Behavioral and neural effects of intra-striatal infusion of anti-streptococcal antibodies in rats

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ARTICLE INFO

ABSTRACT

Group A β-hemolytic streptococcal (GAS) infection is associated with a spectrum of neuropsychiatric disorders. The leading hypothesis regarding this association proposes that a GAS infection induces the production of auto-antibodies, which cross-react with neuronal determinants in the brain through the process of molecular mimicry. We have recently shown that exposure of rats to GAS antigen leads to the production of anti-neuronal antibodies concomitant with the development of behavioral alterations. The present study tested the causal role of the antibodies by assessing the behavior of naïve rats following passive transfer of purified antibodies from GAS-exposed rats. Immunoglobulin G (IgG) purified from the sera of GAS-exposed rats was infused directly into the striatum of naïve rats over a 21-day period. Their behavior in the induced-grooming, marble burying, food manipulation and beam walking assays was compared to that of naïve rats infused with IgG purified from adjuvant-exposed rats as well as of naïve rats. The pattern of in vivo antibody deposition in rat brain was evaluated using immunofluorescence and colocalization. Infusion of IgG from GAS-exposed rats to naïve rats led to behavioral and motor alterations partially mimicking those seen in GAS-exposed rats. IgG from GAS-exposed rats reacted with D1 and D2 dopamine receptors and 5HT-2A and 5HT-2C serotonin receptors in vitro. In vivo, IgG deposits in the striatum of infused rats colocalized with specific brain proteins such as dopamine receptors, the serotonin transporter and other neuronal proteins. Our results demonstrate the potential pathogenic role of autoantibodies produced following exposure to GAS in the induction of behavioral and motor alterations, and support a causal role for autoantibodies in GAS-related neuropsychiatric disorders.

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1. Introduction

Group A β-hemolytic streptococcal (GAS) infection is associated with a spectrum of neurological and neuropsychiatric disorders, including Sydenham's chorea (SC), pediatric autoimmune neuropsychiatric disorders associated with streptococcus (PANDAS), obsessive–compulsive disorder (OCD), and Tourette's syndrome (TS) (Dale, 2005; Husby et al., 1976; Peterson et al., 2000; Swedo et al., 1998, 1994; Taranta and Stollerman, 1956). SC is the classical post-streptococcal neurological disorder occurring weeks to months after GAS infection, and is characterized by involuntary movements and neuropsychiatric disturbances, including obsessive–compulsive symptoms, tics, and emotional lability (Marques-Dias et al., 1997; Swedo et al., 1993).

The leading hypothesis regarding the relationship between GAS infection and neuropsychiatric disorders proposes that a GAS infection induces the production of autoantibodies, which cross-react with neuronal determinants in the brain (especially in the basal ganglia, prefrontal cortex and thalamus) through the process of molecular mimicry (Bonthius and Karacay, 2003; Bronze and Dale, 1993; Church et al., 2002; Cunningham, 2000, 2012; Husby et al., 1976; Kirvan et al., 2003; Pavone et al., 2004; Swedo et al., 1994).

Five criteria should be met to establish a disorder as an autoantibody-mediated autoimmune disorder: (A) presence of autoantibodies in the serum or cerebrospinal fluid (CSF); (B) a therapeutic effect of plasma exchange; (C) presence of antibodies

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at the tissue involved in the disorder pathogenesis; (D) induction of symptoms by immunizing with the antigen in an animal model; and (E) induction of symptoms by passive transfer of antibodies to animals (Archelos and Hartung, 2000). Several studies reported the presence of autoantibodies in the sera and CSF of GAS-related neuropsychiatric disorders patients (Bronze and Dale, 1993; Church et al., 2002; Gause et al., 2009; Husby et al., 1976; Kotby et al., 1998; Singer et al., 1998), and plasma exchange treatment has been reported to alleviate symptoms in these patients (Garvey et al., 2005; Perlmutter et al., 1999), fulfilling criteria A and B. We (Brimberg et al., 2012) and others (Hoffman et al., 2004) have found that exposure of rodents to GAS antigen leads to behavioral alterations, fulfilling Criterion D. Others (Ben-Pazi et al., 2012; Doyle et al., 2012; Hallett et al., 2000; Loiselle et al., 2004; Singer et al., 2005; Taylor et al., 2002; Yaddanapudi et al., 2010) have attempted to demonstrate that passive transfer of Immunoglobulin G (IgG) alters behavior (Criterion E). Some have been successful (Doyle et al., 2012; Hallett et al., 2000; Taylor et al., 2002; Yaddanapudi et al., 2010) while others have not (Ben-Pazi et al., 2012; Loiselle et al., 2004; Singer et al., 2005). The aim of the present study was to extend our previous findings (Brimberg et al., 2012) and test whether intra-striatal passive transfer of antibodies to animals would lead to the induction of a behavioral syndrome similar to those reported (Criterion E).

We have recently shown that exposure of male Lewis rats to GAS antigen led to a syndrome which resembles behavioral, pharmacological, immunological and neural characteristics of GAS-related neuropsychiatric disorders (Brimberg et al., 2012). Behaviorally, GAS-exposed rats showed increased compulsive-like behavior and motor disturbances, similar to the neuropsychiatric symptoms found in GAS-related neuropsychiatric disorders. Moreover, the abnormal behaviors in GAS-exposed rats were attenuated by pharmacological treatments used to treat the corresponding symptoms in human patients (i.e., a selective serotonin reuptake inhibitor (SSRI) and a D2 blocker, respectively). Immunologically, IgG was found in the striatum, prefrontal cortex (PFC) and thalamus of GAS-exposed rats (Brimberg et al., 2012), corresponding to the brain regions implicated in GAS-related neuropsychiatric disorders (Barsottini et al., 2002; Citak et al., 2004; Dilenge et al., 1999; Giedd et al., 2000; Huyser et al., 2009). IgG in sera obtained from GAS-exposed rats demonstrated strong immunoreactivity with D1 and D2 dopamine receptors and activated calcium/calmodulin-dependent protein kinase II signaling, as has previously been found for IgG in sera obtained from SC and PANDAS patients (Kirvan et al., 2003, 2006). In addition, we have found alterations in dopamine and glutamate levels in the medial frontal cortex and basal ganglia of GAS-exposed rats (Brimberg et al., 2012), further supporting a functional effect of the autoantibodies.

The aim of the present study was to directly test the role of the autoantibodies in inducing disease-like symptoms and to further test the involvement of the dopaminergic and the serotonergic systems in the induction of behavioral alterations. To this end, IgG purified from the sera of GAS-exposed rats and from adjuvant-exposed rats was infused directly into the striatum of naïve rats (GAS-I and Control-I rats, respectively) and the behavior of infused rats as well as of naïve rats was assessed (for more details see Fig. 1). Our results show that infusion of IgG from GAS-exposed rats leads to behavioral and motor alterations partially mimicking those seen in GAS-exposed rats. In addition, we show for the first time that IgG from GAS-exposed rats reacts with 5HT-2A and 5HT-2C serotonin receptors in vitro. In vivo, IgG deposits in the striatum of infused rats colocalized with specific brain proteins such as dopamine receptors, the serotonin transporter and other neuronal proteins.

Materials and methods

Animals

Male Lewis rats (Harlan, Jerusalem, Israel), 5 weeks old, were housed in groups of 2–3 per cage under a reversed 12-h light–dark cycle (lights on at 1900–0700 h) with ad libitum food and water. Rats were weighed twice a week. All experimental protocols were carried out according to the guidelines of the Institutional Animal Care and Use Committee of Tel-Aviv University.

GAS-exposure

Streptococcus pyogenes

M protein type 18 (Manfraedo) was obtained from Dr. Allon Moses (Hadassah University Medical Center, Jerusalem, Israel), and grown as previously described (Brimberg et al., 2012). In short, streptococci were grown in 400 ml Todd-Hewitt broth (HyLab, Rehovot, Israel) overnight at 37 °C with rocking at 250 rpm. The next morning, the cells were collected by centrifugation at 5000 rpm for 15 min at 4 °C. The cell pellets (in ~1.5 g) were stored frozen at −20 °C until used.

Mutanolysin-extracted GAS antigen

A whole cell digest of M type 18 Streptococcus pyogenes was prepared as previously described (Brimberg et al., 2012). In short, cell pellets were suspended in phosphate-buffered saline (PBS) containing mutanolysin (Sigma–Aldrich, Rehovot, Israel). Following incubation at 37 °C for 2 h with rocking, the digest was further disrupted by sonication (Microson ultrasonic cell disruptor, Plainveiw, NY). The insoluble material was removed by centrifugation at 12,000 rpm (~25,000g) for 30 min at 4 °C. Protein concentration in the supernatant was determined using the Coomasie-Plus Bradford reagent (PIERCE) according to the supplier recommendations. The supernatant was dialyzed extensively against water (10,000 MWCO, Sigma–Aldrich, Rehovot, Israel) then lyophilized and the powder was stored at −70 °C.

Exposure of donor rats to GAS antigen

The exposure protocol followed Brimberg et al. (2012). Twenty eight rats were handled for 2 min daily for 4 days before the beginning of the exposure protocol. The first exposure was done at 5 weeks of age. Before each injection, rats were lightly anaesthetized with Isoflorene (VetMarket, Petach Tikva, Israel). Each rat in the GAS group was immunized subcutaneously with 200 μl of 1:1 emulsion of PBS containing 1.2 mg of the GAS antigen and Complete Freund's adjuvant (CFA, Sigma–Aldrich, Rehovot, Israel) supplemented with 4 mg/ml of heat-killed mycobacteria H37RA (Difco Laboratories, Detroit, MI). In order to increase the permeability of the blood brain barrier (Linthicum et al., 1982) rats have received an intraperitoneal injection of 1010 heat-killed Bordetella pertussis (Bioport, Lansing, MI, USA) as an additional adjuvant. Two boosts were introduced two and four weeks following the first exposure. Each rat was boosted with 200 μl 1:1 emulsion of incomplete Freund's adjuvant (IFA, Sigma–Aldrich): PBS containing 1.2 mg of the GAS antigen. Control animals were injected with PBS and adjuvants only. Behavioral testing began when the rats were 11 weeks old.

