptide IgG from schizophrenia patients (anti-M₁p IgG) alone or in the presence of $10^{-5}$ M haloperidol, $10^{-5}$ M pilocarpine, $10^{-7}$ M normal IgG or $10^{-3}$ M negative control IgG. Values are mean ± S.E.M. of four pools from nine schizophrenia patients positive for M₁ mAChRs, two pools from five schizophrenia patients negative for M₁ mAChRs and three pools from 10 normal subjects, performed in duplicate in each group. * $p < 0.0001$ vs. baseline. ** $p < 0.0001$ vs. anti-M₁ peptide IgG from schizophrenia patients.

![Graph 1](image1)

**Fig. 1.** (a) Stimulation of NOS activity. Dose–response curve of anti-M₁ peptide IgG from schizophrenia patients alone (●) or in combination with $10^{-7}$ M pirenzepine (○), pre-incubated with $10^{-5}$ M M₁ peptide (□) or schizophrenic IgG eluted from M₁ peptide (non-peptide fraction) (△). (b) Effect of $10^{-5}$ M anti-M₁ peptide IgG from schizophrenia patients (anti-M₁p IgG) alone or in the presence of $10^{-5}$ M haloperidol, $10^{-5}$ M pilocarpine, $10^{-7}$ M normal IgG or $10^{-3}$ M negative control IgG. Values are mean ± S.E.M. of four pools from nine schizophrenia patients positive for M₁ mAChRs, two pools from five schizophrenia patients negative for M₁ mAChRs and three pools from 10 normal subjects, performed in duplicate in each group. * $p < 0.0001$ vs. baseline. ** $p < 0.0001$ vs. anti-M₁ peptide IgG from schizophrenia patients.

PGE₂ production. Dose–response curve of anti-M₁ peptide IgG from schizophrenia patients alone (●) or in combination with $10^{-7}$ M pirenzepine (○), pre-incubated with $10^{-5}$ M M₁ peptide (□) or schizophrenic IgG eluted from M₁ peptide (non-peptide fraction) (△). (b) Effect of $10^{-5}$ M anti-M₁ peptide IgG from schizophrenia patients (anti-M₁p IgG) alone or in the presence of $10^{-5}$ M haloperidol, $10^{-5}$ M pilocarpine, $10^{-7}$ M normal IgG or $10^{-3}$ M negative control IgG. Values are mean ± S.E.M. of four pools from nine schizophrenia patients positive for M₁ mAChRs, two pools from five schizophrenia patients negative for M₁ mAChRs and three pools from 10 normal subjects, performed in duplicate in each group. * $p < 0.0001$ vs. baseline. ** $p < 0.0001$ vs. anti-M₁ peptide IgG from schizophrenia patients.

![Graph 2](image2)

