

## ORIGINAL ARTICLE

# Passive transfer of streptococcus-induced antibodies reproduces behavioral disturbances in a mouse model of pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection

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**Streptococcal infections can induce obsessive-compulsive and tic disorders. In children, this syndrome, frequently associated with disturbances in attention, learning and mood, has been designated pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection (PANDAS). Autoantibodies recognizing central nervous system (CNS) epitopes are found in sera of most PANDAS subjects, but may not be unique to this neuropsychiatric subset. In support of a humoral immune mechanism, clinical improvement often follows plasmapheresis or intravenous immunoglobulin. We recently described a PANDAS mouse model wherein repetitive behaviors correlate with peripheral anti-CNS antibodies and immune deposits in brain following streptococcal immunization. These antibodies are directed against group A  $\beta$ -hemolytic streptococcus matrix (M) protein and cross-react with molecular targets complement C4 protein and  $\alpha$ -2-macroglobulin in brain. Here we show additional deficits in motor coordination, learning/memory and social interaction in PANDAS mice, replicating more complex aspects of human disease. Furthermore, we demonstrate for the first time that humoral immunity is necessary and sufficient to induce the syndrome through experiments wherein naive mice are transfused with immunoglobulin G (IgG) from PANDAS mice. Depletion of IgG from donor sera abrogates behavior changes. These functional disturbances link to the autoimmunity-related IgG1 subclass but are not attributable to differences in cytokine profiles. The mode of disrupting blood–brain barrier integrity differentially affects the ultimate CNS distribution of these antibodies and is shown to be an additional important determinant of neuropsychiatric outcomes. This work provides insights into PANDAS pathogenesis and may lead to new strategies for identification and treatment of children at risk for autoimmune brain disorders. *Molecular Psychiatry* (2010) 15, 712–726; doi:10.1038/mp.2009.77; published online 11 August 2009**

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## Introduction

Infectious and immune factors are broadly implicated in the pathogenesis of childhood neuropsychiatric conditions, including Sydenham's chorea (SC), Tourette's syndrome (TS), obsessive-compulsive disorder (OCD), attention-deficit/hyperactivity disorder (AD/HD) and autism spectrum disorders (ASD).<sup>1–5</sup> Infection with group A  $\beta$ -hemolytic streptococcus (GABHS) is highlighted as a specific trigger in SC, where the characteristic movement disorder and

variable elements of attentional and emotional instability develop in parallel with GABHS-induced autoimmune responses directed against central nervous system (CNS) components.<sup>6,7</sup>

The acronym PANDAS (pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection) is used to describe a subset of children with OCD or chronic tic disorder after GABHS infection.<sup>8,9</sup> PANDAS diagnostic criteria require presence of a lifetime diagnosis of OCD or a tic disorder;<sup>10</sup> in addition, other clinical features are suggested as part of a broader PANDAS classification, including cognitive,<sup>9</sup> attentional,<sup>11</sup> social,<sup>12</sup> eating<sup>13</sup> and mood disturbances.<sup>3,9</sup> A role for GABHS-induced autoimmunity has also been proposed in AD/HD,<sup>11,14</sup> anorexia nervosa,<sup>15,16</sup> major depression<sup>17</sup> and ASD;<sup>18,19</sup> these conditions are also frequently noted in PANDAS populations as comorbid neuropsychiatric disorders.<sup>9</sup>

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Antibodies to basal ganglia are found in SC and PANDAS<sup>4,20–23</sup> and may extend beyond the basal ganglia to include cerebellum and cerebral cortex.<sup>24</sup> However, given that antibodies have also been reported in basal ganglia of healthy individuals,<sup>20</sup> their function in disease is uncertain. Monoclonal antibodies to *N*-acetyl- $\beta$ -D-glucosamine, the dominant epitope of GABHS carbohydrate, and lysoganglioside GM1, a neuronal cell-surface molecule, have been cloned from children with SC. *In vitro*, these antibodies induced the activity of calcium/calmodulin-dependent protein kinase II, a protein involved in learning and behavior.<sup>25</sup> Children with PANDAS as well as classical GABHS-related autoimmune neuropsychiatric disorders such as SC frequently respond to plasma exchange, intravenous immunoglobulin (IVIg)<sup>26</sup> or prophylactic antibiotics.<sup>27</sup>

We previously reported behavioral abnormalities reminiscent of those reported in PANDAS, and antibodies directed against streptococcus M protein in peripheral blood and brain, in autoimmune disease-susceptible mice following immunization with GABHS.<sup>28</sup> Here we extend work in this model, examining whether peripheral anti-CNS antibodies are sufficient to reproduce the syndrome, whether the effect is ablated by depleting immunoglobulin G (IgG) before transfer into naive mice and if additional neurobiologic domains implicated in PANDAS and PANDAS variants may also be compromised, including motor coordination, spatial and olfactory learning and memory, and social interaction.

## Materials and methods

### Animals

Mice were housed at  $24 \pm 1$  °C with 12:12 light/dark cycle commencing at 0600 hours in standard polycarbonate cages with wood chip bedding and wire tops containing food and water (*ad libitum* except where noted); an additional filter top covered each cage. Group housing was used until later phases of behavioral testing: 7 days before resident-intruder testing, mice were moved to individual housing; individual housing was maintained through the remainder of behavioral testing (olfactory discrimination and reversal spatial learning and memory testing). All animals were handled in accordance with Association for Assessment and Accreditation of Laboratory Animal Care International guidelines with the approval of the Institutional Animal Care and Use Committee at Columbia University. Male and female SJL/J mice were used in all experiments (The Jackson Laboratory, Bar Harbor, ME, USA).

### Direct immunization of donor mice

One set of SJL/J mice was directly immunized with either GABHS or phosphate-buffered saline (PBS). Primary immunization was performed when the mice were 4 weeks (GABHS,  $n = 44$ ; PBS,  $n = 38$ ) or 6 weeks (GABHS,  $n = 33$ ; PBS,  $n = 35$ ) of age. Inactivated GABHS homogenate was prepared for use as immu-

nogen from *Streptococcus pyogenes*, group A type 6 bacteria<sup>28</sup> (12348; American Type Culture Collection, Manassas, VA, USA). Homogenates were derived from purified, lysed supernatants of bacteria grown on blood agar plates. GABHS homogenates were stored at  $-70$  °C and re-incubated on blood agar plates to verify the absence of viable bacteria before injections. Each mouse in the GABHS group was immunized subcutaneously with  $\sim 125$   $\mu$ l of 1:1 emulsion of complete Freund's adjuvant (CFA; Sigma-Aldrich, St Louis, MO, USA)/PBS containing 2.5  $\mu$ l of GABHS homogenate. Control mice were immunized with  $\sim 125$   $\mu$ l of CFA/PBS alone. Mice were then boosted three times at 3-week intervals (boost 1, age 7 or 9 weeks; boost 2, 10 or 12 weeks; boost 3, 13 or 15 weeks). Boosts consisted of  $\sim 125$   $\mu$ l of 1:1 emulsion of incomplete Freund's adjuvant (IFA, Sigma-Aldrich)/PBS containing 2.5  $\mu$ l of GABHS homogenate (GABHS donor mice) or  $\sim 125$   $\mu$ l of IFA/PBS alone (PBS donor mice).

### Preparation of pooled GABHS and PBS donor sera and IgG-depleted GABHS and PBS donor serum pools

Individual serum samples were collected at time of sacrifice from male donor mice 2 weeks after the third boost. A total of 24 GABHS sera were combined to prepare a GABHS donor serum pool; 20 individual PBS sera were used for a PBS donor serum pool. Pooled GABHS or PBS donor sera were passed through a Protein G column (GE Healthcare Biosciences Corporation, Piscataway, NJ, USA) to deplete IgG. Excess sera from individual samples, if available, were retained for later analyses.

### Confirmation of IgG depletion and determination of serum Ig subclasses

The efficiency of IgG depletion was confirmed for all IgG subclasses using the Beadlyte multiplex mouse Ig isotyping kit (Upstate Biotechnology, Lake Placid, NY, USA; Supplementary Figure S1). Individual serum samples from 17 of the GABHS and 17 of the PBS donor mice contributing to their respective donor pools were analyzed. Volume limitations precluded individual analysis of seven GABHS and three PBS donor samples. IgG subclass analysis was repeated on GABHS and PBS sera pools. The lyophilized multi-Ig standard was resuspended in isotyping serum diluent and was serially diluted. Standards or serum samples were incubated with the multi-Ig capture bead suspension array in a 96-well filter plate for 2 h at room temperature. Beads were washed and incubated with phycoerythrin-conjugated anti-mouse  $\kappa$ -light chain reporter for 15 min, and washed and resuspended in assay buffer. The median fluorescence intensity of 100 beads per Ig subclass was read using a Luminex 100 instrument (Luminex Corporation, Austin, TX, USA). Concentrations of Ig subclasses in serum samples were interpolated from standard curves.