Preparation of pooled GAS and control donor IgG

Rats were euthanized and blood was collected. After clotting and centrifugation, serum was collected and stored at −70 °C. Protein L resin (Genscript, USA) was packed in a polypropylene column (1 ml) and equilibrated with 10 ml of wash buffer (20 mM Na$_2$HPO$_4$, 0.15 M NaCl, pH 8.0). Sera samples were filtered by pass-
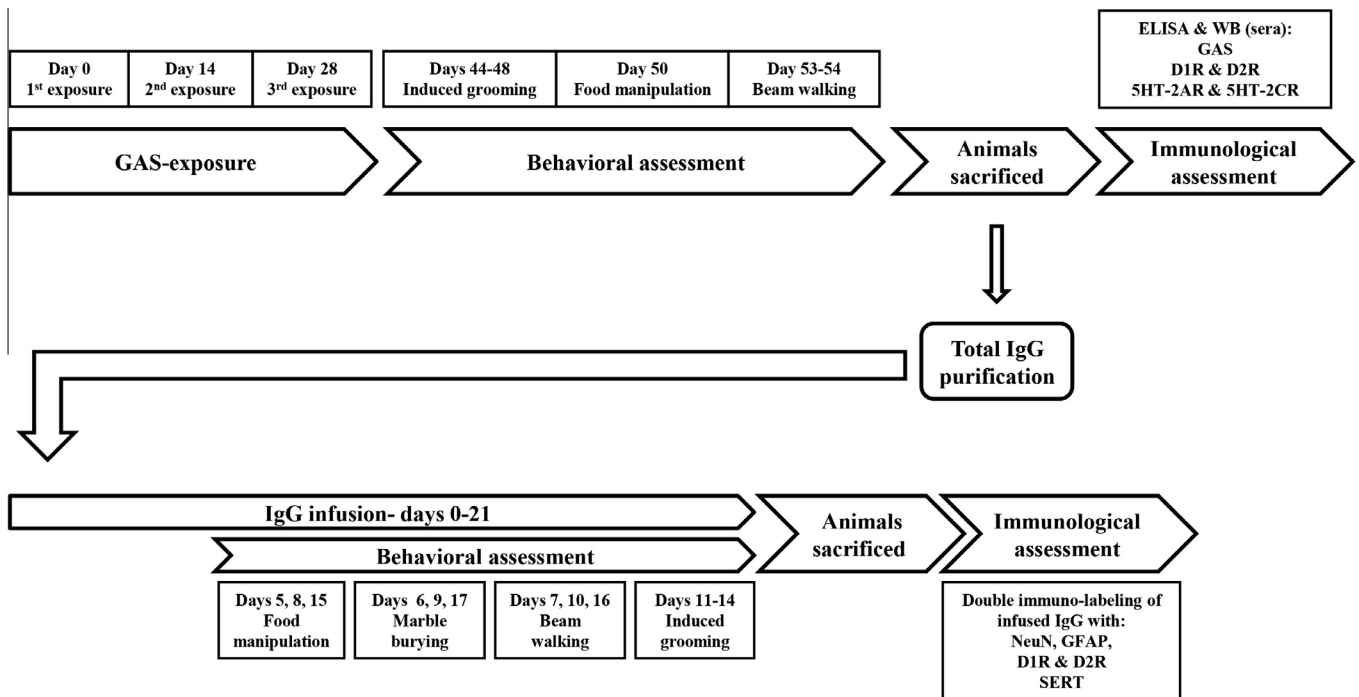
Fig. 1. Time line and behavioral and immunological procedures included in the study assessing the effects of GAS-exposure (upper half) and in the study assessing the effects of IgG infusion (lower half). 5HT-2AR = 5HT-2A serotonin receptor; 5HT-2CR = 5HT-2C serotonin receptor; D1R = D1 dopamine receptor; D2R = D2 dopamine receptor; GFAP = glial fibrillary acidic protein; NeuN = neuronal nuclei; SERT = serotonin transporter; WB = Western blot.

ing them through a 0.45 μm filter, and loaded onto the column. The column was washed with wash buffer. Total IgG was eluted with 10–15 ml elution buffer (0.1 M glycine, pH 2.5), and neutralized to pH 7.4 with neutralization buffer (1 M Tris–HCl, pH 8.5). Samples were dialyzed extensively against PBS (10,000 MWCO, Sigma–Aldrich, Rehovot, Israel). Prior to microinfusion, IgG was sterilized by filtration, pooled and brought to a concentration of 2 mg/ml. IgG concentration was determined by a NanoDrop Spectrophotometer (Thermo Scientific).

Intra-striatal infusion of IgG

Fourteen Lewis rats, 6 weeks old, were anesthetized with an intraperitoneal injection of ketamine and xylazine. Bilateral osmotic pump connector, 28 gauge, stainless steel cannulae (PlasticOne, USA), were implanted into the dorsolateral striatum at the following coordinates (Paxinos and Watson, 1998): 0.9 mm anterior to bregma, ±3.6 mm lateral to the midline, and 4.9 mm ventral to the skull. The cannulae were connected to an osmotic pump (Alzet Corp, Palo Alto, CA), containing 200 μl of IgG (2 mg/ml) purified from the sera of either GAS-exposed rats (GAS-I group, $n = 8$) or control rats (Control-I group, $n = 6$), through a sterile polyethylene tube (containing sterile PBS). The choice of IgG concentration was based on previous studies in which IgG purified from PANDAS, SC or TS patients was infused into the striatum using osmotic pumps (Doyle et al., 2012; Hallett et al., 2000; Taylor et al., 2002). IgG was micro-infused bilaterally for 21 days, at a rate of 0.5 μl/h. A third group of control rats were anesthetized but were not implanted with cannulae (naïve group, $n = 8$). Behavioral testing began 5 days following cannula implantation.

Behavioral assessment

A 22-h food restriction schedule with water freely available was initiated at age 9 weeks for the GAS exposure experiment, and 3 days after surgery for the intra-striatal IgG infusion experiment.

Food manipulation

Rats' ability to manipulate a food pellet was assessed as previously described (Ayalon et al., 2004; Brimberg et al., 2012). In short, one day following a 10 min exposure to a 38 × 21 cm Plexiglas observation box (habituation), food-deprived rats were placed in the box, and 5 min later several Purina rat food pellets (approximately 1 g each) were introduced into the box. Rats were given 10 min to consume the food pellet, and their ability to manipulate it was rated independently by two observers, according to the scale of Kolb and Holmes (1983): (0) Unable to manipulate the food pellet with the forepaws; (1) holds the food pellet against the floor with its forepaws when it eats; (2) eats the food pellet from the floor and sometimes hold the food pellet in its forepaws; (3) picks the food pellet up in its forepaws and partially eats it but drops the food pellet before it is all consumed; and (4) sits up on its hind paws and holds the food pellet in its front paws until it is finished. Half scores were given when the animal behavior was in-between scale definitions. The observer rating behavior was blind to the animal condition.

Beam walking

Motor coordination and balance were assessed by measuring the ability of rats to traverse a 1 m long beam with a width of 5, 2.5 or 1.5 cm (Brimberg et al., 2012; Carter et al., 1999; Urakawa et al., 2007). The beam was placed 1 m above the floor, with one end mounted on a narrow support and the other end attached to the rat's home cage. On Day 1 each rat received three training trials on the 5 cm wide beam. On Day 2, after two training trials on the 5 cm beam, the time it took the rat to traverse this beam and the number of foot slips were recorded. Immediately after, rats were given 2 training trials on the 2.5 and 1.5 cm wide beams and the time it took each rat to traverse these beams on the third trial and the number of foot slips were recorded. The rater was blind to the animal condition.
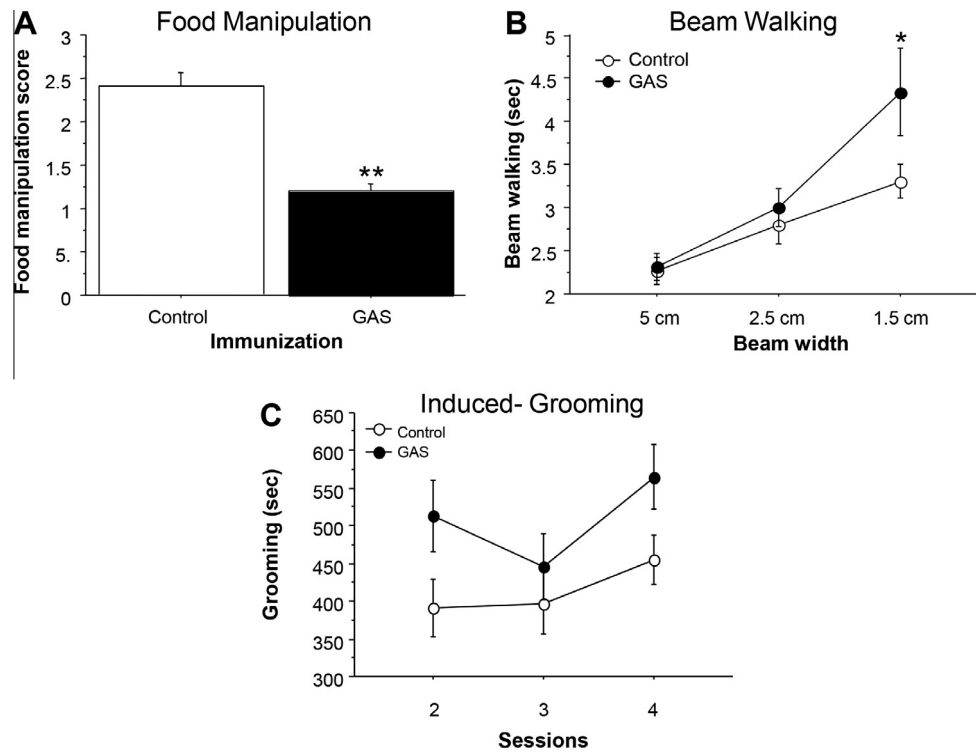
Fig. 2. Effects of streptococcal exposure on (A) food manipulation, (B) beam walking, and (C) grooming. (A) The mean and standard error (SE) of food manipulation scores of GAS (n = 12) and control rats (n = 16). (B) The mean and SE of the time spent on the wide and narrow beams of GAS (n = 12) and control rats (n = 16). (C) The mean and SE of the duration of grooming in three sessions of induced-grooming of GAS (n = 9) and control (n = 16) rats. $^*p < 0.01$, $^{**}p < 0.0001$.