**Fig. 2.** (a) PGE₂ production. Dose–response curve of anti-M₁ peptide IgG from schizophrenia patients alone (●) or in combination with $10^{-7}$ M pirenzepine (○), pre-incubated with $10^{-5}$ M M₁ peptide (□) or schizophrenic IgG eluted from M₁ peptide (non-peptide fraction) (△). (b) Effect of $10^{-5}$ M anti-M₁ peptide IgG from schizophrenia patients (anti-M₁p IgG) alone or in the presence of $10^{-5}$ M haloperidol, $10^{-5}$ M pilocarpine, $10^{-7}$ M normal IgG or $10^{-3}$ M negative control IgG. Values are mean ± S.E.M. of four pools from nine schizophrenia patients positive for M₁ mAChRs, two pools from five schizophrenia patients negative for M₁ mAChRs and three pools from 10 normal subjects, performed in duplicate in each group. * $p < 0.0001$ vs. baseline. ** $p < 0.0001$ vs. anti-M₁ 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\(_1\) IgG, we studied the effect of selective inhibitors of enzymatic pathways commonly associated with M\(_1\) mAChR activation. Figure 5(a–c) shows that the inhibition of phospholipase C (PLC) by U-73122 (5 \(\times\) 10\(^{-6}\)M), inhibition of protein kinase C (PKC) by staurosporine (5 \(\times\) 10\(^{-9}\)M) and inhibition of calcium-calmodulin by TFP (5 \(\times\) 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 \(\times\) 10\(^{-6}\)M) or COX-1 inhibition by FR 122047 (5 \(\times\) 10\(^{-8}\)M) prevented the stimulatory action of M\(_1\)p IgG-induced PGE\(_2\) and MMP-3 production. As control FR 122047 did not modify M\(_1\)p IgG-induced NOS activity (data not shown). COX-2 inhibition by DuP 697 (5 \(\times\) 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\(_1\)p IgG (10\(^{-8}\)M) stimulation for 1 h increased iNOS and cox-1 mRNA levels, with...
no modification of cox-2 mRNA levels. The depleted fraction of anti-peptide IgG had no effect (data not shown). Figure 6 also shows that the authentic agonist pilocarpine (10⁻⁸ M) mimicked the antibody action. Moreover, when M₁p 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 M₁ mAChR. The molecular interaction of schizophrenia antibodies with cerebral frontal cortex M₁ mAChR has the capacity to generate the pro-inflammatory substances NO, PGE₂, 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.
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 M₁ mAChR peptide, but also displayed agonistic activity triggering NO, PGE₂ and MMP-3 production. Studies on signalling events involved in mAChR activation in rat cerebral frontal cortex have implicated stimulation of NOS and PGE₂ (Borda et al. 1998; Orman et al. 2005; Sterin-Borda et al. 2003). The increased production of PGE₂ 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 PGE₂/MMP-3 production triggered by the autoantibodies. Regarding the effect of haloperidol inhibiting M₃p IgG-increased NOS, PGE₂ and MMP-3, we can interpret this as the antipsychotic drug behaving like an inhibitor on M₁ 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 PGE₂/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-M₃p 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-M₃p IgG for the M₁ mAChR subtypes while pilocarpine would bind to other cerebral mAChR subtypes (M₄–M₅); 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 PGE₂ (Orman et al. 2005).

Our data indicate that the endogenous NO signalling system is a key factor in the generation of PGE₂ 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-M₁ 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 PGE₂ 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 M₁ 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 PGE₂/MMP-3 regulation by IgG from schizophrenia patients observed in the present study was mediated mostly by PLA₄ and COX-1, as it was prevented by specific blockade of these two enzymes. Therefore, the activation of rat cerebral frontal cortex M₁ mAChRs by the antibody that triggers PGE₂/MMP-3 production is preceded by NOS activation, which in turn catalyses PLA₄/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 neuro-inflammatory process. By contrast, COX-2 which is
localized in pyramidal neurons is expected to be activated during ischaemia and excitotoxicity (Choi et al. 2009). The fact that M₁ mAChR antibodies are able to interact with neuronal and astrocyte M₁ 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 schizophrenic 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 PGE₂, 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, PGE₂ 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

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**Fig. 7.** Proposed model to explain the mechanism whereby schizophrenic IgG up-regulates iNOS and cox-1 mRNA levels and prostaglandin E₂/metalloproteinase-3 (PGE₂/MMP-3) in cerebral frontal cortex. Schizophrenia anti-M₁ peptide IgG (schizophrenic IgG) acting on G_q-protein activates PLC, mediating production of inositol triphosphate (IP₃) and 1-2 diacylglycerol (DAG). IP₃ triggers intracellular release of calcium stores (Ca²⁺). Free Ca²⁺ binds to calcium-calmodulin and sensitizes PKC activation via DAG. Subsequent PKC translocates to the membrane and the Ca²⁺-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 Ca²⁺ activates phospholipase A₂ (PLA₂) with subsequent activation of COX-1 which induces generation of PGE₂ and MMP-3. Inhibitory agents are indicated in italics.
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.

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Statement of Interest

None.

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