#### *Analysis of Th1 and Th2 cytokines in nondepleted and IgG-depleted GABHS and PBS donor serum pools*

Serum levels in nondepleted and IgG-depleted GABHS and PBS donor serum pools of the cytokines interferon- $\gamma$  (IFN- $\gamma$ ), interleukin (IL)-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-13 and IL-17, and of the chemokines IL-8, MCP-1, IP-10, MIP-1 $\beta$  were analyzed in duplicate using a multiplexed, bead-based cytokine immunoassay (Upstate Beadlyte mouse multi-cytokine/chemokine kit; Millipore, St Charles, MO, USA) and the Luminex 100 detection system (Luminex Corporation), according to the manufacturer's protocol (1:2 dilution in assay kit serum diluent). Concentrations of cytokines and chemokines were interpolated from serial standard curves. Th1/Th2 cytokine ratios were estimated on the basis of IFN- $\gamma$ /IL-4 ratios (serum concentration of IFN- $\gamma$  divided by serum concentration of IL-4).

#### *Passive transfer mice*

Naive 6-week-old male SJL/J mice received through tail vein 100  $\mu$ l of four types of pooled sera, forming the following four groups: (1) GABHS donor sera recipients (GABHS-R mice,  $n=9$ ); (2) PBS donor sera recipients (PBS-R mice,  $n=6$ ); (3) IgG-depleted GABHS donor sera (IgG-depleted GABHS-R mice,  $n=7$ ) and (4) IgG-depleted PBS donor sera (IgG-depleted PBS-R mice,  $n=7$ ). Lipopolysaccharide (LPS, isolated from *Escherichia coli*, strain 055:B5,  $\gamma$ -irradiated and cell culture tested; Sigma-Aldrich) was administered intraperitoneally 15 min (3 mg kg<sup>-1</sup> in 0.2 ml of lactated Ringer's solution) and 48 h (1.5 mg kg<sup>-1</sup> in 0.2 ml lactated Ringer's solution) after tail vein injection of nondepleted or IgG-depleted GABHS or PBS donor sera to increase blood-brain barrier (BBB) permeability transiently.<sup>29-34</sup> Previous pilot work injecting LPS alone established that this dosing schedule was associated with a return to baseline body temperature and weight by 24 h after the second LPS dose, at which time no further LPS-associated sickness behavior was observed.

#### *Behavioral testing*

**Rotarod.** Motor ability and coordination were assessed in donor mice at baseline (postnatal week 4 or 6) and 3 weeks after the first immunization (week 7 for groups first immunized at postnatal week 4; week 9 for groups first immunized at week 6). Passive transfer (nondepleted or IgG-depleted GABHS-R or PBS-R recipient) mice were tested 4 days after injection of either nondepleted or IgG-depleted donor sera. Rotarod performance was tested using an accelerated rotarod apparatus (SmartRod Rotating Rod apparatus; AccuScan Instruments Inc., Columbus, OH, USA) in two sequential, 3-min tests. Mice were first tested on the stationary bar for 3 min; they were then tested on a rotating rod that slowly accelerated over 2 min from 0 to 20 revolutions per minute (r.p.m.), and then decelerated over 1 min to 0 r.p.m. Time spent on the rod under the stationary and rotating conditions, and the speed at which the

animal fell off the rotating rod, was automatically recorded (SmartRod software, AccuScan Instruments, Inc., Columbus, OH, USA).

**Open-field locomotor activity.** An automated system was used to quantitate locomotor activity and repetitive behaviors in a 90-min test (three 30-min periods) in an open-field testing arena (Coulbourn Instruments, Allentown, PA, USA). Locomotor activity was assessed in donor mice at baseline (postnatal week 4 or 6) and at the first post-immunization time point (postnatal week 7 or 9). To account for baseline variability across individual animals in locomotor activity, we normalized post-immunization data (postnatal week 7 or 9) by dividing the number of post-immunization vertical stereotypy moves (rearing) at postnatal week 7 or 9 by the number of baseline (week 4 or 6) vertical stereotypy moves. Passive transfer mice were tested 4 days after receiving either nondepleted or IgG-depleted donor sera (nondepleted or IgG-depleted GABHS-R or PBS-R recipient mice).

**Resident-intruder (territoriality) test.** Aggressive, exploratory and social behaviors were examined in male GABHS and PBS donors using a 10-min resident-intruder test at 17 weeks of age. Passive transfer recipient mice were tested 6 days after injection of either nondepleted or IgG-depleted GABHS or PBS donor sera (nondepleted or IgG-depleted GABHS-R or PBS-R recipient mice). Pairs of resident and intruder mice from different litters were matched for age. All mice were singly housed for at least 7 days before testing. Two hours before testing began, wire tops containing food and water were removed from resident mouse cages, filter top cage lids were repositioned on top of each cage, and cages were placed on a countertop under a video camera in the experimental room. An intruder was then introduced into the cage of the resident for 10 min. An observer masked to the experimental status of the mice manually scored each videotaped test session for the occurrence of agonistic, defensive, exploratory or self-maintenance behaviors within each of four 2.5-min time bins for mice in the resident role: (1) cage exploration (exploratory sniffing, rearing); (2) social investigation of partner (social approach, following, walking around/circling/standing at the side, investigating with the nose, anogenital sniffing, light grooming); (3) defensive/escape (defensive sideways posture, defensive upright posture, crouching, escaping, evading, jumping away from partner) and (4) submission (enduring aggressive behavior such as biting or aggressive grooming).<sup>35</sup> Social confrontation by an unfamiliar male mouse intruding into the home cage of an experimental male mouse typically elicits offensive, aggressive, territorial behaviors from the resident mouse, including approaching and pursuing the intruder, anogenital sniffing and a threaten/attack sequence that forces the intruder into submission.

Intruder mice typically exhibit a variety of defensive/submissive body postures and escape responses.

**Forced-choice olfactory habituation–discrimination test.** Olfactory function was assessed in GABHS and PBS donor animals at 19 weeks of age using a forced-choice, olfactory habituation–discrimination paradigm consisting of four 2-min acquisition/habituation trials followed by one 2-min discrimination (probe) trial. This task was a modification of the paradigm described in Nicot *et al.*,<sup>36</sup> closely paralleling the forced-choice, two-alternative procedure described by Gheusi *et al.*<sup>37</sup> This task assesses the degree to which mice spontaneously habituate to olfactory stimuli by exposing them first to one novel odor along with a neutral, control odor over four trials, and then measuring the magnitude of their response to (discrimination of) a second novel odor, also co-presented with the control odor, in a fifth discrimination (probe) trial. Presentation of a novel odor typically elicits substantial investigation of the new stimulus in control mice; as its novelty recedes during subsequent reexposures to the odor, responses diminish in animals with normal learning and memory capacity. Upon subsequent exposure to a second novel odor in animals that have already habituated to the first novel odor, animals capable of discriminating the second novel odor from the first odor typically again increase their investigation time.

In the forced-choice paradigm, two tubes are placed at randomly assigned, opposite corners of the home cage without disturbing the animal: one tube contains the novel odor (pure vanilla extract for the four habituation trials; pure lemon extract for the discrimination trial; both from McCormick, Sparks, MD, USA); the other tube contains the control odor (distilled water for both the acquisition/habituation and the discrimination trials). Novel odors are diluted 1:10 in distilled water and presented on Whatman 1 filter paper inserted into the base of a 15 ml tube (100  $\mu$ l of diluted odor stimulus). Control tubes consist of 100  $\mu$ l of distilled water on filter paper. Four 2-min presentations of both the first novel odor tube and the control odor tube occur in succession, followed by the fifth 2-min novel odor trial (discrimination probe trial) in which the second novel odor (lemon extract) is presented along with the control odor (distilled water) tube to assess discrimination and specificity. Sessions were videotaped and evaluated by a rater masked to the experimental status of the mice. The amount of time mice spent actively investigating each novel or control odor was measured for each of the five, consecutive 2-min trials. Data were represented as the time (in seconds) spent investigating the novel odor normalized to the number of seconds each mouse spent investigating the control odor within each individual 2-min trial.