Grooming

The assessment of induced-grooming was carried out as previously described (Brimberg et al., 2012; Greer and Capecchi, 2002). Each rat was videotaped individually for 30 min on 4 consecutive days in an empty cage. Ten min after the beginning of each session, the rat was misted with water to induce grooming. The duration of grooming behavior was assessed in the 20 min of the induced-grooming of each day. The rater was blind to the animal condition.

Marble burying

Marble burying was assessed because we have recently found that GAS-exposure increases the number of marbles buried in this test (Lotan et al., in preparation). Rats were placed individually in a cage measuring 37 cm long × 21 cm wide × 18 cm high, containing bedding that was 5 cm in depth, with nine marbles 2.3 cm in diameter arranged in two rows along the short wall of the cage. The number of buried marbles after 15 min was counted. Marbles were considered buried if they were at least one-half covered with bedding. The observer rating behavior was blind to the animal condition.

Activity

Activity was assessed as previously described (Ayalon et al., 2004). In short, rats were individually placed in an activity box (45 cm wide × 65 cm long × 40 cm high) located in a quiet room and allowed 1 h of free exploration. Images from a camera located above each box were analyzed using image analysis software. The software "grabbed" the image from each box every 1 s and compared this image, pixel by pixel, with the image obtained in the previous second. Each white rat was monitored against the darker background. The percentage of pixels that went from dark to light or from light to dark from 1 s to the next ("activity counts") was quantified. This percentage provided a measure of the magnitude

of an animal's displacement or "activity". One-second activity values ranged from 0% (no movement) to approximately 7.5%.

Immunological assessments

Reagents

The following antigens were used in this study: bovine serum albumin (BSA) was purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Human D1 and D2L dopamine receptors, and 5HT-2A and 2C serotonin receptor antigens were purchased from PerkinElmer (Waltham, Massachusetts, USA) and used for both the ELISA and Western blot procedures.

ELISA-GAS

Immunoreactivity to the GAS mutanolysin extracted streptococcal antigen was assessed using ELISA, as previously described (Brimberg et al., 2012). Ninety six well ELISA plates (Nunc) were coated with 5 µg/ml of GAS mutanolysin extracted streptococcal antigen in PBS overnight at 4 °C. Plates were blocked with 300 µl/well of 2% non-fat milk in PBS for 1 h at RT. Diluted rat serum was applied onto the plates in a dilution series (1:500, 1:2500, and 1:125,000) in 0.05% PBST and incubated for 1 h at RT. Following incubation, the plates were washed ×3 with PBST. HRP conjugated donkey anti-rat antibodies (Jackson Immunolaboratories, USA, 1:10,000 dilution in PBST) were added to the wells (100 µl/ well) for 1 h at RT, followed by ×3 washes with PBST. The Plates were developed using the chromogenic HRP substrate TMB (Sigma–Aldrich, Rehovot, Israel) (100 µl/well) and color development was terminated with 1 M H_2SO_4 (50 µl/well). The plates were read at 450 nm.

ELISA and Western blot

Immunoreactivity to D1 and D2 dopamine receptors and to the 5HT-2A and 2C serotonin receptors membrane antigens was as-
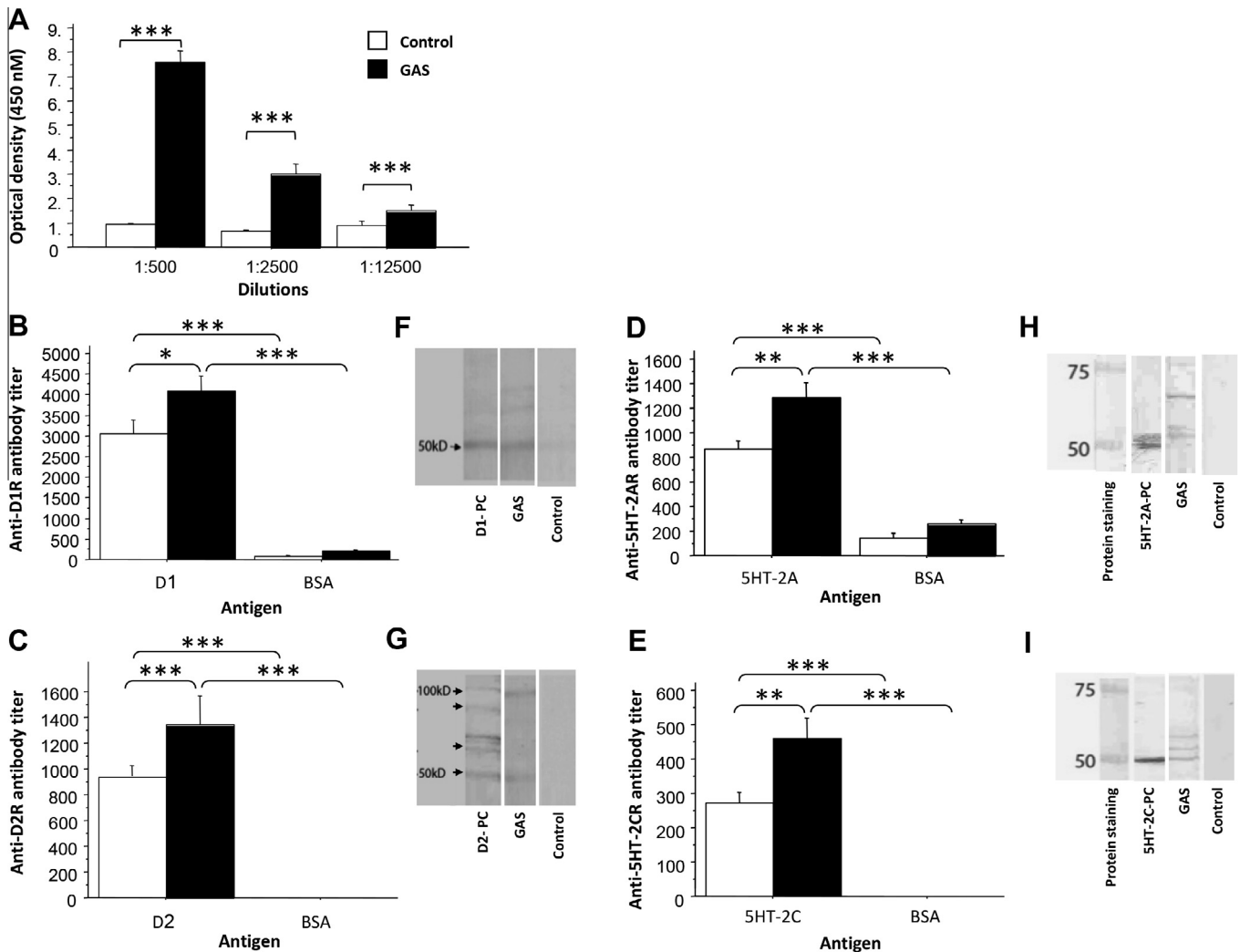
Fig. 3. Effects of group A streptococcal (GAS) exposure on immunoreactivity of rat serum (IgG) with GAS antigen, dopamine receptors and serotonin receptors in the ELISA (A–E) and Western blot (F-I). ELISA results for anti-GAS serum IgG reactivity with: (A) GAS mutanolysin extracted antigen (GAS, n = 12; control, n = 10), (B) D1 dopamine receptor (GAS, n = 11; control, n = 10), (C) D2 dopamine receptor (GAS, n = 13; control, n = 10), (D) 5HT-2A serotonin receptor (GAS, n = 12; control, n = 10), and (E) 5HT-2C serotonin receptor (GAS, n = 12; control, n = 10), of sera taken from GAS and control rats, in comparison to reactivity with BSA. Western blot of pooled sera (IgG) reactivity from GAS-exposed rats compared with sera (IgG) from adjuvant-exposed control rats with: (F) D1 dopamine receptor, (G) D2 dopamine receptor, (H) 5HT-2A serotonin receptor, and (I) 5HT-2C serotonin receptor. D1-PC = anti-D1 receptor positive control; D2-PC = anti-D2 receptor positive control; 5HT-2A-PC = anti-5HT-2A receptor positive control; 5HT-2C-PC = anti-5HT-2C receptor positive control. $^*p < 0.01$, $^{**}p < 0.005$, $^{***}p < 0.0001$.

sessed using ELISA and western blot analysis of sera obtained from GAS-exposed rats:

ELISA. Ninety six well Immunolon 4 microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with 10 μg/ml of each antigen (D1, D2L, and 5HT-2A and 2C receptors membrane antigens; BSA served as a negative control protein) in 0.015 M carbonate buffer (pH 9.6) overnight at 4 °C. Plates were blocked with 1% BSA in PBS for 1 h. Diluted rat serum (50 μl/well) was applied onto the plates in a dilution series and incubated overnight at 4 °C in 1% BSA in PBS. The next day, the plates were washed ×3 with PBST. Alkaline phosphatase-conjugated rabbit anti-rat secondary antibody specific to IgG (Sigma Chemical Co.) was added to the plates diluted 1:1000 in 1% BSA–PBS. Plates were further incubated for 1 h at room temperature and washed with PBST. Plates were developed with 1 mg/ml p-nitrophenyl phosphate colorimetric substrate (Sigma Chemical Co.) and the optical density was determined at 405 nm in a Opsys MR microplate reader (Dynex Technologies, Chantilly, VA). For the titers of antibodies against

the serotonin receptors 5HT-2A and 2C, the following dilutions were performed: 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400; D1: 1:300, 1:600; 1:1200, 1:4800, 1:9600; D2: 1:320, 1:640; 1:1280, 1:5120, 1:10,240; BSA: 1:200, 1:400, 1:800, 1:1600. Titers were determined as the last dilution resulting in an OD of 0.1.

