GABAergic Signaling Is Linked to a Hypermigratory Phenotype in Dendritic Cells Infected by Toxoplasma gondii

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Abstract

During acute infection in human and animal hosts, the obligate intracellular protozoan Toxoplasma gondii infects a variety of cell types, including leukocytes. Pooled to respond to invading pathogens, dendritic cells (DC) may also be exploited by T. gondii for spread in the infected host. Here, we report that human and mouse myeloid DC possess functional γ-aminobutyric acid (GABA) receptors and the machinery for GABA biosynthesis and secretion. Shortly after T. gondii infection (genotypes I, II and III), DC responded with enhanced GABA secretion in vitro. We demonstrate that GABA activates GABA_A receptor-mediated currents in T. gondii-infected DC, which exhibit a hypermigratory phenotype. Inhibition of GABA synthesis, transportation or GABA_A receptor blockade in T. gondii-infected DC resulted in impaired transmigration capacity, motility and chemotactic response to CCL19 in vitro. Moreover, exogenous GABA or supernatant from infected DC restored the migration of infected DC in vitro. In a mouse model of toxoplasmosis, adoptive transfer of infected DC pre-treated with GABAergic inhibitors reduced parasite dissemination and parasite loads in target organs, e.g., the central nervous system. Altogether, we provide evidence that GABAergic signaling modulates the migratory properties of DC and that T. gondii likely makes use of this pathway for dissemination. The findings unvel that GABA, the principal inhibitory neurotransmitter in the brain, has activation functions in the immune system that may be hijacked by intracellular pathogens.

Introduction

Toxoplasma gondii is an obligate intracellular parasite that infects warm-blooded vertebrates. It infects approximately 25% of the global human population [1]. Initial infection occurs orally or congenitally, whereby the formed tachyzoite stages disseminate widely in the organism. Although principally asymptomatic in humans, infection can cause severe neurological complications in immune-compromised individuals, disseminated congenital infections in the developing fetus, and ocular manifestations in otherwise healthy individuals [1]. T. gondii enters host cells by active penetration, a rapid process that is dependent on the actin-myosin cytoskeleton of the parasite, and does not rely on the host cell machinery for uptake [2]. T. gondii can invade and multiply inside any nucleated cell type, including blood leukocytes, and a preference to infect myeloid leukocytes in vitro has been reported [3]. Following primary infection, T. gondii strikes a fine balance between eliciting an effective immune response and establishing a silent, lifelong infection [4–6]. Acute infection triggers a robust Th1 polarized immune response with efficient activation of antigen presenting cells, including dendritic cells (DC) [7,8].

DC are a fundamental component of the immune response but also a putative gate to immune evasion and persistence for pathogens [9]. DC serve as sensors in peripheral tissues that allow processing and presentation of antigens for initiation of adaptive immune responses and pathogen clearance. The mechanisms underlying DC migration are complex and the molecular traffic signals that govern DC migration are not fully understood [10]. One of the hallmarks of mature DC is the expression of the C-C chemokine receptor 7 (CCR7). Binding to its ligands (CCL19 and CCL21) guides the migrating cells to the lymph nodes where adaptive immune response is initiated [11]. In order to avoid clearance by the immune system, intracellular parasites, bacteria, fungi and virus have evolved diverse strategies to subvert this central function of DC [9,12].

Mounting evidence indicates that DC play a pivotal role during T. gondii infection as mediators of essential immune responses [8,13] and as parasite carriers that facilitate the dissemination of the infection [14–17]. In this context, T. gondii induces a hypermotility state in infected DC that contributes to parasite dissemination in vitro.
transmitters in the CNS [19], acting via activation of GABA receptors facilitate parasite dissemination. A phenotype observed in GABAergic cells and that GABA modulates the hypermigratory receptors and transporters have been found in a variety of tissues metastasis [26,27]. GABA, its synthesis enzymes GAD, GABA motogenic function and participate outside the CNS in diverse indicates that neurotransmitters, including GABA, have a mechanism. Dendritic cells are considered the gatekeepers of the immune system but can, paradoxically, also mediate dissemination of the parasite. Previous work has shown that Toxoplasma induces a hypermigratory state in dendritic cells when they become infected. Here, we show that, shortly after infection by the parasite, dendritic cells start secreting γ-aminobutyric acid (GABA), also known as the major inhibitory neurotransmitter in the brain. We show that dendritic cells express GABA receptors, as well as the machinery to synthesize and transport GABA. When GABA synthesis, transport or receptor function was impaired, the migration of infected dendritic cells was impaired. In a mouse model of toxoplasmosis, treatment of infected dendritic cells with GABA inhibitors resulted in reduced propagation of the parasite. This study establishes that GABAergic signaling modulates the migratory properties of dendritic cells and that the intracellular pathogen Toxoplasma gondii sequesters the GABAergic signaling of dendritic cells to assure propagation.

Mouse and human DC express functional GABA A receptors

In an effort to ascertain which GABA A receptor subunits are expressed in mouse DC, we screened the 19 subunits expression profiles in mouse DC and astrocytes. We detected GABA A R α 1, α 2, β 1, β 2, and ρ 1 subunit transcripts in DC, whilst 12 different subunits were detected in primary astrocytes (Table 1, Table S1 for primer sequences). We decided to quantify differential gene transcription in mouse DC following T. gondii infection and variability in the secreted levels of GABA was observed among the donors (Figure 1F). Monocytes challenged with T. gondii also exhibited an increase in GABA secretion (Figure S2A). Representative strains from the three predominant T. gondii genotypes (I, II and III) induced GABA secretion in infected DC (Figure S3). We conclude that upon Toxoplasma-infection, mouse and human myeloid DC exhibit elevated levels of GABA secretion.

Results

Mouse and human DC secrete GABA upon infection with T. gondii

To address the GABAergic response of mouse DC upon infection, GABA was quantified in the cell supernatant. Challenge of DC with freshly egressed T. gondii tachyzoites led to a significant increase of GABA in the supernatant, while heat inactivated parasites, parasite lysate or LPS did not increase GABA secretion relative to non-infected DC (Figure 1A). Moreover, secretion of GABA from DC challenged with freshly egressed tachyzoites rapidly increased over time, even prior to parasite replication, and augmented over 24 h (Figure 1B). In contrast, the GABA precursor glutamate exhibited a modest transient increase in the supernatant following infection, which was redundant by 24 h (Figure S1). We next assessed if GABA secretion was induced in infected DC or uninfected bystander DC. GABA secretion rapidly augmented with MOI over time (Figure 1C) and supernatants from infected DC did not induce significant GABA secretion in DC (Figure 1D). Moreover, fluorescence-activated cell sorting of DC populations challenged with GFP-expressing T. gondii showed that GABA secretion occurred essentially in GFP + cells (Figure 1E).

Non-infected bystander DC and DC in complete medium (CM) are similar. Next, 9 human donors were assessed. Monocyte-derived DC from all donors responded with increased amounts of GABA upon T. gondii infection and variability in the secreted levels of GABA was observed among the donors (Figure 1F). Monocytes challenged with T. gondii also exhibited an increase in GABA secretion (Figure S2A). Representative strains from the three predominant T. gondii genotypes (I, II and III) induced GABA secretion in infected DC (Figure S3). We conclude that upon Toxoplasma-infection, mouse and human myeloid DC exhibit elevated levels of GABA secretion.

[14,15]. Interestingly, this strategy for dissemination appears to be conserved among other members of the Apicomplexan parasite family, e.g. Neospora caninum [18]. Yet, the molecular mechanism controlling the parasite-induced hypermigratory phenotype in DC remains unknown. Given its characteristics, i.e. random directional hypermotility in absence of chemotactic cues, alternative/non-classical pathways are likely to be involved [4].