**Reversal spatial learning and memory.** Working and procedural memory capacity were tested in donor

mice using a simplified, reversal spatial learning paradigm (hole-board memory task with acquisition phase followed by a single reversal probe trial) to assess the role of caudate, cerebellum and hippocampus in ‘procedural’ as well as ‘place’ learning.<sup>38,39</sup> Animals with lesions of caudate<sup>39</sup> or cerebellum<sup>40</sup> show faulty response or ‘procedural’ learning on this type of task; evidence of an intact ‘spatial strategy’ on this task suggests hippocampal integrity.<sup>41</sup>

To enhance motivation, partial food restriction was initiated at week 19 and continued until body weight decreased to 95% of baseline. Testing occurred in a Plexiglas, photobeam-equipped activity-monitoring chamber equipped with ‘nose poke’ floor with holes arranged in a 4  $\times$  4 array (Coulbourn Instruments). Extra-maze cues in fixed positions were arranged to be clearly visible. Fruit Loops cereal pieces (Kellogg’s, Battle Creek, MI, USA) were used as food reward. A single, 20-min habituation session, in which mice were allowed to explore the maze and consume pieces of food randomly scattered around the maze, occurred the day before (day 0) initiation of the 4-day acquisition (training) phase.

During the acquisition phase, consisting of four 3-min trials on each of the 4 training days, a single cereal piece was hidden in the same hole location within the nose poke array. For each 3-min trial, the mouse was placed facing in the same starting direction and allowed to search for 3 min for the food reward (located in the same spatial location on each trial). Freshly powdered cereal, stored in a tightly sealed container, was spread across the entire maze floor so that animals could not simply locate food by smell, and the hole board was thoroughly cleaned between every trial to reduce traces of animal odors or residual food that might serve as positional cues. Retention of the spatial location used in the acquisition phase is tested on day 5, one day after training concludes, in a single, 3-min reversal ‘probe’ trial, using the starting direction opposite to that used during training/acquisition.

In the reversal probe trial, mice turning in the physical direction that led to the location of food during training were designated ‘place learners’ (suggesting intact hippocampal function<sup>39,41</sup>) and mice using the same turning response as that required during training (that is, now leading away from the physical location of the food, as in the acquisition trials mice start from the direction opposite to that used in the probe trial) were designated ‘response learners’ (suggesting intact caudate<sup>39</sup> and/or cerebellar<sup>40</sup> function). Nose poke entries are defined as an entry (‘nose poke’) into any hole, baited or unbaited. A novel nose poke entry is defined as an entry into any hole, baited or unbaited, that has not previously been entered during that test session. The number of novel nose poke entries, the number of nose poke entries into baited or unbaited holes, the time in between novel nose poke entries (interresponse time (IRT) interval, a measure of impulsivity, that is, the inability to hold off on a response even when

unnecessary) and the time from the start of the session to the baited hole (latency to baited hole) were measured. Task errors were defined as the total number of entries into unbaited holes. Working memory ratio was defined as the number of novel nose poke entries into the baited hole (0 or 1, as above, as only the first entry into the baited hole can be considered to be novel; animals failing to enter the baited hole in that trial were scored as 0) divided by the number of nose poke reentries into the baited hole plus the number of novel entries into the baited hole (the number of novel entries was again limited to either 0 or 1 in this simple, single-baited hole task). Reference memory ratio was defined as the number of novel entries into the baited hole (as above, limited to either 0 or 1) plus the number of reentries into the baited hole divided by the total number of entries into the baited and unbaited holes (counting both the first, 'novel' entry as well as any subsequent reentries into either a baited or an unbaited hole).

#### *Immunohistology*

**IgG deposits.** To examine whether nondepleted sera harboring anti-GABHS antibodies bind to CNS targets, and whether IgG depletion of such sera ablated CNS binding, immunohistochemical analysis was pursued. Six days after the injection of nondepleted or IgG-depleted sera, brains were obtained from anesthetized recipient mice following perfusion with PBS and 4% buffered paraformaldehyde in 0.1M phosphate buffer. Brains were postfixed overnight at 4 °C and cryoprotected in 30% sucrose/PBS for 36 h at 4 °C. Serial, coronal cryostat sections (14 μm) were collected on slides, permeabilized with 0.1% Triton X-100 for 1 h and blocked overnight in PBS with 10% normal goat serum (Sigma-Aldrich). Sections were incubated for 1 h with Cy3-conjugated goat anti-mouse IgG (1:200; Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA). The sections were dehydrated serially in increasing concentrations of ethanol and mounted with ProLong Gold antifade reagent with 46-diamidino-2-phenyl indole (Invitrogen, Carlsbad, CA, USA). Images were captured with a Zeiss LSM 510 NLO multiphoton confocal microscope and analyzed using Carl Zeiss Confocal Microscope (AIM) software (Carl Zeiss GmbH, Heidelberg, Germany).

#### *Statistical analysis*

StatView version 5.0.1 software (SAS Institute, Cary, NC, USA) was used for all statistical analyses. As no differences in behavioral pattern were observed between GABHS or PBS donor animals first immunized at either 4 or 6 weeks, data were collapsed across these groups for all reported analyses. Group comparisons were carried out using analysis of variance (ANOVA) for normally distributed data or data that became Gaussian when transformed (one-way ANOVA using immunization type as the between-subject independent variable for the primary analyses); Fisher's protected least-significant difference (PLSD) test was used for *post hoc* comparisons.

Mann–Whitney *U*-tests were used for group comparisons requiring nonparametric analytic approaches. Sex effects were also examined (two-way ANOVA including sex as well as immunization type as between-subject independent variables for Gaussian distributions; individual comparisons of sex effects using individual Mann–Whitney *U*-tests); for tests demonstrating sex-restricted effects, analyses were restricted to that sex. For all tests, statistical significance was assumed where  $P < 0.05$ .

## **Results**

### *GABHS donor mice have impaired motor coordination*

Baseline motor coordination did not differ between GABHS and PBS donor groups. Time maintained on the stationary rod after immunization (week 7/9) was also similar (data not shown). GABHS donor mice had diminished ability to remain on the accelerating rod compared to PBS donor mice ( $n = 27–30$ ; time on accelerating rod: Fisher's PLSD,  $P = 0.033$ ; speed of fall off accelerating rod: Fisher's PLSD,  $P = 0.018$ ; Figures 1a and b; Table 1). Sex influenced coordination capacity, but there were no significant interactions between dose group and sex on the accelerating rod (data not shown).

### *Male GABHS donor mice have increased rearing behavior*

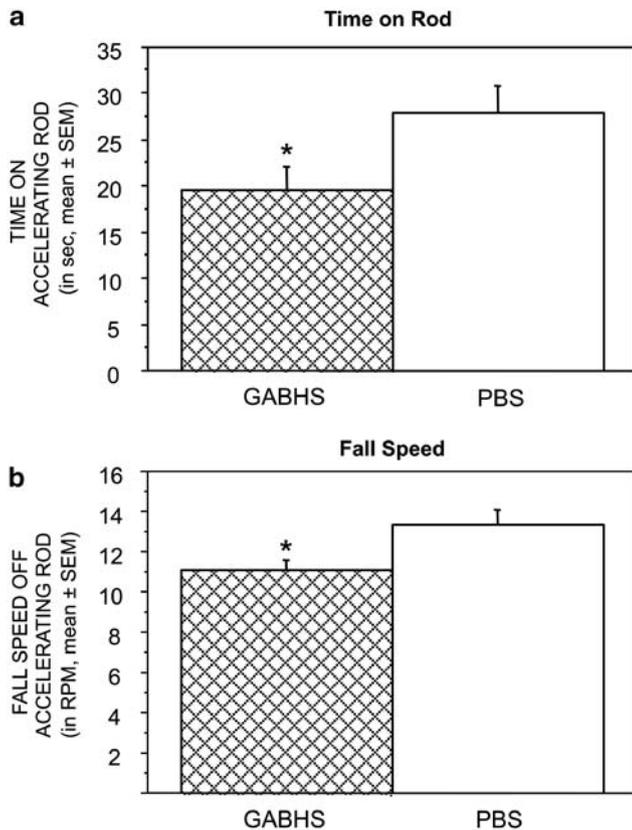
Male GABHS donor mice had increased rearing at the post-immunization time point when compared to male PBS donors ( $n = 6–7$ ; Mann–Whitney *U*,  $P = 0.022$ ; Figure 2). Normalized post-immunization vertical stereotypy moves were similar in female GABHS and PBS donors (data not shown).

### *Male GABHS donor mice behave passively in a resident-intruder task*

Resident GABHS donor mice showed fewer nonsocial activities (exploring the cage environment) and reduced social investigation toward intruder mice than PBS donors showed in the resident role ( $n = 19–20$ ; cage exploration events: Mann–Whitney *U*,  $P = 0.001$ ; social investigation events: Mann–Whitney *U*,  $P = 0.010$ ; Figures 3a and b). GABHS immunization also inhibited aggressive behavior of resident mice toward intruders, as demonstrated by increased submissive and defensive-escape behaviors in resident GABHS mice ( $n = 19–20$ ; submission events: Mann–Whitney *U*,  $P = 0.011$ ; defensive-escape events: Mann–Whitney *U*,  $P = 0.004$ ; Figures 3c and d).