Western blot. D1 and D2 dopamine receptors (Perkin Elmer) were loaded as 10 μg per lane, and 5HT-2A and 2C serotonin receptors (abcam) were loaded as 1 μg per lane in the SDS PAGE gel. Antigens were separated on a 7–10% SDS–PAGE gel using a 5% stacking gel and separated antigens were electro-transferred onto the nitrocellulose membrane. Membranes were blocked overnight at 4 °C in 5% nonfat milk in Tris-Buffered Saline Tween-20 (TBST). To determine the immunoreactivity of the sera to blotted proteins, individual lanes were separated into strips and incubated with a pool of sera from the control or GAS-exposed rats, diluted in PBST (1:150) overnight at 4 °C. Rabbit anti-D1 receptor sera were diluted 1:1000 (R&D systems); rabbit anti-D2 receptor sera were diluted 1:400 (R&D systems); rabbit anti-5HT-2A receptor sera were diluted
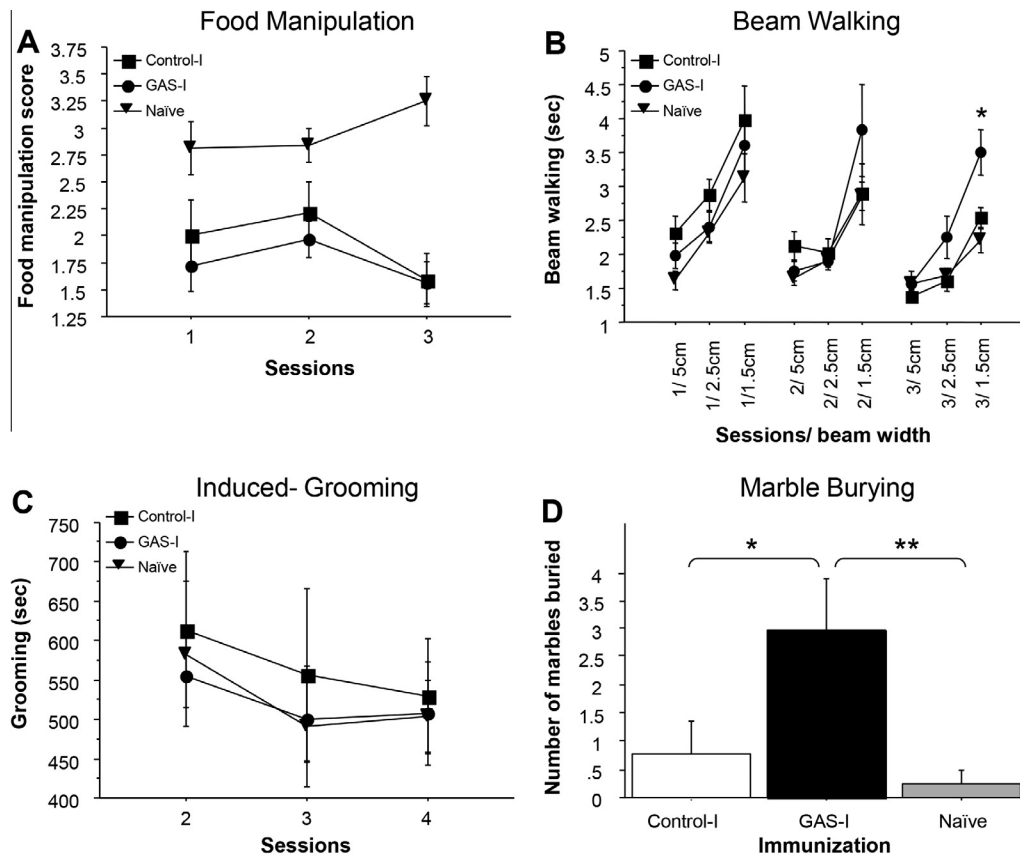
Fig. 4. Effects of passive transfer of IgG from GAS-exposed and control rats to the striatum of naïve rats on (A) food manipulation, (B) beam walking, (C) grooming, and (D) marble burying. (A) The mean and standard error (SE) of food manipulation scores of rats infused with IgG extracted from GAS-exposed rats (GAS-I group, $n = 8$), rats infused with IgG extracted from control rats (Control-I group, $n = 6$) and naïve rats ($n = 8$). (B) The mean and SE of the time spent on the wide and narrow beams of GAS-I rats ($n = 8$), Control-I rats ($n = 6$) and naïve rats ($n = 8$). (C) The mean and SE of the duration of induced-grooming on three sessions of GAS-I rats ($n = 8$), Control-I rats ($n = 5$) and naïve rats ($n = 8$). (D) The mean and SE of the number of marbles buried by GAS-I rats ($n = 8$), control rats ($n = 5$) and naïve rats ($n = 8$). $^*p < 0.05$, $^{**}p < 0.01$.

1:1000 (abcam); rabbit anti-5HT-2C receptor sera were diluted 1:500 (abcam); all dilutions were used based on manufacturer guidelines. The strips were washed in PBST and incubated with HRP-conjugated donkey anti-rat antibodies or with HRP-conjugated goat anti-rabbit antibodies (Jackson Immunolaboratories, USA) at dilutions of 1:5000. Blots were detected using the enhanced chemiluminescence (ECL) Plus Western detection kit (Amersham Pharmacia Biotech). The bands developed from the immunoblot were compared to the relative position of separated proteins on Coomassie blue-stained immobilized nitrocellulose strips. The relative molecular weight of proteins was determined using pre-stained molecular weight protein standards (Bio-Rad).

Preparation of tissue sections

Rats were overdosed with 100 mg/kg sodium pentobarbital, i.p, and perfused intracardially with cold perfusion buffer. Brains were removed and placed in 4% paraformaldehyde over night, after which they were cryoprotected in 30% sucrose solution for at least 48 h. For immunostaining, brains were sectioned in the coronal plane at 16 μm for detecting IgG deposits, and sections were mounted on gelatin-coated slides. Slides were stored at −70 °C.

Immunostaining for IgG deposits and double staining

To assess IgG deposits in the brain, 16 μm sections were incubated in PBS until reaching room temperature. Sections were then heated in citric acid (pH 6, 10 mM) for 5 min and were treated with 0.5% Triton X-100 for 3 min. The sections were blocked (2% BSA

solution, 10% normal horse serum and 0.25% Triton X-100 in PBS) for 1 h in room temperature. Sections were then incubated for 1 h with fluorescent Alexa 488 goat anti rat secondary antibody (1:250, Molecular Probes, Eugene, OR, USA) diluted in blocking solution. Following Immunostaining for IgG deposits, the sections were incubated overnight at 4 °C with one of the following markers labeled with mouse anti-neuron-selective NeuN (anti-neuronal nuclei, 1 : 250; Millipore), rabbit anti-Glial fibrillary acidic protein (GFAP, 1:400, Sigma), rabbit anti anti-D2 dopamine receptor (1:100, Millipore), rabbit anti-D1 dopamine receptor (1:300, Millipore), and rabbit anti-serotonin transporter (1:400, Millipore). Sections were washed three times with PBS, and incubated with fluorescent Alexa 594 goat anti-mouse or anti-rabbit antibodies (1:250; molecular Probes) for 1 h at room temperature. Following immunostaining, sections were coverslipped with mounting media with DAPI (Vector Laboratories, Burlingame, USA) to counterstain the nuclei. The imaging was carried out using a fluorescence microscopy (Nikon Eclipse 80i) and a NIS imaging software (Nikon).

Statistical analysis

We used two-sample Student's *t*-tests to compare means of two independent groups. When more groups were compared, we used analysis of variance (ANOVA; the specific factors for each analysis are given in the Results section) together with post hoc LSD analysis (the specific analyses are detailed in the Results section). We
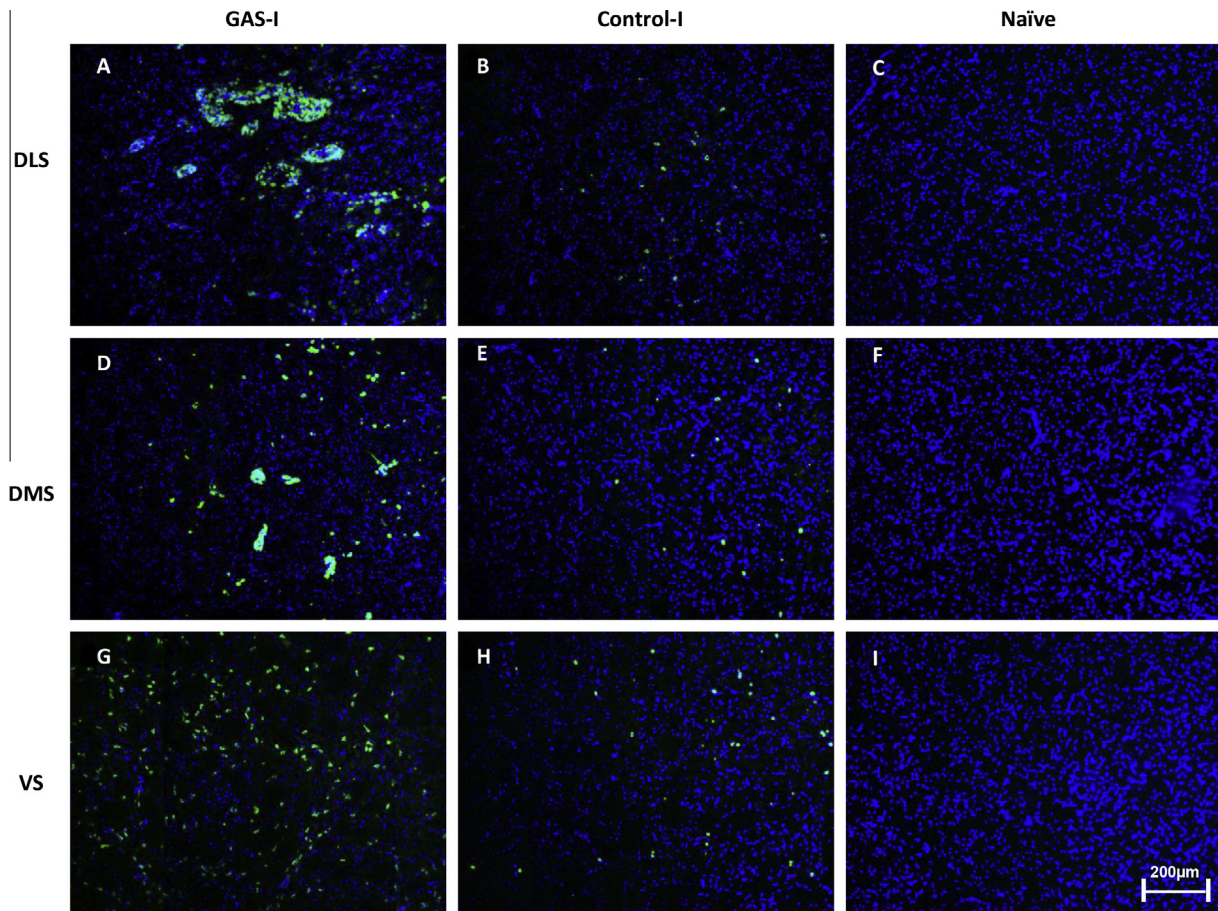
Fig. 5. Representative immunofluorescence images showing IgG deposition in the striatum of a GAS-I rat (A, D, G), Control-I rat (B, E, H) and naïve rat (C, F, I). (A–C) Labeling in the dorso-lateral striatum (DLS, the injection site); (D–F) labeling in the dorso-medial striatum (DMS); (G–I) labeling in the ventral striatum (VS). Tissue sections were incubated with anti-rat alexa antibody 488 for visualization of IgG deposition; Blue signal indicates nuclear counterstaining (DAPI). 10× microscope objective; scale bar = 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