γ-aminobutyric acid (GABA) is one of the major neurotransmitters in the CNS [19], acting via activation of GABA A receptors [20] and to a lesser extent GABA B receptors [21]. GABA is shuttled in and out of cells via GABA transporters (GAT) of the solute carrier family 6 [22]. GABAergic cells synthesize GABA via glutamate decarboxylases (GAD) [23]. In contrast to its role as an inhibitory neurotransmitter, GABA plays an excitatory role during neuronal development [24,25]. In fact, mounting evidence indicates that neurotransmitters, including GABA, have a motogenic function and participate outside the CNS in diverse functions including cell migration, immunomodulation, and metastasis [26,27]. GABA, its synthesis enzymes GAD, GABA receptors and transporters have been found in a variety of tissues outside the CNS, such as the pancreatic islets and testes [28,29].

Using in vitro models and in vivo bioluminescence imaging (BLI) in a mouse model of toxoplasmosis, we demonstrate that DC are GABAergic cells and that GABA modulates the hypermigratory phenotype observed in Toxoplasma-infected DC. During in vitro infections, the GABAergic system of infected DC is likely used to facilitate parasite dissemination.
Figure 1. DC demonstrate GABAergic properties upon *T. gondii* infection. (A) Live *Toxoplasma* infection of mouse DC enhances the release of GABA. DC were incubated for 24 h with complete medium (CM), lipopolysaccharide (LPS, 100 ng/ml), heat inactivated *T. gondii* PTGluc tachyzoites (Hi Tg), sonicated PTGluc tachyzoites (Son Tg) or freshly egressed PTGluc tachyzoites (Live Tg, MOI 1). GABA in the supernatant was quantitatively determined by ELISA as described under Materials and Methods. Values represent means (±SEM) from two experiments performed in quadruplicate. (*) indicates significant differences (P = 0.0045, Mann Whitney U-test). (B) Mouse DC GABA release occurs rapidly following *Toxoplasma* infection. DC were challenged with PTGluc tachyzoites (MOI 1) and GABA was quantitatively determined at indicated time points as described under Materials and Methods. Values represent means (±SEM) from two experiments performed in quadruplicate. (*) indicates significant increase in GABA production compared to non-infected DC (P < 0.0001, GLM ANOVA, Tukeys Pairwise comparison). (C) Effect of multiplicity of infection (MOI) on GABA secretion from DC over time. Mouse DC were challenged with PTGluc tachyzoites at indicated MOI and GABA concentration in the supernatant was quantitatively determined after 3 h or 8 h by ELISA. Values represent means (±SEM) from one representative experiment performed in triplicate. (*) indicates significant differences (P < 0.05, Student's t test). (D) Supernatants from infected DC do not induce secretion of GABA. Graph shows GABA concentrations after exposure of 10^6 non-infected mouse DC to supernatants from infected DC (PTGluc, MOI 1, 24 h, 700 μl). “NI Sup 24 h” indicates GABA ELISA readout from DC in CM for 24 h; “I sup 0-8-24 h” indicate GABA concentrations after exposure of non-infected DC to supernatant from infected DC for 0, 8 and 24 h respectively. “I Sup 6 h + CM 8–24 h” indicate that non-infected DC were exposed to supernatant from infected DC for 6 h; the supernatant was then washed away and substituted by CM for 8 and 24 h respectively before assessment of GABA concentrations. Values represent means (±SEM) from one representative experiment performed in triplicate. Performed three times with similar result. (*) indicates significant differences (P < 0.05, Student’s t test). (E) Supernatants from infected DC do not induce secretion of GABA. Graph shows GABA concentrations after exposure of 10^6 non-infected mouse DC to supernatants from infected DC (PTGluc, MOI 1, 24 h, 700 μl). “NI Sup 24 h” indicates GABA ELISA readout from DC in CM for 24 h; “I sup 0-8-24 h” indicate GABA concentrations after exposure of non-infected DC to supernatant from infected DC for 0, 8 and 24 h respectively. “I Sup 6 h + CM 8–24 h” indicate that non-infected DC were exposed to supernatant from infected DC for 6 h; the supernatant was then washed away and substituted by CM for 8 and 24 h respectively before assessment of GABA concentrations. Values represent means (±SEM) from one representative experiment performed in triplicate. Performed three times with similar result. (*) indicates significant differences (P < 0.05, Student’s t test). (F) Infected (GFP+)(+) DC exhibit elevated GABA secretion but not uninfected (GFP-) by-stander DC. DC were challenged with freshly egressed GFP-expressing tachyzoites (PTGluc, MOI 1) for 6 h followed by fluorescence-activated cell sorting as indicated under Materials and Methods. For all conditions, cells were washed, replated in CM and secreted GABA (1 × 10^6 DC/condition) was quantified after 16 h incubation as indicated under Materials and Methods. “CM” indicates DC in complete medium, “Toxo” indicates DC challenged with *T. gondii* that were subsequently subjected to cell sorting, illustrated by contour plot. Values represent means (±SD) from three independent experiments performed in duplicate. Significant differences were observed between GFP+ and GFP- cell populations (P < 0.0001, Paired t-test). (F) Human monocyte-derived DC are GABAergic. DC were infected with PTGluc tachyzoites (MOI 1) and GABA in the supernatant was analyzed after 24 h as described under Materials and Methods. Values represent means (±SEM) from each individual donor (n = 9) performed in quadruplicate.

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Targeting GABA synthesis and transport reduces transmigration of infected DC in vitro

To investigate the effects of Toxoplasma infection on the GABAergic system, expression levels of the GABA transporter GAT4 and the GABA synthesizing enzymes, GAD65 and GAD67, were assessed in DC. A rapid induction of GAT4 transcription was observed shortly after infection (Figure 3A). In contrast, expression of GAD65 was detected in both non-infected and infected DC at similar levels (Figure 3A), whereas GAD67 expression was not detectable in either group (data not shown). Moreover, addition of GAD inhibitor (SC) and GAT4 inhibitor (SNAP) to infected DC nearly abolished or significantly reduced, respectively, the secreted levels of GABA in the supernatant from infected DC (Figure 3B). Inhibitor treatments did not significantly affect intracellular parasite replication in vitro (Figure S5) or the GABA signal detected in complete medium containing extracellular parasites (Figure S6). We next assessed the impact of the GABAergic inhibitors on the transmigration of infected DC. Both GAT4 (SNAP) and GAD inhibition (SC) had a significant inhibitory effect on the transmigration of infected DC, and transmigration was significantly restored following incubation of the inhibitor-treated cells in supernatant from infected DC at similar levels (Figure 3C). In contrast, monocytes did not exhibit this inhibitory effect on the transmigration of infected DC, and significant effects on CCR7 expression were observed (Figure 6D). Interestingly, directionality towards CCL19 was observed for infected DC, similar to that observed upon LPS maturation (Figure 4B). It is also notable that Toxoplasma-infected DC ((−) chemokine, Fig. 4 B) outranged LPS-matured non-infected DC ((+) chemokine, Figure 4A) in migration distances and velocity (Figure 4A, B, C). We conclude that Toxoplasma-infected DC exhibit a hypermigratory phenotype in vitro and that hypermotile Toxoplasma-infected DC maintain the ability to chemotax in vitro.

Next, we determined whether targeting the GABAergic system affected the migratory and chemotactic responsiveness of non-infected DC (Figure 5A, B) and Toxoplasma-infected DC (Figure 5D, E) in vitro. Overall, inhibition of GABA synthesis (SC, GAD inhibitor) or GABA transport (SNAP, GAT4 inhibitor) led to a significant decrease in the velocity and the accumulated distance covered by DC (Figure 5C, F). Interestingly, the ability to respond with directionality towards CCL19 was not abolished by inhibiting GABA transport or synthesis but, as a consequence of the reduction in velocity, the overall chemotactic response was diminished (Figure 5). No significant influence of a GABA gradient on the directionality of DC motility was observed for non-infected and infected DC (data not shown). In summary, present data show that inhibition of the GABAergic signaling system significantly reduces the velocity of infected DC in vitro and thereby the magnitude of the chemotactic response in vitro.