### *GABHS donor mice have deficits in olfactory discrimination*

GABHS and PBS donor mice habituated similarly to odorants presented repetitively during the acquisition phase of the forced-choice olfactory test. Olfactory discrimination was reduced in GABHS mice, with less time spent investigating novel odors in the discrimination trial compared to PBS donors ( $n = 3–5$ ; Fisher's PLSD,  $P = 0.020$ ; Figure 4).



**Figure 1** Impaired motor coordination in group A  $\beta$ -hemolytic streptococcus (GABHS) donor mice. Motor coordination of GABHS and phosphate-buffered saline (PBS) donor mice was assessed using an accelerating rotarod test. The length of time the mouse was able to remain on the rod as it accelerated to 20 r.p.m. (a) and the speed at which mice fell off the accelerating rod (b) were measured. GABHS donor mice remained on the rotating rod for shorter intervals, and fell off the rotating rod at slower rotation speeds than PBS donor mice (Fisher's protected least-significant difference (PLSD); \* $P < 0.05$ ). Error bars indicate s.e.m.

#### Male GABHS donor mice have superior spatial and reversal learning and memory performance

By day 3 of acquisition/training in the hole-board task, working memory ratios and task errors of male PBS donors became relatively stable across the four daily trials. Male GABHS donors, in contrast, had greater working memory ratios and fewer task errors during the first two trials on day 3 of acquisition/training relative to their performance on the latter two trials on that day. Working memory of male GABHS donors was also enhanced during the acquisition phase relative to male PBS donors ( $n = 10-13$ ; Mann-Whitney  $U$ ,  $P = 0.006$ ; Figure 5a). The number of acquisition phase task errors tended to be lower in GABHS donor mice ( $n = 10-13$ ; Mann-Whitney  $U$ ,  $P = 0.075$ ; Figure 5b, Supplementary Figure 2). More impulsive responding was observed in GABHS donor mice, however, with reduced IRT intervals ( $n = 11-13$ ; Mann-Whitney  $U$ ,  $P = 0.008$ ; Figure 5c).

At the reversal task (reversal of the starting position relative to the baited hole in a single probe trial on the fifth day of hole-board spatial learning and memory testing), GABHS donor mice showed superior ability to locate the baited hole despite the change in spatial context. Working and reference memory ratios were higher in GABHS donor mice relative to control donor mice ( $n = 12$ ; working memory ratio, probe trial: Mann-Whitney  $U$ ,  $P = 0.023$ ; Figure 5d; reference memory ratio, probe trial: Mann-Whitney  $U$ ,  $P = 0.033$ ; Figure 5e). The time required to locate the baited hole also tended to be lower in GABHS donor mice relative to PBS donors at the reversal trial ( $n = 12$ ; Mann-Whitney  $U$ ,  $P = 0.052$ ; Figure 5f).

#### IgG1 levels are increased in GABHS serum

The Ig subclass profile was examined in sera from GABHS and PBS mice. GABHS mice had markedly higher total serum IgG1 than PBS mice ( $n = 17$ ; Mann-Whitney  $U$ ,  $P = 0.025$ ; Figure 6); however, IgG2b and IgG3 subclass responses were similar ( $P = \text{NS}$ ; Figure 6).

#### Th1/Th2 cytokine ratios in donor serum pools are unaltered by IgG depletion

To examine the potential influence of cytokines present in the peripheral circulation on CNS function,<sup>42</sup> we compared concentrations of Th1 and Th2 cytokines across donor pools as a function of immunization type (GABHS, PBS) and IgG depletion (nondepleted, depleted). Concentration of the Th1 cytokine, IFN- $\gamma$ , was similar in GABHS and PBS donor serum pools (mean serum IFN- $\gamma$  concentration, in  $\text{pg ml}^{-1}$ ,  $\pm$  s.e.m.: GABHS,  $2.01 \pm 0.23$ ; PBS,  $2.06 \pm 0.67$ ; Mann-Whitney  $U$ ,  $P = \text{NS}$ ). IL-4 serum concentrations (Th2 cytokine) were significantly elevated in GABHS donor pools as compared with PBS pools (mean serum IL-4 concentration, in  $\text{pg ml}^{-1}$   $\pm$  s.e.m.: GABHS,  $0.63 \pm 0.11$ ; PBS,  $0.25 \pm 0.09$ ; Mann-Whitney  $U$ ,  $P = 0.029$ ). IFN- $\gamma$ /IL-4 (Th1/Th2) cytokine ratios were lower in GABHS donor pools (mean serum IFN- $\gamma$ /IL-4 ratio  $\pm$  s.e.m.: GABHS,  $3.28 \pm 0.20$ ; PBS,  $9.51 \pm 1.82$ ; Mann-Whitney  $U$ ,  $P = 0.021$ ). IgG depletion did not substantially alter IFN- $\gamma$ /IL-4 ratios measured in GABHS donor samples (range before depletion, 2.81–3.09; range after depletion, 3.53–3.70) or PBS donor samples (range before depletion, 5.16–10.81; range after depletion, 8.36–13.71).

#### Immunoglobulin deposits are found in brains of naive mice after passive transfer of GABHS donor sera

Six days after passive immunization with nondepleted or IgG-depleted GABHS into tail veins, which was followed by two intraperitoneal LPS injections, 48 h apart, brains of mice receiving sera from GABHS donor mice ( $n = 3$ ) exhibited selective binding of IgG to hippocampal dentate gyrus (DG) granule cells and cells in the periventricular area, at the level of the caudate nucleus (Figures 7a and e). Depletion of the IgG fraction from these GABHS sera ( $n = 3$ ) ablated labeling of DG granule cell neurons and of cells in the

**Table 1** Summary of findings in PANDAS passive transfer mouse model

Parameter	GABHS donor mice <sup>a</sup> (n = 6–32)	Recipients of nondepleted GABHS donor sera <sup>b</sup> (n = 3–9)	Recipients of IgG-depleted GABHS donor sera <sup>c</sup> (n = 3–7)
<i>Rotarod</i>			
Time on rod	↓	—	—
Fall speed	↓	—	—
<i>Open field</i>			
Vertical plane stereotypy moves (rearing)	↑ <sup>d</sup>	↑	↓
<i>Resident–intruder<sup>e</sup></i>			
Exploration	↓	(↓)	(↑)
Social investigation	↓	(↓)	(↑)
Submission	↑	(↑)	(↓)
Defensive-escape	↑	(↑)	(↓)
<i>Olfactory learning</i>			
Habituation	—	ND	ND
Discrimination	↓	ND	ND
<i>Reversal learning and memory</i>			
Acquisition phase (day 3)			
Working memory ratio	↑ <sup>d</sup>	ND	ND
Task errors	↓ <sup>d</sup>	ND	ND
Interresponse time	↓ <sup>d</sup>	ND	ND
Probe trial			
Working memory ratio	↑ <sup>d</sup>	ND	ND
Reference memory ratio	↑ <sup>d</sup>	ND	ND
Latency to first baited hole	↓ <sup>d</sup>	ND	ND
<i>Brain regions with IgG deposits</i>			
Cerebellum	↑	—	—
Striatum	↑	—	—
Hippocampus	—	↑	—
Periventricular area	—	↑	—

Abbreviations: GABHS, group A  $\beta$ -hemolytic streptococcus; IgG, immunoglobulin G; —, no significant differences or trends for that parameter; ND, not determined.

Arrows indicate direction of effect in the first group relative to the indicated comparison group, where significant. Arrows within parentheses indicate direction of nonsignificant trends.

<sup>a</sup>GABHS donor mice, as compared with PBS donor mice.

<sup>b</sup>Nondepleted GABHS donor sera recipient mice, as compared with nondepleted PBS donor sera recipient mice.

<sup>c</sup>IgG-depleted GABHS donor sera recipient mice, as compared with nondepleted GABHS donor sera recipient mice.

<sup>d</sup>Effect observed in males only.

<sup>e</sup>Females not tested.