considered values as significant when $p < 0.05$. All data are presented as means ± SEM.

Results

Effects of exposure to GAS antigen

Altered behavior in Lewis rats exposed to GAS antigen

Fig. 2A–C presents the behavior of GAS-exposed and control (adjuvant-exposed) rats in the food manipulation, beam walking and induced-grooming assays. GAS-exposed rats were impaired in manipulating food (Fig. 2A, $t(26) = 5.726$, $p < 0.0001$), and in traversing the narrow (1.5 cm) but not the wider (2.5 and 5.0 cm) beams, as evident in a longer time to traverse the beam (Fig. 2B, Exposure × Beam Width mixed ANOVA: Exposure: $F(1,26) = 3.484$, $p = 0.0733$; Beam Width: $F(2,52) = 20.814$, $p < 0.0001$; Exposure × Beam Width interaction: $F(2,52) = 2.479$, $p = 0.0936$; see Fig. 2B for the results of the post hoc comparisons). There were no differences between the groups in the number of foot slips (data not shown, p's > 0.3). In addition, GAS rats exhibited increased grooming in the induced-grooming assay (Fig. 2C, Exposure × Session mixed ANOVA: Exposure: $F(1,23) = 5.145$, $p < 0.05$; Session: $F(2,46) = 2.818$, $p = 0.0701$; Exposure × Session interaction: $F(2,46) = 0.548$, $p = 0.5821$). GAS rats did not have any gross indications of skin lesions, nor did they show loss of hair, which could indicate an increase in spontaneous grooming.

Sera from GAS-exposed rats reacted with dopamine and serotonin receptors in the ELISA and Western immunoblot

As expected, IgG antibodies in sera of GAS-exposed rats were significantly elevated against the GAS cell wall antigen, the immunogen (Fig. 3A, $F(1,20) = 99.53$, $p < 0.0001$). In addition, we found in the ELISA (Fig. 3B and C) and confirmed in the Western blot (Fig. 3F and G) that the streptococcal exposure led to induction of antibodies against the dopamine receptors D1 and D2, and that this reactivity was specific when compared to BSA (Fig. 3B, D1: Exposure × Protein mixed ANOVA: Exposure: $F(1,38) = 5.519$, $p < 0.05$; Protein: $F(1,38) = 187.845$, $p < 0.0001$; Exposure × Protein interaction: $F(1,38) = 3.095$, $p = 0.0866$; Fig. 3C, D2: Exposure × Protein mixed ANOVA: Exposure: $F(1,40) = 3.137$, $p = 0.082$; Protein: $F(1,40) = 97.616$, $p < 0.0001$; Exposure × Protein interaction: $F(1,40) = 3.137$, $p = 0.082$, see Fig. 3B and C for the results of the post hoc analysis). In addition to replicating our previous findings (Brimberg et al., 2012), we have found in the ELISA (Fig. 3D and E) and confirmed in the Western blot (Fig. 3H and I), elevated antibodies against the 5HT-2A and the 5HT-2C serotonin receptors. This reactivity was specific when compared to BSA (Fig. 3D, 5HT-2A: Exposure × Protein mixed ANOVA: Exposure: $F(1,39) = 12.232$, $p < 0.005$; Protein: $F(1,39) = 133.090$, $p < 0.0001$; Exposure × Protein interaction: $F(1,39) = 3.919$, $p = 0.0548$; Fig. 3E, 5HT-2C: Exposure × Protein mixed ANOVA: Exposure: $F(1,38) = 8.197$, $p < 0.01$; Protein: $F(1,38) = 125.484$, $p < 0.0001$; Exposure × Protein interaction: $F(1,38) = 8.197$, $p < 0.01$, see Fig. 3D and E for the results of the post hoc analysis).
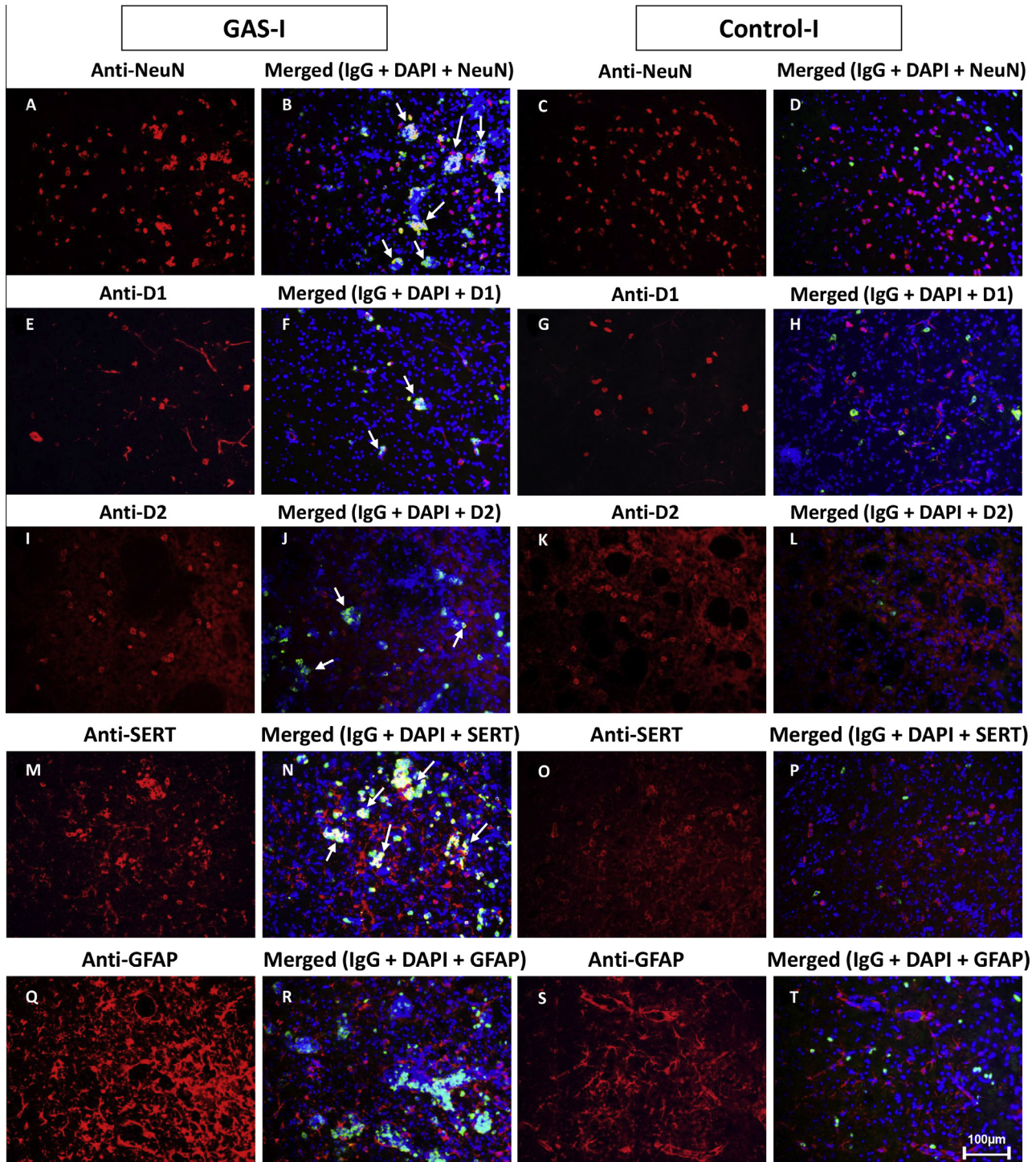
Fig. 6. Representative immunofluorescence images taken from the dorso-lateral striatum (DLS, the injection site) of a GAS-I rat (A–B, E–F, I–J, M–N, Q–R), and a control-I rat (C–D, G–H, K–L, O–P, S–T) showing labeling with markers for neurons (NeuN, A–D), D1 dopamine receptor (E–H), D2 dopamine receptor (I–L), serotonin transporter (SERT, M–P), and astrocytes (GFAP, Q–T), and colocalization of these markers with IgG deposition (B, D, F, H, J, L, N, P, R, T). Tissue sections were incubated with anti-rat alexa antibody 488 for visualization of IgG deposition; IgG against NeuN was labeled with anti-mouse alexa antibody 594; D1 and D2 dopamine receptors, SERT and GFAP were labeled with anti-rabbit alexa antibody 594. Blue signal indicates nuclear counterstaining (DAPI). 20× microscope objective; scale bar = 100 μm. The arrows point to colocalization between the infused IgG and the relevant marker. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Effects of intra-striatal passive transfer of purified total IgG from sera of GAS-exposed rats