Human and mouse DC exhibit upregulation of CCR7 upon T. gondii infection in vitro

We next assessed the relative expression of the CCL19 ligand CCR7 on human and mouse DC by flow cytometry. First, the chemotactic responses observed with mouse DC were confirmed using human monocyte-derived DC (Figure 6A). Additionally, monitoring of infected and uninfected DC in suspensions challenged with T. gondii showed that the chemotactic response occurred preferentially in the infected (RFP+) DC population (Figure 6A, central panel). In line with this result, DC challenged with T. gondii or treated with LPS exhibited a relatively higher expression of CCR7 compared to DC in complete medium (Figure 6B). The analyses of DC populations challenged with T. gondii showed that upregulation of CCR7 occurred essentially in infected (RFP+) DC (Figure 6B, central panel). An upregulation of CCR7 was consistently observed in infected DC from 7 different human donors (Figure 6C). For mouse DC, a small but significant upregulation of CCR7 was observed in infected DC (Figure 6E). In the presence of GABAergic inhibitors (SC, SNAP), overall non-significant effects on CCR7 expression were observed (Figure 6D and E). We also assessed the effects of GABAergic inhibitors (SC, SNAP) on the expression of co-stimulatory molecules and hanced random directional motility in absence of chemokine (Figure 4B), with a significant increase in velocity compared to non-infected DC (Figure 4C). Interestingly, directionality towards CCL19 was observed for infected DC, similar to that observed upon LPS maturation (Figure 4B). It is also notable that Toxoplasma-infected DC ((−) chemokine, Fig. 4 B) outranged LPS-matured non-infected DC ((+) chemokine, Figure 4A) in migration distances and velocity (Figure 4A, B, C). We conclude that Toxoplasma-infected DC exhibit a hypermigratory phenotype in vitro and that hypermotile Toxoplasma-infected DC maintain the ability to chemotax in vitro.

GABAergic signaling modulates motility and chemotaxis of infected DC in vitro

To determine whether the GABAergic system also affected DC motility and chemotaxis, infected and non-infected DC were allowed to migrate along a concentration gradient of CCL19 in a chemotaxis chamber system. Non-infected DC exhibited a low level of random directional motility in absence or presence of chemokine and LPS-stimulation of non-infected DC resulted in a distinct directional migration towards CCL19 (Figure 4A). In contrast, Toxoplasma-infected DC exhibited a dramatically en-

### Table 1. Characterization of GABA<sub>A</sub> receptor subunit gene expression in murine bone marrow-derived DC related to primary murine astrocytes.

| Cell type | a1 | a2 | a3 | a4 | a5 | a6 | β1 | β2 | β3 | γ1 | γ2 | γ3 | δ | θ | ε | π | p1 | p2 | p3 |
|-----------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| DC        | x  | x  | x  | x  | x  | x  | x  | x  | x  | x  | x  | x  | x  | x  | x  | x  | x  | x  |
| Astrocytes| x  | x  | x  | x  | x  | x  | x  | x  | x  | x  | x  | x  | x  | x  | x  | x  | x  | x  |

A PCR screen of the 19 GABA<sub>A</sub> subunits was performed using mouse DC cDNA and mouse astrocyte cDNA as a positive control as indicated under Materials and Methods.

A The transcripts of GABA<sub>A</sub> receptor subunits α3, β3, and γ1 were consistently quantified, and mean Ct values from non-infected DC cDNA were 37.0; 30.8; and 34.9 respectively. The transcripts of subunits α5 and β1 could not be consistently quantified.

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Figure 2. DC express GABA_{A} receptor subunits and GABA activates GABA_{A} receptor-mediated currents in DC. (A) GABA_{A} \( \alpha_3 \), \( \beta_3 \) and \( p_1 \) subunit expression is modulated in DC upon challenge with *T. gondii*. Mouse DC were incubated with PTGluc tachyzoites (MOI 3) or CM for 2 h or 8 h. RNA was extracted and cDNA synthesized as detailed in Materials and Methods. Following RT-qPCR, GABA_{A} \( \alpha_3 \), \( \beta_3 \) and \( p_1 \) subunit expression was determined using the \( \Delta \Delta C_t \) method, with GAPDH as the reference gene, and non-infected DC GABA_{A} \( \alpha_3 \), \( \beta_3 \) and \( p_1 \) expression as the calibrator, respectively. Y-axis indicates relative expression against non-infected DC calibrator. Values represent means (±SEM) from two or three independent experiments performed in triplicate. (B–C) Immunocytochemistry of non-infected DC (B) and DC challenged with GFP-expressing *T. gondii* tachyzoites (green) (C) stained with GABA_{A} receptor \( \beta_3 \) subunit monoclonal antibody (red) and DAPI (blue). In C, upper micrograph shows replicating tachyzoites (green) and lower micrograph shows tachyzoites (green) shortly after invasion. Stainings were performed as indicated under Materials and Methods. Scale bar: 10 \( \mu \)m. (D–H) GABA activates GABA_{A} receptor-mediated currents in DC. Patch-clamping of Toxoplasma-infected and non-infected DC was performed as indicated under Materials and Methods. Whole-cells currents were evoked by application of 1 \( \mu \)M or 1 mM GABA to mouse DC (n=7; D, E, F) or human DC (n=7; G, H). In symmetrical chloride solution the currents were inward at negative potentials (D, E, G, H; \( -80 \) mV) outward at positive potentials (F; +40 mV) and blocked by SR-95531, a GABA_{A} competitive antagonist (H). D, E, F and H show results from infected (12 h) DC whereas G was recorded from a non-infected DC.

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GABAergic inhibition in adoptively transferred infected DC reduces the dissemination of *T. gondii* in vivo

Previously we have demonstrated that the adoptive transfer of *T. gondii*-infected DC leads to rapid dissemination of parasites as well as exacerbation of infection compared to infection with free parasites [14]. To assess whether GABAergic inhibition of *T. gondii*-infected DC had an impact on the aforementioned *in vivo* dissemination, mice were inoculated i.p. with freshly egressed luciferase-expressing tachyzoites or with tachyzoite-infected DC. Photonic emissions were measured by BLI daily for 5 days [30]. Infected DC were pretreated with a combination of inhibitors against GAD and GAT4 shown to have prolonged (24 h) inhibition on transmigration of DC *in vitro*. Interestingly, GABAergic inhibition of infected DC resulted in a significant reduction (≈2.8 fold) in total parasite photonic counts compared to non-treated infected DC by day 4 post infection (\( P = 0.0003 \), GLM ANOVA, Figure 7A and B). Furthermore, the photonic counts from the combination treated group were equivalent to levels observed during free tachyzoite infection (\( P = 0.05 \), GLM ANOVA, Figure 7A and B). Analyses of adoptively transferred uninfected DC pretreated with GABAergic inhibitors showed similar numbers of treated and non-treated DC in the spleen and the peritoneum (Figure S8).

To determine the presence of parasites in different organs, photonic emissions were assessed *ex vivo* in the spleen, MLN and brain (Figure 7C). Special assessment of the brain showed significant differences in photonic emissions on days 1–2 with important variability between mice (Figure 7D). To quantify parasitic loads in target organs, plaque assays were performed. Overall, higher parasitic loads were observed in mice challenged with non-treated infected DC compared to mice challenged with infected DC treated with GABAergic inhibitors (Figure 7E). Altogether, and in line with observations *in vitro*, this indicates that treatment of infected DC with GAD- and GAT4-inhibitor (SC, SNAP) results in a significant reduction in the dissemination of *T. gondii*, and subsequently a reduction of the parasitic loads during the course of infection in mice.