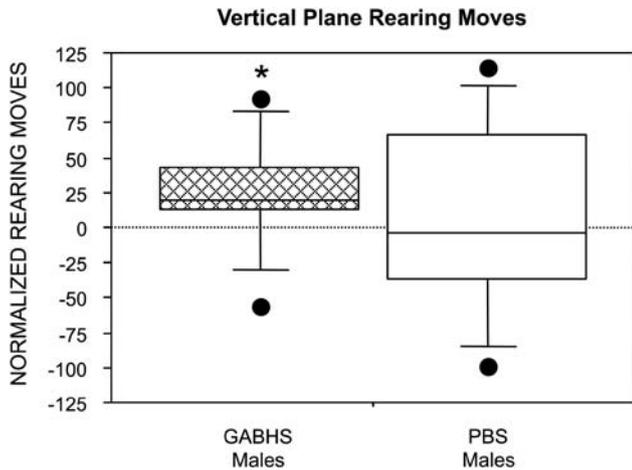
periventricular area in the brains of recipient mice (Figures 7b and f). No labeling was observed after passive transfer of PBS donor sera either without ( $n=3$ ) or with ( $n=3$ ) IgG depletion (Figures 7c, d, g and h). Anti-IgG immunoreactivity did not differ for any passive transfer groups in the cerebellum, striatum or olfactory cortex (data not shown).

*Passive transfer of GABHS donor sera to naive mice reproduces the increased rearing and passive social behavior of donor mice but has no impact on motor coordination*

Repetitive rearing behavior was slightly increased in naive, recipient mice injected with sera from GABHS donor mice as compared to mice injected with PBS

donor sera ( $n=3-5$ ; Mann–Whitney  $U$ ,  $P=0.058$ ; Figure 8). Depletion of IgG from GABHS donor sera before passive transfer blocked the increase in repetitive rearing behaviors observed after passive transfer of nondepleted GABHS donor sera ( $n=5-6$ ; Mann–Whitney  $U$ ,  $P=0.024$ ; Figure 8). No significant differences were observed in rearing behavior of mice receiving nondepleted PBS donor sera as compared with passive transfer recipients of IgG-depleted PBS donor sera (Figure 8).

Resident mice injected with GABHS donor sera showed a trend toward reduced social investigation and increased defensive-escape behaviors relative to resident mice receiving nondepleted PBS donor sera ( $n=8-9$ ; social investigation events: Mann–Whitney



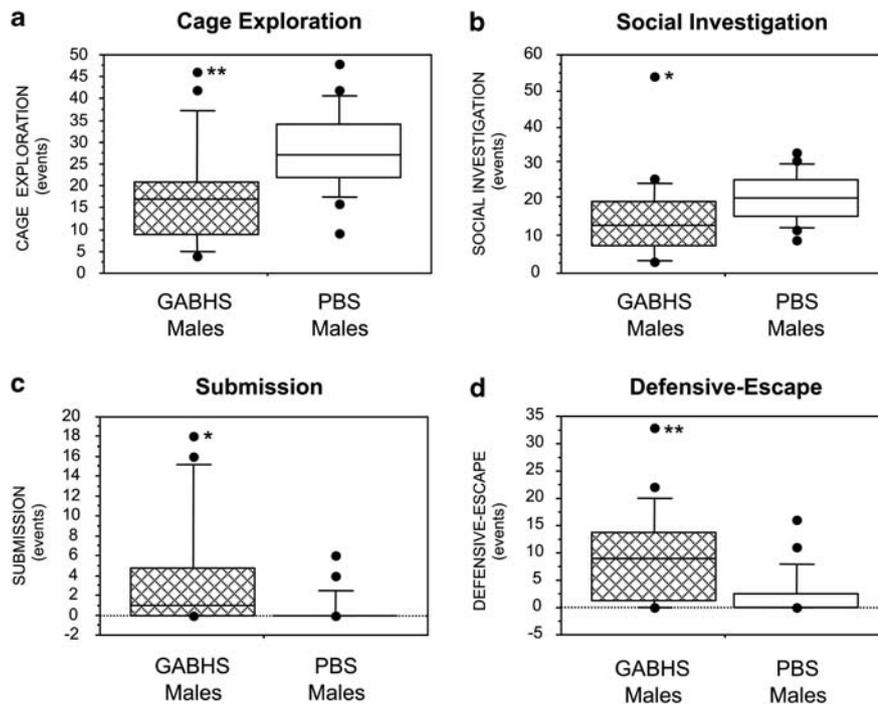
**Figure 2** Increased repetitive rearing behaviors in male group A  $\beta$ -hemolytic streptococcus (GABHS) donor mice. Repetitive motor behaviors of GABHS and phosphate-buffered saline (PBS) mice were measured in an open-field paradigm. Data are presented as the number of vertical plane stereotypy moves (rearing) exhibited by mice at the first post-immunization time point, normalized to the number of vertical plane stereotypy moves observed at preimmunization baseline. Normalized rearing moves were increased in male GABHS donor mice as compared to male PBS mice (Mann–Whitney  $U$ ;  $*P < 0.05$ ). Height of box plot shows interquartile range; horizontal line, median; error bars, range; circles, outliers.

$U$ ,  $P = 0.066$ ; Figure 9a;  $n = 6-9$ ; defensive-escape events: Mann–Whitney  $U$ ,  $P = 0.108$ ; Figure 9b). Depletion of IgG from GABHS donor sera before injection tended to normalize social investigation ( $n = 7-9$ ; Mann–Whitney  $U$ ,  $P = 0.079$ ; Figure 9a) toward levels observed in PBS sera recipients (non-depleted and IgG-depleted PBS-R mice). A similar trend was found with defensive-escape behaviors on IgG depletion ( $n = 7-9$ ; Mann–Whitney  $U$ ,  $P = 0.39$ ; Figure 9b).

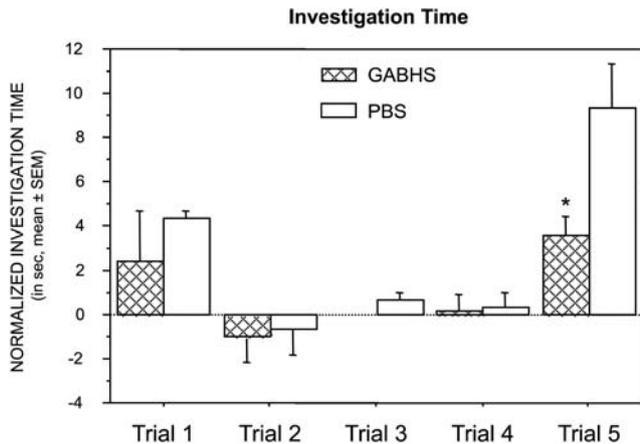
GABHS and PBS passive transfer groups (non-depleted and IgG-depleted GABHS-R and PBS-R mice) did not differ from one another in motor coordination measures (rotarod test; data not shown).

## Discussion

We established a mouse model of PANDAS to dissect mechanisms by which immunologic responses to GABHS cause CNS dysfunction. GABHS mice, like PANDAS children, have complex disturbances of movement and behavior, and antibodies that bind to CNS targets. Based on observations that plasmapheresis and IVIg are therapeutic in PANDAS, we predicted implication of humoral autoimmunity in this mouse model. This prediction was sustained when passive transfer of serum from GABHS donor



**Figure 3** Reduced territoriality and increased passive behaviors in male group A  $\beta$ -hemolytic streptococcus (GABHS) donor mice in a social interaction test. Exploration of cage environment (a), social investigation (b), submission (c) and defensive-escape (d) behaviors of male GABHS and phosphate-buffered saline (PBS) donor mice were tested in a social interaction (resident–intruder) paradigm. Cage exploration and social investigation were reduced in resident GABHS donor mice as compared to resident PBS mice (Mann–Whitney  $U$ ;  $*P < 0.05$ ,  $**P < 0.005$ ). Submissive and defensive-escape behaviors were increased in GABHS donor mice in the resident role as compared to PBS mice in the resident role (Mann–Whitney  $U$ ;  $*P < 0.05$ ,  $**P < 0.005$ ). Height of box plot shows interquartile range; horizontal line, median; error bars, range; circles, outliers.



**Figure 4** Olfactory discrimination deficits in group A  $\beta$ -hemolytic streptococcus (GABHS) donor mice. GABHS and phosphate-buffered saline (PBS) donor mice were habituated over four trials to a novel odor (acquisition/habituation trials). A second novel odor was presented in the fifth (discrimination) trial. Data are presented as the time spent investigating the novel odor normalized to time investigating the control odor (water). GABHS donor mice spent less time investigating the novel odor in the discrimination trial compared with control mice (Fisher's protected least-significant difference (PLSD); \* $P < 0.05$ ). Error bars indicate s.e.m.

mice into naive mice replicated several aspects of the syndrome observed in GABHS mice.