Increased compulsive-like behavior and motor deficits in recipient rats

Fig. 4 presents the behavior of naïve rats, of rats intra-striatally infused with total IgG purified from GAS-exposed rats (GAS-I), and of rats intra-striatally infused with total IgG purified from adjuvant-exposed rats (Control-I) in the food manipulation (Fig. 4A), beam walking (Fig. 4B), induced-grooming (Fig. 4C) and marble-burying assays (Fig. 4D). There were no significant differences between the three groups in the time spent grooming in the induced-grooming test (Fig. 4C, Condition × Session mixed ANOVA:
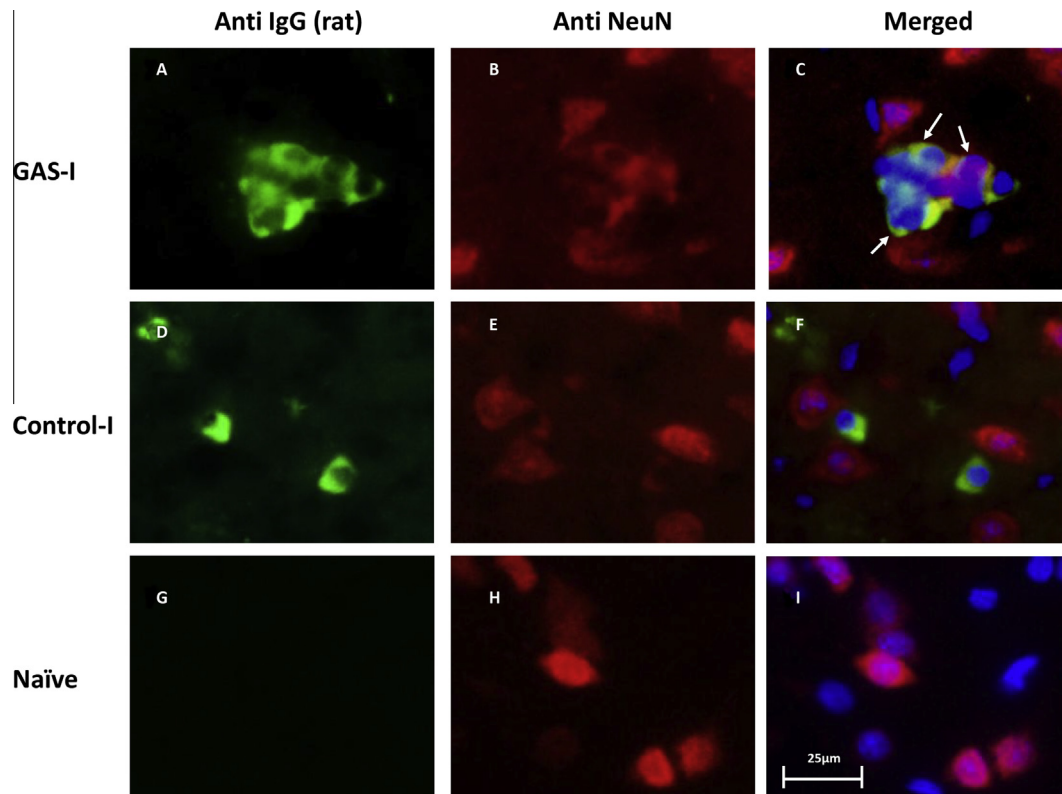
Fig. 7. Representative immunofluorescence images showing IgG deposition in the striatum of a GAS-I rat (A–C), Control-I rat (D–F) and naïve rat (G-I) and double labeling with a marker for neurons (NeuN). Figures A, D and G display labeling of rat IgG; Figures B, E and H display NeuN labeling; Figures C, F and I display colocalization of the rats IgG and NeuN. Tissue sections were incubated with anti-rat alexa antibody 488 for visualization of IgG deposition; IgG against NeuN was labeled with anti-mouse alexa antibody 594. Blue signal indicates nuclear counterstaining (DAPI). 40× microscope objective; scale bar = 25 μm. The arrows point to colocalization between the infused IgG and NeuN. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

all p's > 0.4), and both the GAS-I and Control-I groups were impaired in manipulating food compared to the naïve group (Fig. 4A, Condition × Session mixed ANOVA: Condition: $F(2,19) = 11.132$, $p < 0.0005$; Session: $F(4,38) = 2.008$, $p = 0.1482$; Condition × Session interaction: $F(4,38) = 4.723$, $p < 0.005$, see Fig. 4A for the results of the post hoc analysis), suggesting a non-specific effect of the intra-striatal infusion. In contrast, the GAS-I group performed significantly different from the Control-I and naïve groups in the beam walking and marble burying assays. Specifically, on the last session of beam walking, GAS-I rats required more time to traverse the narrow (1.5 cm) but not the wider (2.5 and 5 cm) beams, compared to the naïve and Control-I groups which performed similarly (Fig. 4B). This difference reflects the shortening of the time to traverse the narrow beam over sessions in the naïve and Control-I groups, which was not evident in the GAS-I group (Fig. 4B, Condition × Beam Width × Session mixed ANOVA: Condition: $F(2,19) = 2.64$, $p = 0.0974$; Beam Width: $F(2,38) = 97.423$, $p < 0.0001$; Session, $F(2,38) = 19.641$, $p < 0.0001$; Condition × Beam Width, $F(4,38) = 3.411$, $p < 0.05$; Condition × Session: $F(4,38) = 3.808$, $p < 0.05$; Session × Beam Width: $F(4,76) = 0.802$, $p = 0.5277$; Condition × Beam Width × Session: $F(8,76) = 0.765$, $p = 0.6342$, see Fig. 4B for the results of the post hoc analysis). A similar pattern of results was evident in the number of falls (data not shown). In addition, rats in the GAS-I group buried more marbles compared to the Control-I and naïve groups, which did not differ (Fig. 4D, $F(2,18) = 5.250$, $p < 0.05$, see Fig. 4D for the results of the post hoc analysis). The increased burying in the GAS-I group was not a result of a non-selective increase in behavioral output, as no significant differences were found between the three groups in activity level (in fact, the naïve group tended to be more active, ANOVA: $F(2,19) = 3.432$, $p = 0.0534$, data not shown).

Immunofluorescence of IgG deposits and colocalization with specific brain proteins

Using an immunohistochemical analysis, we assessed the pattern of *in vivo* IgG reactivity in the striatum. We have found IgG labeling in neurons in the striatum of GAS-I rats, in comparison to a much weaker IgG pattern in the Control-I rats. No IgG was observed in the striatum of naïve rats (Fig. 5). DAPI staining of cell nuclei overlaid the IgG reactivity in the figures.

Double immunolabeling shows that IgG clusters in GAS-I rats colocalized with NeuN (labeling neurons, Figs. 6B and 7C), with the D1 (Figs. 6F and 8C) and D2 (Figs. 6I and 9C) dopamine receptors and with the serotonin transporter (Figs. 6N and 10C). Much less staining was observed in the Control-I rat brains (Figs. 6D, H, L, P and 7F, 8F, 9F and 10F). Interestingly, double immunolabeling of GFAP (labeling astrocytes) and IgG (Fig. 6Q–T) revealed that astrocytes encircled the IgG clusters in the striatum of GAS-I rats (Figs. 6R and 11C), whereas this pattern was not observed in Control-I rats (Figs. 6T and 11F).

Discussion

Our study evaluated the role of antibodies produced following exposure of rats to GAS in the induction of neuropsychiatric abnormalities. The first part of the study replicated and extended our previous findings on the behavioral and biological alterations fol-
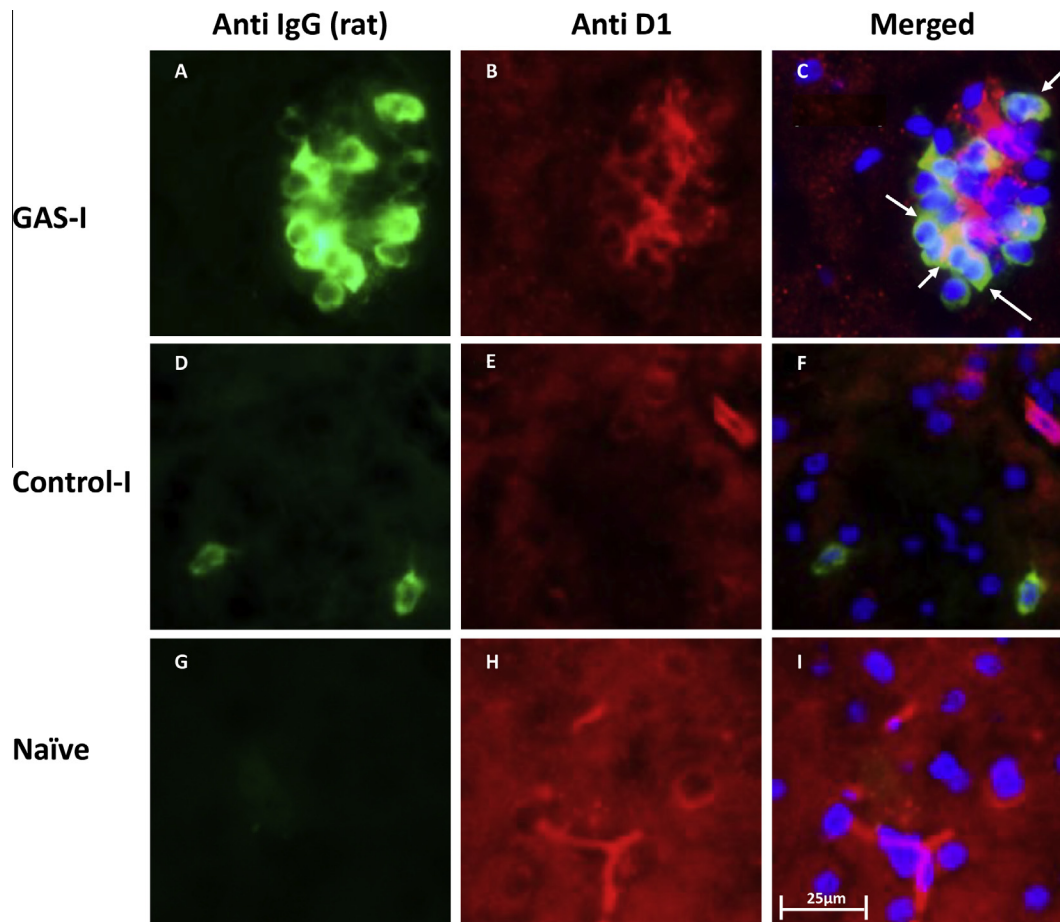
Fig. 8. Representative immunofluorescence images showing IgG deposition in the striatum of a GAS-I rat (A–C), Control-I rat (D–F) and naïve rat (G–I) and double labeling with anti-D1 dopamine receptor antibody. Figures A, D and G display labeling of rat IgG; Figures B, E and H display labeling of the D1 dopamine receptor; Figures C, F and I display colocalization of the rats IgG and the D1 dopamine receptor. Tissue sections were incubated with anti-rat alexa antibody 488 for visualization of IgG deposition; IgG against the D1 dopamine receptor was labeled with anti-rabbit alexa antibody 594. Blue signal indicates nuclear counterstaining (DAPI). 40× microscope objective; scale bar = 25 μm. The arrows point to colocalization between the infused IgG and D1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lowing exposure of rats to GAS extract (Brimberg et al., 2012; Cox et al., 2013). Behaviorally, GAS-exposed rats demonstrated increased grooming in the induced-grooming assay, suggestive of compulsive-like behavior, and were impaired in the food manipulation and beam walking tasks, suggesting impairments in fine motor control and gait, respectively. It should be noted, however, that the increased time it took GAS rats to traverse the narrow but not the wider beams in the beam-walking assay, may reflect increased anxiety of GAS rats rather than an impairment in gait. While we have previously found that GAS rats bury more marbles (Lotan et al., unpublished observations), a behavior which has been suggested to reflect both increased compulsive-like and anxiety-like behavior (for review see, Albelda and Joel, 2012), we did not find a significant difference between GAS and control rats in the proportion of time spent in the open arms of a plus-maze ($p = 0.7$, Brimberg et al., 2012), a commonly used test of anxiety-like behavior. Immunologically, sera from GAS-exposed rats reacted with the D1 and D2 dopamine receptors, as we have previously reported (Brimberg et al., 2012). A novel finding of the present study is that sera from GAS-exposed rats also reacted with the 5HT-2A and 2C serotonin receptors. It should be noted that sera from adjuvant-exposed control rats exhibited background reactivity against the four receptors in the ELISA, but the higher receptor specific reactivity of sera IgG from GAS rats was confirmed by Western blots, where the adjuvant control rat sera was negative and the anti-GAS sera recog-