Discussion

In the present study, we report that GABAergic signaling is closely linked to a hypermigratory phenotype in DC, which is induced by *T. gondii* infection [14]. Furthermore, we demonstrate challenged with GFP-expressing *T. gondii* tachyzoites (green) (C) stained with GABA_{A} receptor \( \beta_3 \) subunit monoclonal antibody (red) and DAPI (blue). In C, upper micrograph shows replicating tachyzoites (green) and lower micrograph shows tachyzoites (green) shortly after invasion. Stainings were performed as indicated under Materials and Methods. Scale bar: 10 \( \mu \)m. (D–H) GABA activates GABA_{A} receptor-mediated currents in DC. Patch-clamping of Toxoplasma-infected and non-infected DC was performed as indicated under Materials and Methods. Whole-cells currents were evoked by application of 1 \( \mu \)M or 1 mM GABA to mouse DC (n=7; D, E, F) or human DC (n=7; G, H). In symmetrical chloride solution the currents were inward at negative potentials (D, E, G, H; \( -80 \) mV) outward at positive potentials (F; +40 mV) and blocked by SR-95531, a GABA_{A} competitive antagonist (H). D, E, F and H show results from infected (12 h) DC whereas G was recorded from a non-infected DC.

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Discussion

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that mouse and human myeloid DC possess functional GABA receptors and are capable of producing and secreting GABA. Interestingly, challenge of DC with T. gondii consistently resulted in a significant increase in the levels of extracellular GABA over time in mouse DC and in DC derived from different human donors. The secretion of GABA was not related to DC activation or maturation following exposure to LPS, parasite lysates, supernatants from infected DC or uptake of heat-inactivated parasites, but linked to the live infection by T. gondii. Our data indicates that DC secrete GABA as a consequence of infection by the parasite and that non-infected bystander DC only provide a minor contribution to the total secreted amounts. Also, the absence of a distinct

Figure 3. The GABAergic system is modulated in Toxoplasma-infected DC to facilitate hypermotility. (A) GAT4 expression is up-regulated following Toxoplasma infection whilst GAD65 is expressed in both non-infected and Toxoplasma-infected mouse DC. DC were incubated with PTGluc tachyzoites (MOI 3) or CM for 2 h or 8 h. RNA was extracted and cDNA synthesized as detailed in Materials and Methods. Following RT-qPCR, GAT4 and GAD65 differential expression was determined using the ΔΔCt method, with GAPDH as the reference gene, and non-infected DC GAT4 and GAD65 expression as the calibrator, respectively. Values represent means (±SEM) from two or three independent experiments performed in triplicate. (B) GAD and GAT4 antagonists inhibit GABA secretion in DC. Mouse DC were incubated with PTGluc tachyzoites (MOI 1) for 24 h under different conditions. CM indicates complete medium; SC (Semicarbazide, GAD inhibitor, 50 μM); SNAP (SNAP-5114, GAT4 inhibitor, 50 μM). GABA levels were analyzed using a GABA ELISA kit as described in Materials and Methods. Values represent means (±SEM) from two independent experiments performed in quadruplicate. (*) indicates a significant decrease in GABA production compared to non-infected DC in CM (P<0.002, Mann Whitney U-Test). (**) indicates a significant decrease in GABA production compared to untreated Toxoplasma-infected DC (P<0.008, Mann Whitney U-test). (C) The inhibitory effects of GAT4 and GAD65 antagonists on Toxoplasma-infected mouse DC transmigration can be restored following the addition of GABA enriched supernatant from infected DC. DC were infected for 6 h with PTGluc tachyzoites (MOI 3) or PTGluc tachyzoites plus SNAP (50 μM) or SC (50 μM) as described in Materials and Methods. DC were then transferred to transwells, and allowed to transmigrate in the presence of CM, SNAP (50 μM), SC (50 μM), or SNAP/SC plus supernatant from infected DC. Cell migration was determined using a Neubauer hemocytometer. Values represent means (±SEM) from two independent experiments done in triplicate. (*) indicates significant differences (P<0.01, One-way ANOVA). (D) The inhibitory effects of the GAT4 antagonist SNAP on infected DC transmigration can be fully restored following the addition of exogenous GABA. Mouse DC were infected for 6 h with PTGluc tachyzoites (MOI 3) or PTGluc tachyzoites plus SNAP (50 μM) or SC (50 μM) as described in Materials and Methods. DC were then transferred to transwells, and allowed to transmigrate in the presence of CM, SNAP (50 μM), SC (50 μM), or SNAP plus supernatant from infected DC. Cell migration was determined using a Neubauer hemocytometer. Values represent means (±SEM) from two independent experiments done in triplicate. (*) indicates significant differences (P<0.01, One-way ANOVA), ns: non-significant. (E) Toxoplasma-induced DC transmigration is partially inhibited by GABA_A and GABA_B receptor inhibitors. Mouse DC were infected for 6 h with PTGluc tachyzoites (MOI 3) or PTGluc tachyzoites plus Muscimol (GABA_A Agonist – 300 μM), Bicuculline (GABA_A Antagonist – 50 μM), Baclofen (GABA_B Agonist – 500 μM), or CGP35348 (GABA_B Antagonist –500 μM) as described in Materials and Methods. DC were then transferred to transwells, and allowed to transmigrate in the presence of complete medium (CM), Muscimol (GABA_A Agonist – 300 μM), Bicuculline (GABA_A Antagonist – 50 μM), Baclofen (GABA_B Agonist – 500 μM), or CGP35348 (GABA_B Antagonist – 500 μM). Cell migration was determined using a Neubauer hemocytometer. Values represent means (±SEM) from two independent experiments done in triplicate. (*) indicates significant differences for GABA_A receptor antagonist and GABA_B receptor antagonist (P<0.0001 and P<0.001 respectively, 2-way ANOVA).

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A

(-) Chemokine

52%<br>
48%

(+) Chemokine

55%<br>
50%

(-) LPS

Non-infected DC

(+) LPS

50%<br>
87%

CCL19

B

(-) LPS

58%<br>
42%

(+) LPS

16%<br>
84%

Toxo-DC

C

CCL19

CM

C

Non-infected DC

Toxo-DC

| Velocity (um/s) x 10^-2 |
|-------------------------|
| 4                      |
| 3.5                    |
| 3                      |
| 2.5                    |
| 2                      |
| 1.5                    |
| 1                      |

* * *

LPS CCL19

- - + + - - + + - - + + - - + +
Figure 4. *Toxoplasma*-infected mouse DC exhibit hypermotility and chemotaxis. (A) Non-infected mouse DC or (B) *Toxoplasma*-infected mouse DC (PTGluc, MOI 1/Toxo-DC) were incubated with LPS (200 ng/ml). After 24 h, cells were collected and placed in a chemotaxis chamber ± CCL19 gradient and tracked for 60 min as described under Materials and Methods. The tracking data presented is a composite of two independent experiments (n = 60 cells). The overall directionality of migration is depicted in the rose-diagram in the upper right corner of each single track summary. Percentages represent the proportion of cells migrating towards or away from chemotactrant or complete medium (CM). For the condition (+) chemokine, the triangle indicates the placement of the CCL19 chemokine gradient in each chemotaxis chamber. For (−) chemokine, CM was added to both chambers. (C) Velocity analysis of non-infected mouse DC or *Toxoplasma*-infected mouse DC in presence or absence of the chemokine CCL19. Bars indicate velocity (+SEM) from two independent experiments. (*) indicate significant differences (P < 0.001, Mann Whitney U-test).