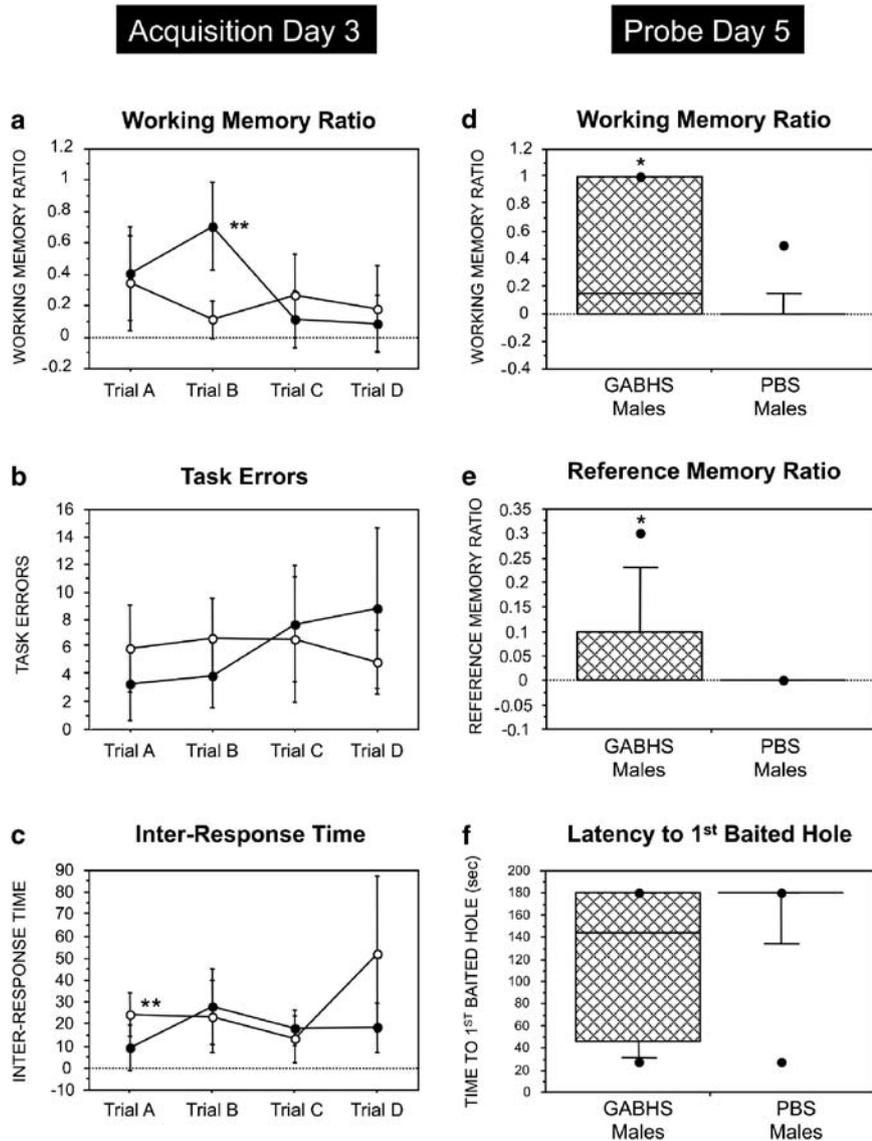
Our depletion studies suggest that IgG is the active component of GABHS donor sera. Whereas injection of GABHS donor sera increases repetitive rearing in open-field testing, and reduces aggression and social behaviors in a resident-intruder paradigm, administration of IgG-depleted GABHS sera does not. Furthermore, the similarity of rearing, social and territorial behaviors in recipients of nondepleted and depleted PBS donor sera indicates that the IgG found in the peripheral sera of PBS donor mice is functionally different than the IgG present in the sera of GABHS-exposed mice. Although serum cytokines may also affect behavior,<sup>42</sup> depletion of IgG abrogated the behavioral changes seen in recipients of GABHS donor sera without altering Th1/Th2 cytokine ratios.

Antibody subclasses may influence pathogenicity. In SC, antibodies directed against basal ganglia proteins primarily represent the IgG1 or IgG3 subclass.<sup>43,44</sup> In GABHS mice, antibody responses are predominantly IgG1. There are no reports of Ig subclass profiles in children with PANDAS. Thus, we do not know whether the IgG1 subclass predominance found in GABHS mice and in humans with SC faithfully replicates PANDAS.

Autoimmune CNS syndromes originating with a peripheral humoral immune response require an insult that compromises the BBB, facilitating trafficking of cross-reactive antibodies into brain.<sup>29,30,45</sup> Many factors influence BBB integrity, including trauma and

infection. Our choice of LPS for the GABHS model was based on work in a mouse model of neuropsychiatric systemic lupus erythematosus (SLE), wherein systemic exposure to LPS and serum autoantibodies with reactivity to DNA and *N*-methyl-D-aspartate receptors from human SLE patients results in memory deficits and preferential death of hippocampal neurons,<sup>29,30,46</sup> and the observation that LPS causes only a transient breach in the BBB without persistent brain injury.<sup>33</sup> The choice of LPS may have influenced pathology independent of antibody specificity. Whereas LPS in current work and other animal models of autoimmune CNS disorders<sup>29,30</sup> is associated with hippocampal IgG deposits, epinephrine administration leads preferentially to IgG deposits in amygdala with changes in emotional regulation.<sup>45</sup> Thus, differences in the distribution of IgG deposits in GABHS donor vs GABHS-R mice may reflect the use of different agents to disrupt BBB (CFA for GABHS donors; LPS for GABHS-R mice). Whereas our initial report of GABHS mice (CFA exposure) described IgG deposits in cerebellum,<sup>28</sup> passive transfer of GABHS mouse serum (LPS exposure) led to IgG deposits in hippocampal and periventricular regions. Consistent with this differential CNS distribution of IgG deposits, motor coordination deficits were observed in GABHS donors, but not in recipients of pooled sera from those donor mice.

In previous work with GABHS mice we suggested that antibodies induced by GABHS antigens cross-react with CNS epitopes, altering neuronal function to trigger repetitive behaviors.<sup>28</sup> GABHS-immunized mice with antibodies to deep cerebellar nuclei (DCN) present in peripheral sera had increased rearing in open-field and hole-board tests and IgG deposits in DCN. Mice with increased anti-DCN immunoreactivity in their sera, and more IgG deposits in DCN, also had the highest immunoreactivity to GABHS proteins on western blots. In addition, serum from a GABHS mouse reacted with normal mouse cerebellum in nondenaturing western blots and immunoprecipitated C4 complement protein and  $\alpha$ -2-macroglobulin.<sup>28</sup> Striatal IgG deposits were also found (Yaddanapudi *et al.*, unpublished observations). Similarly, serum antibodies directed against GABHS that cross-react with basal ganglia neurons are described in both SC<sup>25,47</sup> and PANDAS.<sup>48</sup> It was not evident, however, whether these antibodies alone could cause behavior abnormalities when introduced through the peripheral circulation. Altered humoral immunity, including higher autoantibody levels, is reported in a wide range of behavioral syndromes, including movement disorders,<sup>43,49</sup> schizophrenia,<sup>50,51</sup> autism<sup>52</sup> and neuropsychiatric SLE, but the repertoire of such antibodies is diverse, and often represents binding to unidentified brain antigens.<sup>30,46</sup> Peripheral autoantibodies are also identified in healthy controls in some studies.<sup>53</sup> Our passive transfer studies showed preferential binding of anti-GABHS antibodies to DG granule cells and to cells present in the periventricular area. The strongest