nized receptor specific bands. The higher background in the control sera in the ELISA but not in the Western blot may be expected for these G protein-coupled receptors, because only in the Western blot are these receptors completely separated from the membrane (Baragli et al., 2007; Renart et al., 1979; Reynolds and Tanford, 1970; Towbin et al., 1979). The higher background in the ELISA may therefore reflect either lower avidity antibodies in the control sera that react with the receptor–membrane complex, or immune complexes from the immunization that may also create nonspecific binding. Our conclusion is supported by the lack of significant differences between reactivity of sera from adjuvant-exposed rats and naïve rats against D1 and D2 dopamine receptors in the ELISA (p's > 0.14, Brimberg et al., unpublished observations) and by the present findings of very low binding of IgG in the striatum of rats that were infused with IgG from adjuvant-exposed rats compared with the IgG binding in the striatum of rats that were infused with IgG from GAS-exposed rats.

In the second part of our study, antibodies purified from GAS-exposed rats and infused into the striatum of naïve rats (GAS-I) were found to replicate some of the behavioral effects of exposure to GAS. Specifically, on the last session of testing, GAS-I rats were impaired in traversing a narrow, but not a wide, beam, compared to naïve rats and to rats infused with antibodies purified from adjuvant-treated control rats (Control-I). The specificity of the impairment to the narrow beam, seen also in GAS-exposed rats
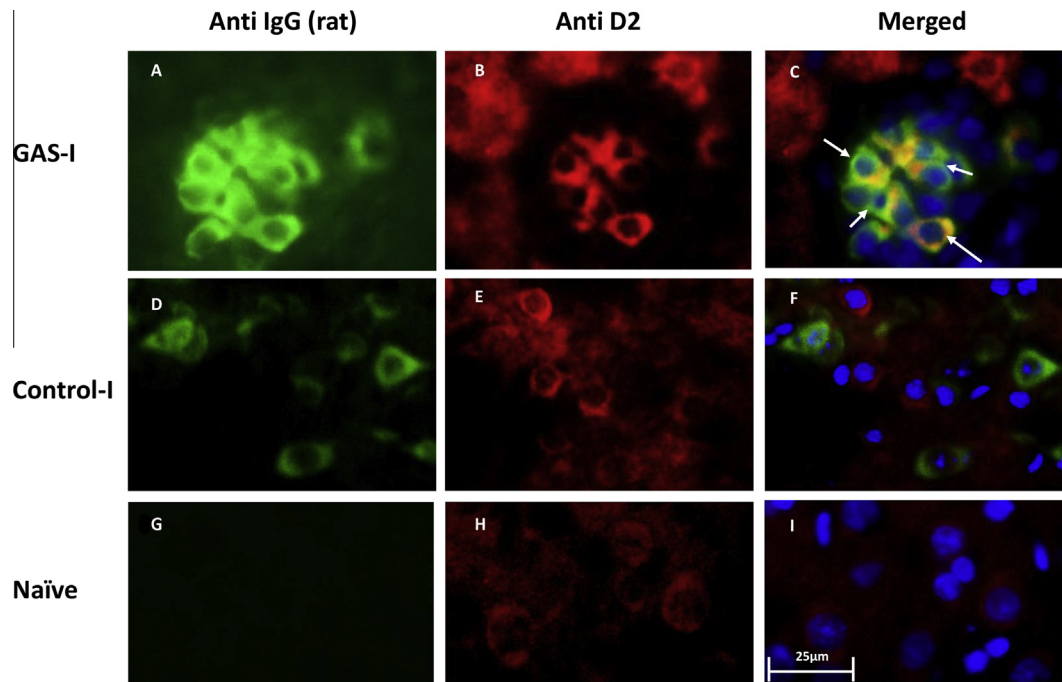
Fig. 9. Representative immunofluorescence images showing IgG deposition in the striatum of a GAS-I rat (A–C), Control-I rat (D–F) and naïve rat (G–I) and double labeling with anti-D2 dopamine receptor antibody. Figures A, D and G display labeling of rat IgG; Figures B, E and H display labeling of the D2 dopamine receptor; Figures C, F and I display colocalization of the rats IgG and the D2 dopamine receptor. Tissue sections were incubated with anti-rat alexa antibody 488 for visualization of IgG deposition; IgG against the D2 dopamine receptor was labeled with anti-rabbit alexa antibody 594. Blue signal indicates nuclear counterstaining (DAPI). 40× microscope objective; scale bar = 25 μm. The arrows point to colocalization between the infused IgG and D2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
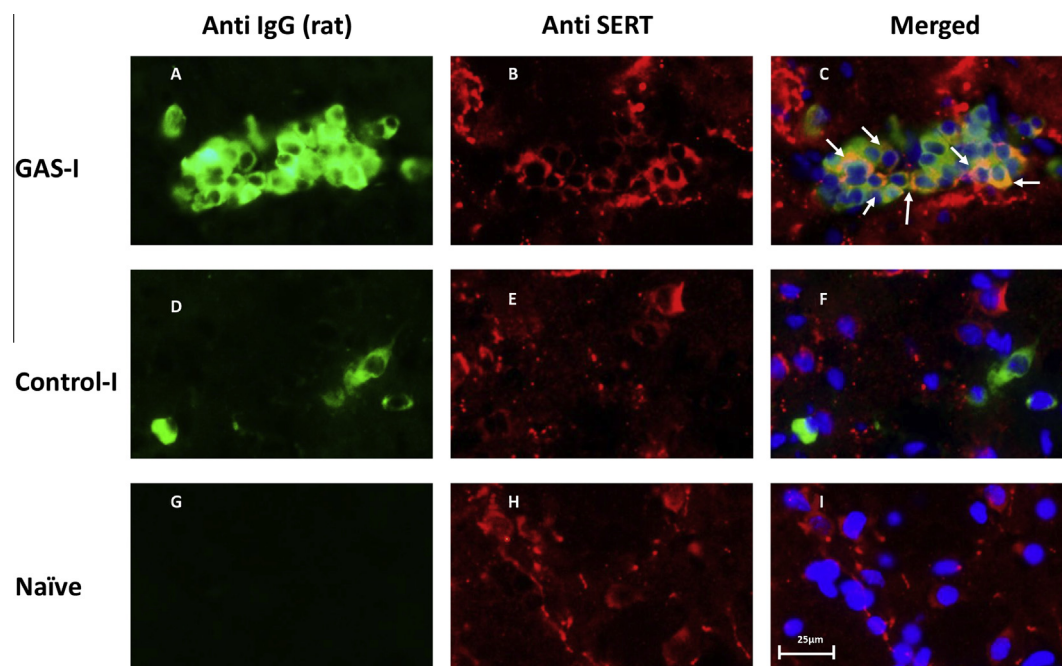