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Figure 5. DC motility and chemotaxis are affected by the GABAergic system. (A, B) Non-infected mouse DC or (D, E) *Toxoplasma*-infected mouse DC (PTGluc, MOI 1/Toxo-DC) were treated with GAD inhibitor (SC, Semicarbazide, 50 μM) or GAT4 inhibitor (SNAP, SNAP5114, 50 μM) with LPS (200 ng/ml). After 24 h, cells were collected and placed in a chemotaxis chamber ± CCL19 gradient and tracked for 60 min as described under Materials and Methods. The tracking data presented is a composite of one to two experiments performed in duplicate (n = 60 cells). The overall directionality of migration is depicted in the rose-diagram in the upper right corner of each single track summary. Percentages represent the proportion of cells migrating towards or away from chemotactrant or complete medium (CM). For the condition (+) chemokine, the triangle indicates the placement of the CCL19 chemokine gradient in each chemotaxis chamber. For (−) chemokine, CM was added to both chambers. (C, F) Velocity analysis of non-infected DC (A, B) and *Toxoplasma*-infected DC (D, E) in presence or absence of GABAergic inhibition (CM, complete medium; SC, GAD inhibitor; SNAP, GAT4 inhibitor) ± gradient with the chemokine CCL19. Bars indicate velocity (+SEM) from two independent experiments. (*) indicate significant differences (P < 0.001, Mann Whitney U-test).

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Figure 6. Chemotactic responses and relative expression of CCR7 in human and mouse DC. (A) Chemotaxis tracking analyses of human monocyte-derived DC. DC in complete medium (non-infected), DC challenged with freshly egressed RFP-expressing tachyzoites (Toxo, PRU-RFP, MOI 1, 24 h) or exposed to LPS (200 ng/ml, 24 h) were placed in chemotaxis chambers with a CCL19 gradient and tracked for 60 min as described under Materials and Methods. In the central panel, cells were separated into RFP⁺ (red tracks) and RFP⁻ (black tracks). The tracking data is a composite of two experiments performed in duplicate (n = 60 cells). The overall directionality of migration is depicted in the rose-diagrams of each single track summary. (B) Histograms show CCR7 expression of human monocyte-derived DC treated as in (A). CCR7 stainings (solid lines), isotype control stainings (dashed lines) and flow cytometry were performed as indicated under Materials and Methods. Displayed data are representative of 4 independent experiments and 7 donors. (C) Human monocyte-derived DC exhibit a significant upregulation of CCR7 upon T. gondii infection. Diagram shows, for each individual donor (n = 7), mean fluorescence intensity (MFI) analyzed by flow cytometry. Mean ± SEM is indicated for each group. CM: DC in complete medium; LPS: LPS-treated DC; DC challenged with T. gondii (PRU-RFP) were separated on RFP-positivity. (*) indicates
modulation on the secreted levels of the GABA-precursor glutamate is indicative of a selective effect on GABA synthesis and secretion.

We found that a determined subset of GABA<sub>δ</sub> receptors subunit genes was transcribed in mouse DC (α<sub>6</sub>, α<sub>5</sub>, β<sub>1</sub>, β<sub>3</sub> and ρ<sub>1</sub>) in contrast to the broader expression in astrocytes. This finding is consistent with the concept that most GABA<sub>δ</sub> receptor pentamers are composed of at least two α- and two β-type subunits while the final subunit type may vary [31]. This also strongly suggests that genetic control plays a major role in the choice of transcribed subunit variants. In fact, such differential expression has been implicated in the changes in responsiveness and function of GABA<sub>δ</sub> receptors [32,33]. Moreover, factors that affect GABAergic signaling, e.g. infection, may simultaneously induce up- and down-regulation of specific GABA<sub>δ</sub> subunits through epigenetic mechanisms [34].

Here, we demonstrate for the first time that GABA evokes GABA<sub>δ</sub> receptor-mediated currents in T. gondii-infected DC and in non-infected DC. While transcript levels of the α<sub>δ</sub>, β<sub>3</sub> and ρ<sub>1</sub> subunits were modulated upon infection, functional patch-clamping data indicates that GABA<sub>δ</sub> receptors are constitutively expressed in DC. In line with this, immunocytochemical analyses indicated expression of the GABA<sub>δ</sub> receptor β<sub>3</sub> subunit in infected and non-infected DC. As individual receptor subunits do not necessarily reflect the number of functional receptor pentamers or combinations of pentamers that are expressed in a particular cell, it remains unknown how individual subunits relate to the receptor function in DC. Thus, further studies are needed to characterize and quantify the precise receptor subset composition of immune cells, and whether sensitivity to GABA is modulated upon infection.

Recently, human monocytes were shown to express the GABA<sub>δ</sub> β<sub>3</sub> subunit and functional GABA<sub>δ</sub> receptors were described in a human myelomonocytic cell line (α<sub>δ</sub>, β<sub>3</sub>, γ<sub>1</sub> and δ subunits) [35]. In contrast to DC, monocytes do not exhibit enhanced transmigration upon T. gondii-infection in vitro [36]. Here, we report that monocytes respond with GABA secretion upon T. gondii infection. Altogether, this raises the questions if receptor activation through secreted GABA, e.g. an autocrine loop, is needed for migratory activation or if different subsets of receptors are expressed in DC compared to monocytes. Whether these intriguing phenotypic differences between monocytes and DC depend on GABA<sub>δ</sub> receptor expression levels, functional receptor subunit composition or capacity to rapidly secrete GABA awaits further investigation.

Induction of GABA secretion in infected DC was confirmed in strains from the three predominant genotypes of T. gondii, but it is unlikely that the maintenance and expression of GABA receptors and the GABAergic system in DC are exclusively a result of evolutionary pressure from T. gondii. Thus, additional functions for the GABAergic system in DC are likely to be discovered.

Mounting evidence indicates that T. gondii modulates the host’s pathways for cell migration to facilitate its dissemination and establishment of a chronic infection. Our studies demonstrate that targeting the GABAergic machinery of host DC, i.e. GABA biosynthesis, transport, or ligand channel activation in vitro resulted in impaired ability to transmigrate, most prominently using inhibitors targeting host cell GAD and GAT4. Furthermore, the
A recently proposed model envisages a complex interplay between ambient GABA, GABA$_A$ receptor activation, and chloride transport as regulators of interneuron migration [64]. It is conceivable that the DC GABAergic system may be working in a similar manner in relation to the hypermigratory phenotype exhibited by $T. gondii$-infected DC. Our model comprises: 1) DC invasion by $Toxoplasma$, resulting in increased GABA production; 2) GABA is shuttled out of the cell by GABA transporters, leading to an autocrine effect in activating GABA$_A$ receptors; 3) chloride ion efflux by GABA$_A$ receptor channels and subsequent calcium influx maintain DC in a depolarizing migratory state. The effector mechanisms leading to increased GABA production in DC as a consequence of $T. gondii$-infection await further investigation. It has been shown that $T. gondii$-infection can lead to extensive transcriptional regulation of host genes in DC [65] and a modulated transcription of GAT4 and of GABA$_A$ $\alpha_3$, $\beta_3$ and $\rho_1$ subunits was observed in infected DC. We found no evidence of significant production of GABA by extracellular parasites but a modulation of the DC biosynthesis of GABA by intracellular parasites cannot be excluded.

In summary, we provide substantial evidence that the DC GABAergic system plays a significant role in the maintenance of the $T. gondii$-induced hypermigratory phenotype observed in infected DC. To the best of our knowledge, this constitutes the first report showing that the GABAergic system can be utilized by an intracellular pathogen to modulate host cell motility and potentiate systemic dissemination. It remains to be seen whether other pathogens also utilize the GABAergic system to facilitate the establishment of an infection. Further investigation of the specific molecules and pathways involved will enable a greater understanding of the diverse roles that the GABAergic system may play outside the CNS.