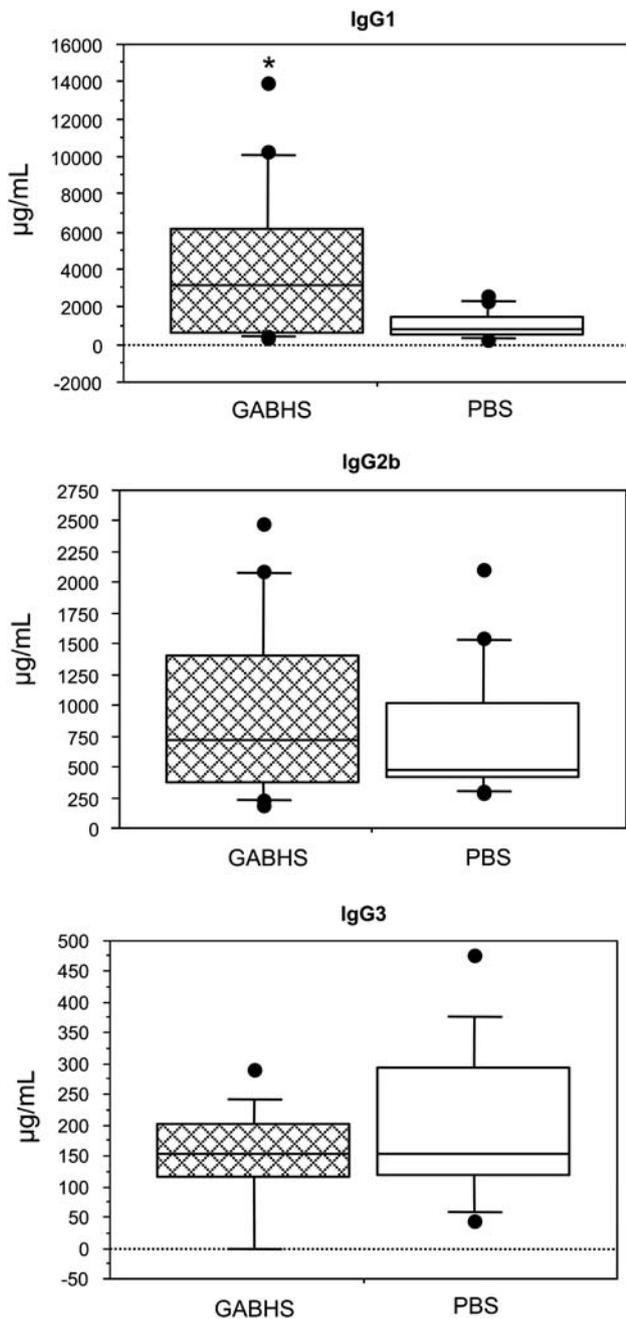


**Figure 5** Superior spatial and reversal learning and memory performance in male group A  $\beta$ -hemolytic streptococcus (GABHS) donor mice. Working and procedural memory capacity of GABHS and phosphate-buffered saline (PBS) donor mice were tested in a reversal spatial learning paradigm (hole-board memory task with 4-day acquisition phase followed by a single reversal probe trial on the fifth day). During the acquisition phase, GABHS donor males (closed circles) had greater working memory ratio (**a**), made fewer task errors (**b**) and exhibited reduced interresponse time (IRT) intervals (**c**) as compared to PBS donor males (open circles) (training day 3, Mann–Whitney  $U$ ; \*\* $P < 0.01$ ). Error bars represent 95% confidence intervals. At the reversal task, administered in a single probe trial on the fifth day (at the start of the probe trial, mice are positioned so that they face the direction opposite to the starting direction used in all acquisition trials; start sites are defined relative to the physical location of the single baited hole), GABHS donor mice had greater working memory (**d**) and reference memory ratios (**e**) relative to PBS donors and found the first baited hole more quickly (**f**) (Mann–Whitney  $U$ , \* $P < 0.05$ ). Height of box plot shows interquartile range; horizontal line, median; error bars, range; circles, outliers.

anti-IgG immunoreactivity within the hippocampus was to neurons of the inner granule cell layer. Given that hippocampus and periventricular zone harbor neuronal progenitor cells in both adult rodents and humans,<sup>54</sup> an antibody response concentrated in these regions might have implications for neuronal plasticity.

The working diagnostic criteria for PANDAS, first proposed by Swedo *et al.* in 1998,<sup>9</sup> and largely unchanged a decade later,<sup>55</sup> focus on presence of a

prepubertal diagnosis of OCD or tic disorder and occurrence of neurologic abnormalities during clinical exacerbations (tics, choreiform movements). A wide range of neuropsychiatric comorbidities are acknowledged as common,<sup>9,11,17</sup> comorbid diagnoses most frequently identified in PANDAS populations include AD/HD and major depression.<sup>9,17</sup> Clinical disturbances observed most often during PANDAS exacerbations include emotional lability, anxiety, impulsivity, attentional problems and deterioration



**Figure 6** Increased IgG1 levels in group A  $\beta$ -hemolytic streptococcus (GABHS) donor sera. Immunoglobulin subclasses were assayed in serum samples from GABHS and phosphate-buffered saline (PBS) donor mice using multiplexed, bead-based immunoassay. Results indicate mean concentration ( $\mu\text{g ml}^{-1}$ ). Serum IgG1 levels were increased in GABHS donor mice as compared to PBS donors (Mann-Whitney  $U$ ;  $*P < 0.05$ ). Height of box plot shows interquartile range; horizontal line, median; error bars, range; circles, outliers.

in handwriting.<sup>9,11</sup> The association of this heterogeneous set of neuropsychiatric findings with PANDAS, and the familial overlap reported for OCD, TS and ASD,<sup>56–58</sup> raises the possibility of common immune pathways in their pathogenesis.<sup>2,59,60</sup>

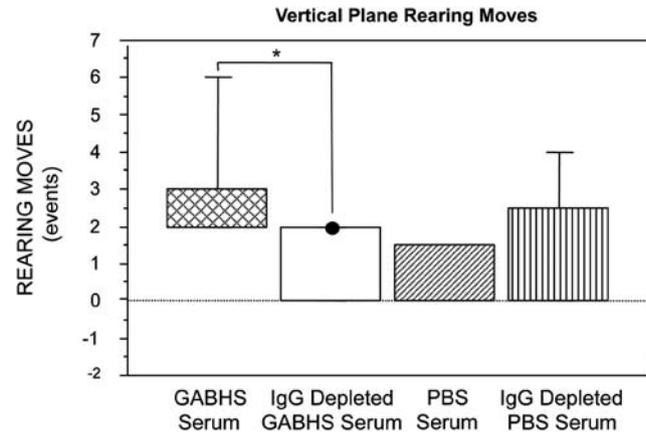
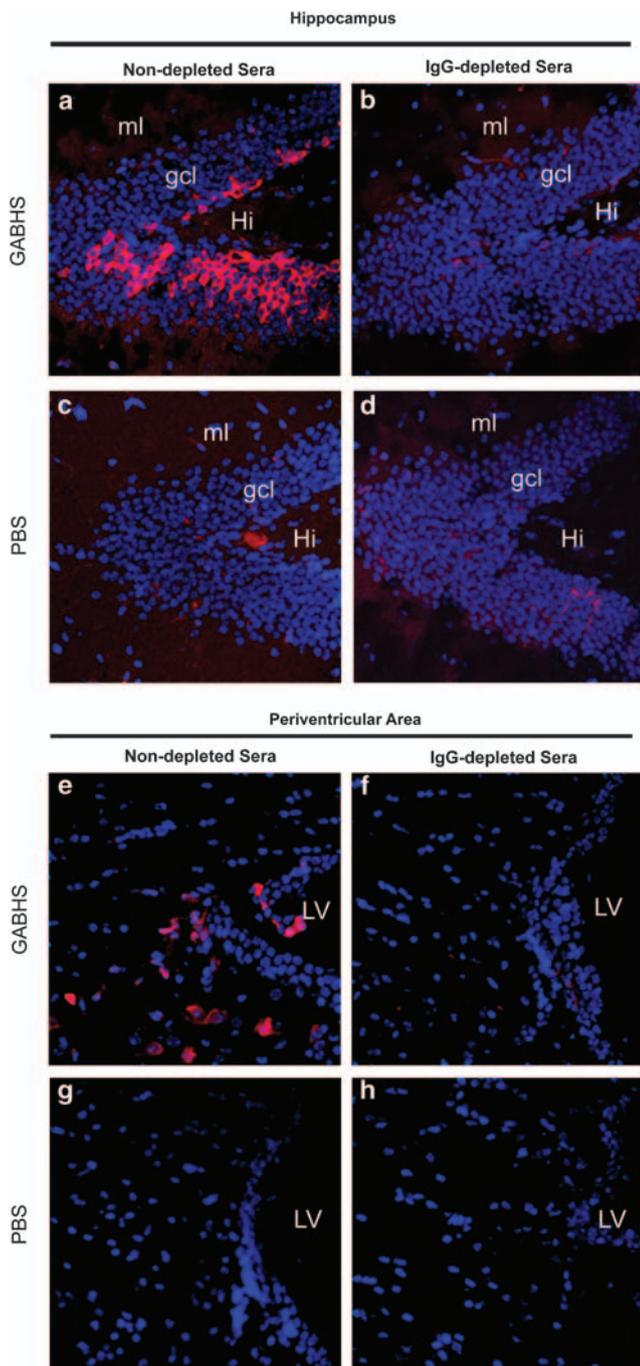
Features of GABHS donor mice and their passive serum transfer recipients are consistent both with the original PANDAS diagnostic criteria<sup>9</sup>—primarily focused on obsessive-compulsive and tic behaviors—as well as with the broader concept of PANDAS arising from subsequent reports.<sup>9,17</sup> Repetitive behaviors in the open-field and hole-board tasks, observed in GABHS donor and passive transfer mice, are reminiscent of tics, obsessions and compulsions. Similarly, deficits in motor coordination are found in both GABHS mice (poor rotarod performance) and PANDAS children (dysgraphia). These impairments are not associated with global cognitive dysfunction. PANDAS children have normal intelligence. GABHS donor mice have superior task acquisition and improved capacity for context-independent performance in the spatial task reversal trial. Indeed, success in task acquisition may capitalize on repetitive tendencies. Persistent rehearsal of the hole-board task through repetition may have enhanced opportunities for GABHS donors to incorporate the spatial context during task acquisition, allowing them to locate successfully the baited hole in the reversal trial. It also implies intact hippocampal function in GABHS donor mice, who, unlike recipients of their sera, have no hippocampal IgG deposits.<sup>28</sup> We were intrigued to find evidence suggestive of a deficit in social interaction in both GABHS donors and GABHS-R passive transfer mice. The low rate of social approaches and the more passive and defensive behaviors in male resident GABHS donor and passive recipient mice in the resident–intruder test of social interaction and territoriality may reflect extension of abnormalities to socioemotional domains and involvement of related (limbic) circuitry. Indeed, territorial behavior and intermale aggression are associated with hippocampal connectivity patterns.<sup>61</sup> The combination in GABHS donor mice of high levels of repetitive behaviors in open-field testing and the hole-board learning and memory task, combined with deficits in social interaction and territoriality in the resident–intruder test, is reminiscent of the repetitive behaviors and aberrant reciprocal social interactions of ASD.<sup>62</sup>