Fig. 10. Representative immunofluorescence images showing IgG deposition in the striatum of a GAS-I rat (A–C), Control-I rat (D–F) and naïve rat (G–I) and double labeling with anti-serotonin transporter (SERT) antibody. Figures A, D and G display labeling of rat IgG; Figures B, E and H display labeling of SERT; Figures C, F and I display colocalization of the rats IgG and SERT. Tissue sections were incubated with anti-rat alexa antibody 488 for visualization of IgG deposition; IgG against the SERT was labeled with anti-rabbit alexa antibody 594. Blue signal indicates nuclear counterstaining (DAPI). 40× microscope objective; scale bar = 25 μm. The arrows point to colocalization between the infused IgG and SERT. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in the present and previous (Brimberg et al., 2012) studies, suggests that the effect of anti-GAS antibodies was specific to gait, while sparing motivation and gross motor control. However, as detailed above, the increased time to traverse the narrow beam may

have reflected increased anxiety of GAS-I rats rather than a motor impairment. Because the difference between the GAS-I and the two control groups was due to the shortening of the time to traverse the narrow beam in the latter groups, it is possible that the differ-
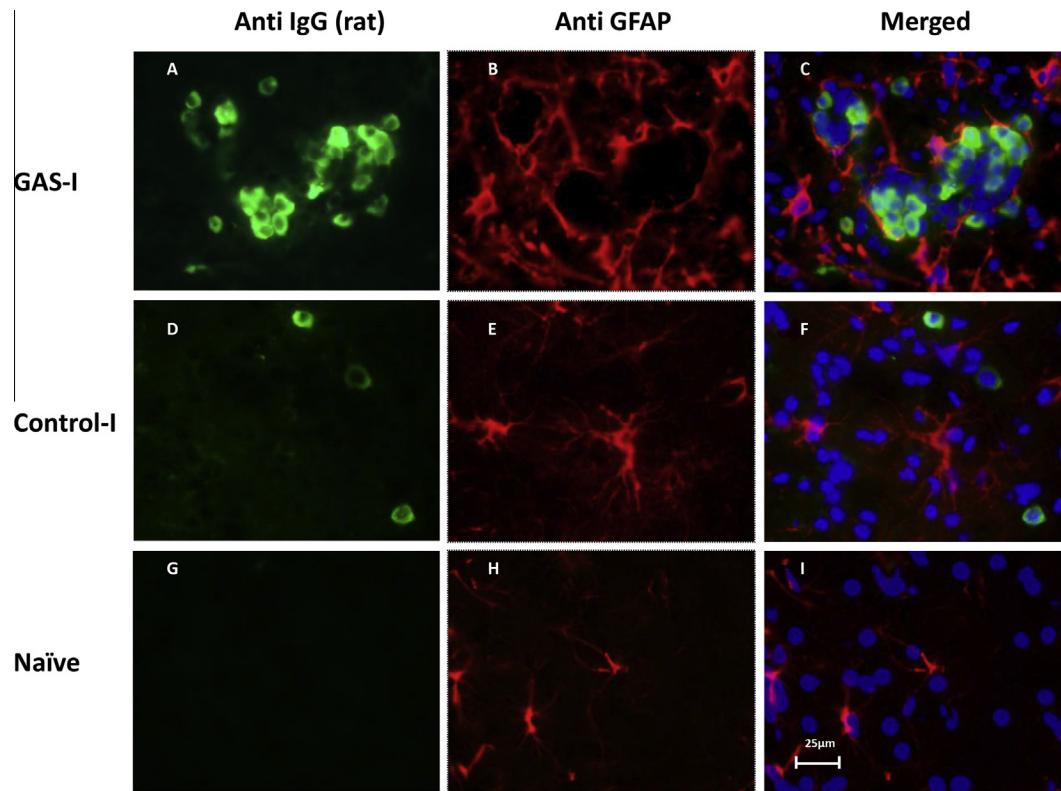
Fig. 11. Representative immunofluorescence images showing IgG deposition in the striatum of a GAS-I rat (A–C), Control-I rat (D–F) and naïve rat (G–I) and double labeling with a marker for astrocytes (GFAP). Figures A, D and G display labeling of rat IgG; Figures B, E and H display GFAP labeling; Figures C, F and I display colocalization of the rats IgG and GFAP. Figures Tissue sections were incubated with anti-rat alexa antibody 488 for visualization of IgG deposition; IgG against GFAP was labeled with anti-rabbit alexa antibody 594. Blue signal indicates nuclear counterstaining (DAPI). 40× microscope objective; scale bar = 25 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ence reflects impaired motor learning of GAS-I rats. Alternatively, this difference may reflect the gradual development of behavioral alteration in GAS-I rats following the accumulation of antibodies, which counteracted the effects of training. A selective and specific effect of IgG infusion was also seen in the marble burying assay. GAS-I rats buried more marbles compared to Control-I and naïve rats, and this increased burying could not be attributed to a non-specific increase in activity level, because the latter was not significantly different between the three groups (in fact, the two infused groups tended to be less active than the naïve group). As we have recently found increased marble burying also in GAS-exposed rats (Lotan et al., in preparation), these results suggest that anti-GAS antibodies caused a specific increase in marble burying. GAS-I rats did not show, however, increased grooming in the induced-grooming assay, in contrast to GAS-exposed rats in the present and previous (Brimberg et al., 2012) studies. In addition, although GAS-I rats were impaired in manipulating food compared to naïve rats, so were Control-I rats, suggesting a non-specific effect of the intra-striatal infusion in this task. One of the limitations of our study is that antibodies were passively transferred intra-striatally which in itself may have led to some non-specific effects due either to mechanical disruption of striatal functioning following infusion of fluids, or an effect of the infusion of IgG, as Control-I rats were also infused with antibodies (against the adjuvant mycobacteria injected into the rats from which sera were collected). Taken together, our new evidence demonstrates that intra-striatal infusion of antibodies (IgG) purified from GAS-exposed rats mimics part of the behavioral syndrome that is induced by exposing rats to GAS antigen, and that some of these behavioral effects are specific to anti-GAS antibodies.

Our results are in line with previous studies, which reported the emergence of stereotypic behaviors and motor abnormalities following infusion of either sera or IgG purified from sera of SC, PANDAS or TS patients to the striatum of naïve rats (Doyle et al., 2012; Hallett et al., 2000; Taylor et al., 2002). Yet, for reasons currently unknown, other studies that used a similar approach failed to induce behavioral alterations (Ben-Pazi et al., 2012; Loiselle et al., 2004; Singer et al., 2005). See (Doyle et al., 2012; Singer et al., 2005) for a discussion of possible causes for the inconsistent results in these studies. Our results are also in line with the finding that systemic passive transfer of anti-streptococcal sera from GAS-exposed mice to naïve mice (concomitant with Lipopolysaccharides (LPS) administration to break the blood brain barrier) resulted in the development of motor and behavioral disturbances similar to those seen in the GAS-exposed mice (Yaddanapudi et al., 2010). Taken together with our present results, these collective studies support a role for antibodies in the induction of behavioral alterations.

Our results further suggest a role for antibodies that target proteins involved in the functioning of the dopaminergic and serotonergic systems. Specifically, we have found anti-D1 and D2 dopamine receptor antibodies, as well as anti-5HT-2A and 2C serotonin receptor antibodies in the sera of GAS-exposed rats. Several lines of evidence point to the involvement of these neurotransmitter systems in the pathophysiology of GAS-related neuropsychiatric disorders. Dysfunction of the dopaminergic system has been implicated in motor disturbances (e.g., Parkinson's disease, Huntington's disease, and Alzheimer's disease (Jahanshahi et al., 2013; Michel et al., 2013; Reeves et al., 2010) as well as in OCD (Denys et al., 2004; Hesse et al., 2005; Nikolaus et al., 2010; Olver et al., 2009), and dysfunction of the serotonergic system has been

implicated in OCD and anxiety disorders (Flaisher-Grinberg et al., 2008; Hesse et al., 2005, 2011; Nikolaus et al., 2010; Perani et al., 2008). Dopaminergic and serotonergic drugs are used to treat motor and psychiatric symptoms, respectively, in patients affected by GAS-related neuropsychiatric disorders (Demiroren et al., 2007; Gabbay and Coffey, 2003; Moretti et al., 2008; Murphy et al., 2010; Swedo and Grant, 2005; Swedo et al., 1993). Finally, immunoreactivity to different components of the serotonergic system, such as serotonin and serotonin receptors, has been reported in sera from panic disorder and rheumatoid arthritis patients (Coplan et al., 1999; Maes et al., 2013; Schott et al., 2003; Tanaka et al., 2003), and sera from SC and PANDAS patients reacted with D1 and D2 receptors (Brimberg et al., 2012; Cox et al., 2013; Dale et al., 2012). Interestingly, Dale et al. (2012) have recently found antibodies against surface D2 receptors in the sera of patients suffering from basal ganglia encephalitis, which is characterized by motor (including, parkinsonism, dystonia and chorea), and psychiatric symptoms (including, emotional lability, attention deficit and psychosis), which are also found in GAS-related neuropsychiatric disorders.

The pattern of IgG deposition in the striatum of GAS-I rats also supports the hypothesis that antibodies that target the dopaminergic and the serotonergic systems play a functional role in the induction of behavioral alterations. Specifically, IgG purified from GAS-exposed rats and infused into the striatum of naïve rats were colocalized with neurons, with cells expressing D1 dopamine receptors, with cells expressing D2 dopamine receptors and with cells expressing the serotonin transporter. Astrocytes encircled clusters of neurons containing IgG.

Colocalization of IgG with neurons in the striatum is in accordance with previous studies showing that antibodies found in sera of GAS-related neuropsychiatric disorders, such as SC, PANDAS, OCD, and TS, reacted with basal-ganglia neurons (Church et al., 2002; Cox et al., 2013; Gause et al., 2009; Husby et al., 1976; Kiessling et al., 1993; Kotby et al., 1998; Martino et al., 2011; Singer et al., 1998). There is less data regarding a possible role of astrocytes in GAS-related neuropsychiatric disorders. Kingston and Glynn (1971) found a cross-reaction of sera taken from rabbits previously exposed to GAS with astrocytes. It is possible that astrocytes encircled the antibody neuronal cell cluster in order to delimit their effect or remove them from the brain tissue.

Our results suggest a potential pathogenic role of autoantibodies produced following exposure to GAS in the induction of behavioral alterations, reminiscent of the outcomes of exposure to GAS in both patients and rats, thus establishing the fifth condition required to establish a disorder as an antibody-mediated autoimmune disorder. Our evidence illustrates induction of symptoms by intra-striatal passive transfer of IgG to animals. Additional studies are required in order to assess the exact mechanisms by which the autoantibodies produced in GAS-related neuropsychiatric disorders are pathogenic.

Financial disclosures

Acknowledgments

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