Materials and Methods

Ethics statement

All protocols involving animals were approved by the Regional Animal Research Ethical Board, Stockholm, Sweden, following proceedings described in EU legislation (Council Directive 86/609/EEC). The Regional Ethics Committee, Stockholm, Sweden, approved protocols involving human cells. All donors received written and oral information upon donation of blood at the Karolinska University Hospital. Written consent was obtained for utilization of white blood cells for research purposes. The ethics committees approved this consent procedure.

Parasites and cell lines

Tachyzoites from the green fluorescence protein (GFP) and luciferase-expressing $T. gondii$ line PTGluc (type II, cloned from ME49/PTG-GFPS65T) [30], RH-LDMluc (Type I, cloned from RH-GFP65T) [30], CTGluc (type III) [66] and PRU-RFP [67] were maintained by serial 2-days passage in human foreskin fibroblast (HFF) monolayers. HFFs were propagated in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) with 10% fetal bovine serum (FBS), gentamicin (20 $\mu$g/ml, Gibco), glutamine (2 mM, Gibco) and HEPES (0.01 M, Gibco) referred to as complete medium (CM).

Mice

C57BL/6 mice (6–10 weeks old) were maintained and bred at the animal facility of the department of Microbiology, Tumor and Cell Biology, Karolinska Institutet (Stockholm, Sweden). For bioluminescence assays, male BALB/c mice (6–8 weeks old) were


purchased from Charles River (Sulzfeld, Germany) and maintained under pathogen-free conditions.

**In vitro generated DC, monocytes and astrocytes**

Mouse bone marrow-derived DC were generated as described [68]. Briefly, cells from bone marrow of C57BL/6 mice were grown in CM containing 20% supernatant from the GM-CSF-secreting cell line 3X63 or 10 ng/ml recombinant mouse GM-CSF (Peprotech). Loosely adherent cells were harvested on day 6. To generate human monocyte-derived DC, buffy coats obtained from healthy blood donors at the Karolinska University Hospital Blood Center were treated with 1 ml RosetteSep (StemCell Technologies) per 15 ml of buffy coat, followed by centrifugation on Lymphoprep (Axis.Shield PoC AS) gradients. The population, defined as monocytes, exhibited CD14+ (DakoCytomation) and <1% CD3+/19+ (BD Biosciences) as evaluated by flow cytometry (FACSCalibur, BD Biosciences). DC were generated as described previously [69]. Briefly, purified cells were cultured 7 days in CM supplemented with 100 ng/ml GM-CSF (Peprotech) and 12.5 ng/ml IL-4 (R&D Systems). DC were typified by expression of CD1a, CD11b, CD14 (DakoCytomation), CD80, CD83, CD86, HLA-DR, CD11a, CD18 (BD Biosciences). Primary astrocytes were generated from cortices from 1–3 day-old C57BL/6 mice as previously described [47].

**Immunocytochemistry**

Human monocyte-derived DC were cultured on poly-L-lysine-coated glass coverslips (Sigma) and challenged with freshly egressed tachyzoites (PTGhuc, MOI 1) for 16 h. After fixation (3% paraformaldehyde; Sigma) and blockade (5% FBS; Gibco), the cells were stained with mouse-anti human β1 (NeuroMab; clone N87/25; UC Davis/NIH NeuroMab Facility; 1:250). Anti-mouse Alexa Fluor-conjugate (Invitrogen) was used as secondary antibody. Slides were mounted using VectaShield with DAPI (Vector Laboratories) and assessed by epifluorescence microscopy (Leica DMRB).

**Methods for electrophysiology**

DC (5×10⁶ cells) were collected and centrifuged for 2 min at 100 g. The supernatant was removed, the pellet washed with the extracellular solution, and centrifuged for 2 min at 100 g. The pellet was then resuspended using 100 μl of the extracellular solution. Nanion’s Port-a-Patch chip technology (Nanion, Germany) was used to voltage-clamp the cells. A cell suspension of 3 μl was dispensed into the extracellular chamber containing the recording chip of 2–3.5 MΩ. Currents were recorded at holding potentials of −80 mV. GABA (1 μM and 1 mM) or GABA plus 100 μM SR95531 (GABA_A antagonist, Sigma-Aldrich) plus 100 μM SR95531 (GABA_A antagonist, Sigma-Aldrich) was used to record GABA responses. The whole-cell configuration was established and currents were recorded at holding potential of −80 mV. GABA (1 μM and 1 mM) or GABA plus 100 μM SR95531 (GABA_A antagonist, Sigma-Aldrich) was used to record GABA responses. External solution in mM: 145 NaCl, 5 KCl, 1 MgCl_2, 1.8 CaCl_2, 10 TES (pH 7.3) and 297 mM/kg. Internal recording solution in mM: 50 NaCl, 10 NaCl, 60 Cs-Fluoride, 20 EGTA, 10 TES (pH 7.3) and 284 mM/kg. Extracellular recording solution in mM: 80 NaCl, 3 KCl, 10 MgCl_2, 35 CaCl_2, 10 HEPES (pH 7.3) and 296 mM/kg. All patch-clamp recordings were performed at room temperature (20–22 °C). Patch-clamp recordings were done using an Axopatch 200B amplifier, filtered at 2 kHz, digitized on-line at 10 kHz using an analogue-to-digital converter and analyzed with pClamp software (Molecular Devices, USA).

**RNA analysis with real time qPCR**

Total RNA was extracted using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen). All primers were initially screened for signal detection using cDNA and conventional PCR. Following sequence confirmation, gene transcription levels were monitored. Using the SYBR Green Master Mix (Applied Biosystems) PCR reactions were carried out on the Applied Biosystems 7500 Fast Real-Time PCR System using the following program: 10 min holding at 95°C, 40 cycles of 95°C for 15 s, 60°C for 1 min. A dissociation stage (95°C for 15 s and 60°C for 1 min, 95°C for 15 s) was added to generate a melting curve for data analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. The data was analyzed using Sequence Detection Software v.1.3.1 (Applied Biosystems) and fold changes in expression were calculated using the comparative ΔCT method against the non-infected DC control. Primers against the 19 GABA_AR subunits (Table S1) were designed using OligoPerfect Designer software (Invitrogen) and Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and validated using OligoAnalyzer (http://eu.idtdna.com/Analyzer/Applications/OligoAnalyzer). The primers were purchased from Invitrogen or Qiagen.

**GABA and glutamate ELISA**

DC were plated at a density of 1×10⁶ cells per well and incubated with freshly egressed T. gondii tachyzoites (MOI 1) or with soluble extracts and reagents in a total volume of 700 μl CM for 24 h. Freshly egressed tachyzoites were heat-inactivated at 56°C for 30 min. Parasite lysates were obtained by harvesting supernatants after sonicating 1×10⁶ tachyzoites for three 30 s pulses (Soniprep 150). Reagents were purchased from Sigma-Aldrich unless stated otherwise, and added at the following final concentrations: Semicarbazide (SC, 50 μM); (S)-SNAP-5114 (SNAP; 50 μM); LPS (100 ng/ml). The cells were centrifuged at 4000 g for 2 min, and the medium supernatant collected and stored at -70°C until analysis. Samples were extracted, derivatized, and incubated with antisera according to manufactures protocol (GABA or Glutamate Research ELISA kits, Labor Diagnostica Nord, Nordhorn, Germany). GABA and glutamate concentrations were quantitatively determined by ELISA, monitored at 450 nm (Multiskan EX, Labsystem Oy, Finland).