Host factors influencing the risk of developing PANDAS after streptococcal infection, including sex and age, may also be pertinent to this mouse model. Consistent with the excess representation of boys in PANDAS,<sup>9</sup> our findings were largely restricted to, or most accentuated in, GABHS-exposed males. Swedo *et al.*<sup>9</sup> also note that risk for PANDAS wanes after puberty, especially in men. Although no substantive differences in outcomes were found when immunization was initiated at 4 vs 6 weeks, we did not compare outcomes among pre-, peri- or postpubertal mice. It is likely that age influences peripheral autoantibody generation, and possibly which IgG subclass dominates the immune response. The BBB is developed by the fourth postnatal week in SJL/J mice, the youngest age we tested; nonetheless, the threshold for disruption of BBB by blood-borne factors may

differ with age, altering access of peripherally synthesized IgG molecules to CNS.

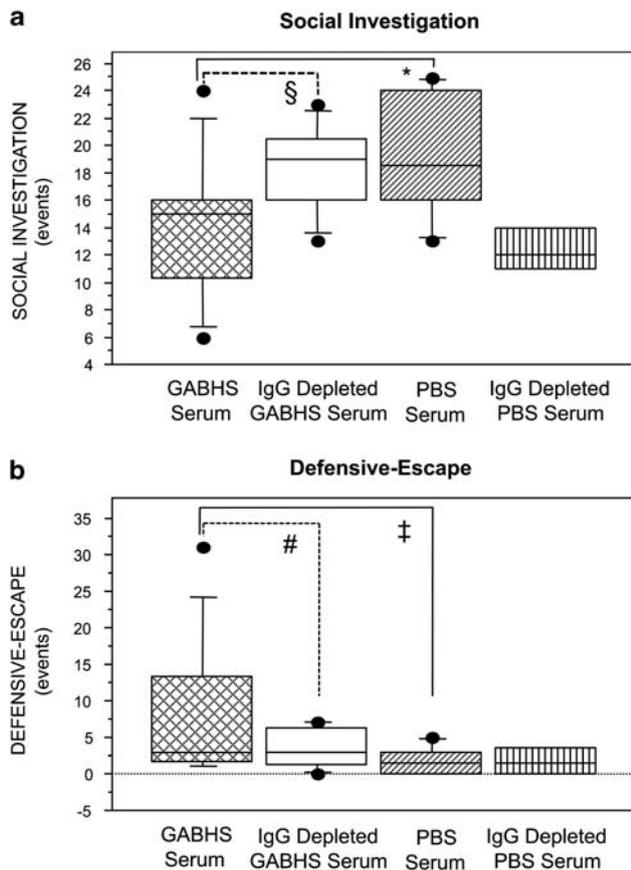
Our study supports the hypothesis that some neuropsychiatric syndromes may be triggered by direct action of GABHS-associated antibodies on brain. Whether environmental factors other than GABHS can lead to similar effects is as yet undefined. Serum antibodies induced by GABHS in this mouse model, when provided access and opportunity to bind to epitopes in brain, may serve as neuronal agonists or antagonists, contributing to development of behavioral disturbances. A loss of BBB integrity is

an important cofactor in immune-mediated CNS syndromes and might occur as a consequence of specific or nonspecific responses to psychosocial or physical stressors, xenobiotics or infectious agents. The function of altered circulating immunomodulators (cytokines, chemokines, acute-phase proteins),



**Figure 8** Increased repetitive rearing behavior in recipient mice injected with group A  $\beta$ -hemolytic streptococcus (GABHS) donor sera. Naive male SJL/J mice were injected with either pooled GABHS or phosphate-buffered saline (PBS) donor sera, or immunoglobulin G (IgG)-depleted GABHS or PBS donor sera. In open-field tests, mice injected with nondepleted GABHS donor sera showed increased repetitive rearing behaviors (vertical plane stereotypy moves) as compared with mice receiving IgG-depleted GABHS donor sera (Mann–Whitney  $U$ ;  $*P < 0.05$ ) and tended to rear more than recipients of nondepleted PBS donor sera (Mann–Whitney  $U$ ;  $P = 0.058$ ). Rearing counts of mice receiving IgG-depleted GABHS donor sera did not differ from those observed in mice receiving either nondepleted or IgG-depleted PBS donor sera. Height of box plot shows interquartile range; horizontal line, median; error bars, range; circles, outliers.

**Figure 7** Immunoglobulin deposits in the brains of recipient mice passively injected with group A  $\beta$ -hemolytic streptococcus (GABHS) donor sera. Brains from naive SJL/J mice injected with either pooled GABHS or phosphate-buffered saline (PBS) donor sera, or immunoglobulin G (IgG)-depleted GABHS or PBS donor sera, were assessed for presence of IgG deposits using immunofluorescence techniques. Hippocampus (a–d); periventricular area (e–h). Recipient mice injected intravenously with nondepleted GABHS donor sera had evidence of IgG binding to dentate gyrus granule cell neurons in the hippocampus (a) and cells in the periventricular area (e), as revealed by anti-IgG staining (red signal). IgG staining in the hippocampal neurons (b) and in the periventricular area (f) was abrogated in mice injected with IgG-depleted GABHS donor sera. Anti-IgG immunoreactivity was absent in the brains of mice injected with nondepleted (c and g) or IgG-depleted PBS donor sera (d and h). Blue signal indicates nuclear counterstaining. gcl, granule cell layer of dentate gyrus; ml, molecular layer; Hi, hilus; LV, lateral ventricle. Scale bars, 40  $\mu$ m.



**Figure 9** Recipient mice injected with group A  $\beta$ -hemolytic streptococcus (GABHS) sera have less territoriality and more passive behavior in a social interaction test. Naive male SJL/J mice were injected with either pooled GABHS or phosphate-buffered saline (PBS) donor sera, or immunoglobulin G (IgG)-depleted GABHS or PBS donor sera. Social investigation (a) and defensive-escape (b) behaviors of mice were tested in a social interaction (resident-intruder) paradigm. Resident mice injected with GABHS donor sera tended to have reduced social investigation and increased defensive-escape behaviors as compared to control mice injected with PBS donor sera (social investigation, Mann-Whitney  $U$ ;  $*P=0.066$ ; defensive-escape, Mann-Whitney  $U$ ;  $\ddagger P=0.108$ ). Social investigation behaviors were higher and defensive-escape behaviors lower in resident recipient mice injected with GABHS donor sera depleted of IgG relative to the levels observed in mice receiving nondepleted GABHS donor sera, but did not achieve significance (social investigation, Mann-Whitney  $U$ ,  $\S P=0.079$ ; defensive-escape, Mann-Whitney  $U$ ;  $*P=0.39$ ). No differences were observed in social investigation or defensive-escape behaviors in the control group injected with IgG-depleted PBS donor sera as compared to resident recipient mice injected with nondepleted PBS sera. Height of box plot shows interquartile range; horizontal line, median; error bars, range; circles, outliers.

neuroendocrine factors or catecholamines in regulating entry of peripheral antibodies into CNS deserves closer attention.<sup>63–65</sup> It will be crucial to understand the conditions abrogating BBB integrity in PANDAS patients, permitting autoantibodies to reach CNS

targets. Delineation of the characteristics of these autoantibodies (Ig class/subclass; specificity, affinity and avidity for select epitopes) and other parameters that may control their binding to brain components once in CNS will be critical in defining the pathogenesis of PANDAS and related immune-mediated neuropsychiatric syndromes.

### Conflict of interest

The authors declare no conflict of interest.

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