**In vitro migration assays and replication assays**

DC and monocytes were plated at a density of 1×10⁶ cells per well and incubated with freshly egressed T. gondii tachyzoites (at indicated MOI) or with soluble extracts and reagents in CM for 6 h. Cells were then gently transferred into triplicate transwell filters (8 μm pore size; BD) at a density of 2×10⁵ per well, and incubated as indicated. Reagents were added at the following final concentrations: Muscimol (300 μM); Bicuculline (50 μM); Baclofen (500 μM); CGP35348 (500 μM); Semicarbazide (50 μM); SNAP 5114 (50 μM); LPS (100 ng/ml); GABA (0.5 μM). When indicated, parasite viability and replication was assessed by plaquing assays on HFF monolayers and vacuole counts in DC as previously described [47]. DC integrity was monitored by propidium iodide staining. In the GAD/GAT4 migration restoration experiment, GABA supernatant was obtained from infected DC which had been incubated for 24 h in CM to enrich for DC derived GABA or commercially available exogenous GABA was added to the transwell medium following DC transfer. Migrated DC were quantified using a neubauer hemocytometer.

**Chemotaxis assays, imaging and analysis**

Non-infected DC or Toxoplasma-infected DC (PTGhuc/MOI 1) were incubated ± LPS (200 ng/ml) as well as ± SNAP5114 (50 μM) or Semicarbazide (50 μM) for 24 h. Following incubation, DC were mixed with Collagen I solution (3 mg/ml, Gibco),
Figure 7. Decreased dissemination and parasite load after adoptive transfer of *Toxoplasma*-infected DC treated with GABAergic inhibitors. (A) BALB/c mice were challenged with 5 x 10^6 cfu of freshly egressed PTGluc tachyzoites (Toxo), 5 x 10^6 cfu of tachyzoite-infected DC (Toxo-DC) or 5 x 10^6 cfu tachyzoite-infected DC treated with SNAP and SC (Toxo-DC + SNAP&SC). Photonic emissions were assessed by BLI on five consecutive days after inoculation of mice. Images show progression of infection and parasite biomass expansion in mice. Control shows non-infected mouse. Color scales indicate photon emission (photons/s/cm^2/sr) during a 180 s exposure. Data are from one set of mice and representative of two independent experiments with three to five mice per group. (B) Total photon emission analysis from individual mice on days 1–5 post inoculation showed a decrease in parasite tissue burden in mice inoculated with GAD and GAT4 inhibitor-treated DC infected with *T. gondii* when compared with burdens from mice inoculated with untreated DC infected with *T. gondii*. Photonic emissions were assessed (photons/s/ROI) during a 180 s exposure. Data are from two independent experiments with 3–5 mice per group. Asterisks indicate a significant increase in photon emissions on day 4 and 5 post infection from mice in the “Toxo-DC” group compared to mice in the “Toxo” or “Toxo-DC (+SNAP&SC)” groups (P<0.0004, GLM ANOVA, Tukeys Pairwise comparison). ROI – region of interest. (C) *Ex vivo* photonic emissions from spleen, MLN and brains of mice BALB/c mice challenged with 10^5 cfu of tachyzoite-infected DC (Toxo-DC) or 10^5 cfu tachyzoite-infected DC treated with SNAP and SC (Toxo-DC + SNAP&SC). Color scales indicate photonic emissions (photons/s/cm^2/sr) during a 180 s exposure as indicated under Materials and Methods. Representative images from day 2 and day 5 post-inoculation are shown. White arrows (lower panel) indicate signal from the brain tissue. (D) Box-plot and whiskers graph represents the lower, upper quartiles, median and minimum–maximum of photonic emissions *ex vivo* from the brains of infected mice. The white bars indicate brains from mice infected with tachyzoite-infected DC (Toxo-DC) and the dark grey bars indicate brains from mice infected with tachyzoite-infected DC treated with SNAP and SC (Toxo-DC + SNAP&SC) (n=4/group/day). Non-significant differences were observed between the treated and untreated groups (P>0.05; Kruskal Wallis test) with significant differences on days 1 and 2 (P<0.05, Student’s t test, indicated by asterisks). (E) Parasite load in spleen, MLN and brain on days 1–6 post inoculation quantified by plaquing assays as indicated under Materials and Methods. BALB/c mice were challenged with 10^5 cfu of tachyzoite-infected DC (Toxo-DC, open circles) or 10^5 cfu tachyzoite-infected DC treated with SNAP and SC (Toxo-DC + SNAP&SC, filled triangles). Mean parasite tissue load for each group is indicated (open and filled lines respectively) and dashed lines represent trendlines for the two groups (n=4/group/day). Significant differences in parasite load between groups were observed for spleen and brain (P<0.022 and P<0.02 respectively, Kruskal-Wallis test). Non-significant differences were observed in MLN (P>0.05, Kruskal-Wallis test) with significant differences between groups on days 2, 4, 5, 6 (P<0.05, Student’s t-test).

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7.5% NaHCO₃ (Invitrogen) and 10× Minimum Essential Medium (MEM; Invitrogen). Approximately 7.5 × 10⁴ cells were loaded into μ-slide 3D chemotaxis chamber slides (Ibidi) and placed at 37 °C, 5% CO₂ to allow gel formation. Then, to establish a gradient, 1.25 μg/ml CCL19 (R&D Systems) or GABA (0.5 μM or 5 μM) were added to one chamber reservoir whilst the other reservoir was filled with CM. Control experiments used CM in both reservoirs. Cell migration was monitored using a Zeiss AxioImager Z1 microscope and AxioVision software (version 4.7.2). Images were taken every 60 s for 60 min. Cell tracking and chemotaxis analysis were performed using ImageJ (http://imagej.nih.gov/ij/) with Manual Tracking (Cordelieres, Institute Curie) and Chemotaxis Tool (Ibidi) plugins.

Flow cytometry
CCR7 expression of human monocyte-derived DC was studied using FITC-labeled anti-CCR7 and mouse IgG2a isotype control antibodies (R&D Systems). Marine bone marrow-derived DC were stained with CCL19-Fc (eBiosciences), CD4, CD80, CD86, MHC II, CD18 antibodies (BD Biosciences) as indicated by the manufacturer. Data were generated using a Cyan ADP (Beckman Coulter) or a FACSCalibur (BD Bioscience) flow cytometer. Fluorescence-activated sorting of Toxoplasma-infected cells was performed at the Center for Cell Analysis, Karolinska institutet on a FACS Aria (BD Bioscience) system. Dead cells were gated out by SYTOX Blue stain (Invitrogen) and pre-sorting infection rates were 43–63%. Data analysis was done with FACSDiva software, version 6.1.3 (BD Bioscience).

Adoptive transfers of DC
DC were challenged with freshly egressed PTGluc tachyzoites for 6 h at MOI 1. Extracellular parasites were removed following three washes at 80 g. Following infection and resuspension in PBS, 5 × 10⁴ colony forming units (cfu) were adoptively transferred into male BALB/c mice. Total numbers of cfu injected into animals was confirmed by plaquing assays [70]. SNAP 5114 (50 μM) and Semicarbazide (50 μM) were added upon DC infection for 5 h. When indicated, such groups were treated with an additional 50 μM combination therapy of SNAP 5114 and Semicarbazide for 1 h, and added to PBS DC suspension prior to injection. DC were stained with 5 μM 5,6,6-[4-chloromethyl]benzoyl-amino-tetramethylrhodamine (CMTMR; Molecular Probes) or 5 μM carboxyfluorescein diacetate, succinimidyl ester (CFSE; Invitrogen) as indicated by the manufacturer. Stained cells (±50 μM SC+50 μM SNAP for 6 h) were then injected i.p. into BALB/c mice. After 12 h, the spleens were harvested, homogenized and cells were analyzed by flow cytometry (FACS Calibur). An intraperitoneal lavage was performed and cells were similarly analyzed by flow cytometry.

In vivo bioluminescence imaging (BLI)
BLI was performed as described [30]. Briefly, BALB/c mice inoculated i.p. with freshly egressed PTGluc tachyzoites, or with PTGluc-infected DC ± Semicarbazide and SNAP 5114 were injected i.p. with 1.5 mg D-luciferin potassium salt (Caliper Life Sciences, Hopkinson, MA, USA) and anaesthetized with 2.3% isoflurane prior to BLI. Ten min after injection of D-luciferin, biophotonic images were acquired at a binning of 8 (medium) for 180 s with an In Vivo Imaging System Spectrum CT (Caliper Life Sciences). For ex vivo imaging of organs, mice were injected i.p. with 1.5 mg D-luciferin and euthanized after 10–15 min. Organs were extracted as assessed as above. Analysis of images and assessment of photons emitted from a region of interest (ROI) was performed with Living Image software (version 4.2; Caliper Life Sciences).

Plaquing assays
Plaquing assays were performed as described [14]. Briefly, organs were extracted and homogenized under conditions that did not affect parasite viability. The number of parasites was determined by plaque formation on HFF monolayers.

Statistical analysis
All statistics were performed using Minitab version 15 (Minitab Inc, PA, USA).

Online supplemental material
Table S1 shows the 19 subunit primer pair sequences used to screen DC and astrocyte cDNA for GABAAR subunit transcripts and primer sequences for GAD 63, GAD 67, GAT4 and GAPDH cDNA. Figure S1 shows the ELISA-determined glutamate levels from non-infected and Toxoplasma-infected DC supernatant. Figure S2 shows GABA secretion and transmigration by monocytes upon challenge with T. gondii. Figure S3 shows GABA secretion of DC challenged with T. gondii type I, II and III strains. Figure S4 shows immunocytochemistry of infected and non-infected DC. Figure S5 shows parasite replication in DC in the presence of GABAergic inhibitors. Figure S6 shows the effects of GABAergic inhibitors on tachyzoite-infected DC and extracellular tachyzoites. Figure S7 shows effects of GABAergic inhibition on activation and maturation markers of DC. Figure S8 shows flow cytometric analyses of DC in vivo after GABAergic inhibition.

Supporting Information
Figure S1 DC glutamate secretion is transiently affected by Toxoplasma infection. Mouse DC were challenged with PTGluc tachyzoites (MOI 1) and glutamate levels were analyzed at indicated time points using a glutamate ELISA kit as described in Materials and Methods. Values represent means (±SEM) from two experiments performed in quadruplicate. (*) indicates a statistically significant increase in glutamate levels following infection at 3 h (P = 0.034) and 8 h (P < 0.001). However, there were no significant differences in glutamate levels by the end of the time course (GLM ANOVA, Tukeys pairwise comparison, P > 0.05). (TIF)

Figure S2 GABA secretion and transmigration by monocytes upon challenge with T. gondii. (A) Monocytes respond with GABA secretion upon challenge with T. gondii. Freshly isolated human monocytes from four donors (D1–D4) were incubated with PTGluc tachyzoites (MOI 1) for 24 h. GABA in the supernatant was quantitatively determined by ELISA as described in Materials and Methods. For each donor, values represent mean (±SD) performed in triplicate. (B) Transmigration of freshly isolated monocytes. Monocytes were challenged with PTGluc tachyzoites (MOI 3) or PTGluc tachyzoites plus SNAP (GAT4 inhibitor, 50 μM) and SC (GAD inhibitor, 50 μM) as described in Materials and Methods. Monocytes were then transferred to transwells, and allowed to transmigrate in the presence of CM (complete medium) or inhibitors (SNAP, 50 μM + SC, 50 μM). Cell migration was determined using a neutubauer hemocytometer. Values represent means (±SD) from two different human donors done in triplicate. (*) indicates significant difference (P < 0.05, Student’s t-test): ns: non-significant. (TIF)
**Figure S3** GABA secretion of DC challenged with *T. gondii* type I, II and III strains. Mouse DC were challenged with RH-LDHuc (type I), PTGluc (type II) or CTGluc (type III) at indicated MOIs. GABA in the supernatant was quantitatively determined after 24 h by ELISA as described under Materials and Methods. One representative experiment performed in duplicate is shown. Performed twice with similar result. Non-significant differences were observed between strains (P>0.05, Student’s t-test).

(TIF)

**Figure S4** Infected and non-infected DC express the GABA_A receptor β3 subunit. Micrographs show immunocytochemistry of DC challenged with GFP-expressing *T. gondii* tachyzoites (PTGluc, green) stained with GABA_A receptor β3 subunit monoclonal antibody (red) and DAPI (blue). Stainings were performed as indicated under Materials and Methods. Scale bar: 10 μm.

(TIF)

**Figure S5** Parasite replication in the presence of GABAergic inhibition of DC. Replication of tachyzoites in DC assessed by vacuole size counts at the indicated time points as described in Materials and Methods. Non-significant differences were observed between samples in complete medium (CM) and samples treated with a combination of SC (GAD inhibitor, 50 μM) and SNAP (GAT4 inhibitor, 50 μM) (P>0.05; Mann-Whitney U test). Geneticin, a blocker of polypeptide synthesis in eukaryotic cells, was used as a reference control. Significant differences were observed in samples treated with geneticin (P<0.05; Mann-Whitney U test).

(TIF)

**Figure S6** GABAergic inhibitors target host cell-derived GABA production and transport. Extracellular PTGluc tachyzoites (Toxo) or tachyzoite-infected mouse DC (MOI 1; Toxo-DC) were incubated for 24 h in CM under different conditions. SC (GAD inhibitor, 50 μM); SNAP (GAT4 inhibitor, 50 μM); GABA levels were analyzed using a GABA ELISA kit as described in Materials and Methods. Values represent means (±SE) from two independent experiments performed in quadruplicate. (*) indicates a significant decrease in GABA production compared to untreated *Toxoplasma*-infected DC (P<0.008, Mann Whitney U test).

(TIF)

**Figure S7** Co-stimulatory molecule stainings and maturation markers of DC in presence of GABAergic inhibitors. Mouse bone marrow-derived DC were treated as indicated for 12–16 h and stained as described under Materials and Methods. "Untreated" and "LPS" indicate DC in complete medium or exposed to LPS (200 ng/ml), respectively; "Toxo" indicates DC challenged with freshly egressed GFP-expressing PTGluc tachyzoites (MOI 1). "SC + SNAP" indicates treatment with SC (GAD inhibitor, 50 μM) and SNAP (GAT4 inhibitor, 50 μM). Results are representative of two or three independent experiments with similar results.

(TIF)

**Figure S8** Prevalence and dissemination of adoptively transferred DC upon GABAergic inhibition. BALB/c mice were inoculated i.p with 5×10^6 untreated DC labeled with CMTMR and 5×10^6 untreated DC labeled with CFSE (top panel) or with 5×10^6 untreated CMTMR-labeled DC and 5×10^6 SC+SNAP treated DC, labeled with CFSE. After 12 h, an intraperitoneal lavage was performed and spleens were extracted and processed for flow cytometry as indicated in Materials and Methods. Dot plots of CMTMR (γ-axis) and CFSE (x-axis) show numbers of SC+SNAP treated DC as compared to the untreated population, in both the spleen and peritoneal lavage. Numbers indicate the relative portion of each quadrant related to the total population. Double-positive (CMTMR/CFSE) cells may indicate uptake in phagocytic cells. Data shown are from one experiment, and are representative of the results from 2 independent experiments.

(TIF)

**Table S1** Primer pair sequences used to screen DC and astrocyte cDNA for 19 GABA_A subunit transcripts and primer sequences for GAD 1, GAD 2, GAD 3, GAD 4, and GAPDH cDNA. Primers were designed as indicated under Materials and Methods.

(DOCX)

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**Author Contributions**

Conceived and designed the experiments: AB JMF JMW RBGA BB. Performed the experiments: JMF RBGA JMW ZJ RPAW BR AB. Analyzed the data: JMF RBGA SKM ZJ BB RPAW BR AB. Wrote the paper: AB JMF JMW.

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