Increased GABA(A) channel subunits expression in CD8(+) but not in CD4(+) T cells in BB rats developing diabetes compared to their congenic littermates

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Increased $GABA_A$ channel subunits expression in $CD8^+$ but not in $CD4^+$ T cells in BB rats developing diabetes compared to their congenic littermates.

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Abstract

GABA (γ-amninobutyric acid), the main inhibitory neurotransmitter in the central nervous system is also present in the pancreatic islet β cells where it may function as a paracrine molecule and perhaps as an immunomodulator of lymphocytes infiltrating the pancreatic islet. We examined $CD4^+$ and $CD8^+$ T cells from diabetes prone ($DR^{lyp/lyp}$) or resistant ($DR^{+/+}$) congenic biobreeding (BB) rats for expression of $GABA_A$ channels. Our results show that BB rat $CD4^+$ and $CD8^+$ T cells express α1, α2, α3, α4, α6, β3, γ1, δ, ρ1 and ρ2 $GABA_A$ channel subunits. In $CD8^+$ T cells from $DR^{lyp/lyp}$ animals the subunits were significantly upregulated relative to expression levels in the $CD8^+$ T cells from $DR^{+/+}$ rats as well as from $CD4^+$ T cells from both $DR^{lyp/lyp}$ and $DR^{+/+}$ rats. Functional channels were formed in the T cells and physiological concentrations of GABA (100 nM) decreased T cell proliferation. Our results are consistent with the hypothesis that GABA in the islets of Langerhans may diminish inflammation by inhibition of activated T lymphocytes.

Key words: GABA, diabetes, lymphocytes, $GABA_A$ subunits, proliferation, immunomodulation.

1. **Introduction**

Neurotransmitters are not only effector molecules in the nervous system but are also emerging as modulators of the immune system where they may modulate the function of immune cells such as lymphocytes (Bjurstom et al., 2008). In recent years reports have emerged indicating that immune cells may express $GABA_A$ channels but what physiological role they have is not clear (Alam et al., 2006; Bhat et al., 2010; Bjurstom et al., 2008; Reyes-Garcia et al., 2007; Tian et al., 2004). Lenmark and colleagues (Michalkiewicz et al., 2004) have generated congenic Biobreeding (BB) rats that develop spontaneously type 1 diabetes ($DR^{lyp/lyp}$) and on the same genetic background BB rats that are diabetes resistant ($DR^{+/+}$). The diabetic prone ($DR^{lyp/lyp}$) rats shares characteristics such as hyperglycemia, hypoinsulinemia and weight loss with human type 1 diabetes (Elder and Maclaren, 1983; Hawkins et al., 2005). A unique feature of the BB rat is that lymphophenia is a prerequisite for diabetes (Markholst et al., 1991) in addition to cells of the immune system that are thought to be actively involved in the destruction of the pancreatic islet β cells (Ramanathan and Poussier, 2001). Glutamic acid decarboxylase (GAD), which catalyses the formation of GABA from glutamate is a major autoantigen in type 1 diabetes (Lernmark et al., 1978). The signal molecule GABA binds to $GABA_A$ receptors ($GABA_A$ channels) and opens their chloride channel. GABA is the predominant inhibitory neurotransmitter in the mammalian central nervous system. Outside the nervous system GABA is produced by the pancreatic islet β cells and is present in blood in low concentrations. Pancreatic α cells and some blood cells express $GABA_A$ channels (Gladkevich et al., 2006; Olsen and Sieghart, 2008). In T lymphocytes it is known that neurotransmitters´ receptors can be regulated by the T cell receptor activation, cytokines or the neurotransmitters themselves (Bjurstom et al., 2008;

Levite, 2008; Tian et al., 2004). But what is unclear is the role these receptors/channels have in normal T cell function and disease. The $GABA_A$ channels are heteropentamers and to-date 19 $GABA_A$ channel subunits have been cloned (Birnir and Korpi, 2007). It is possible that extracellular GABA may be immunomodulatory in the endocrine pancreas and blood and affect the fate and function of cells that express high-affinity $GABA_A$ channels. In this study we examined if $CD4^+$ and $CD8^+$ T lymphocytes isolated from mesenteric lymph nodes from prediabetic and diabetic $DR^{lyp/lyp}$ and age matched $DR^{+/+}$ rats express $GABA_A$ channel subunits, make functional channels and if GABA can modulate proliferation of the cells. Our results show that in BB rats, the $GABA_A$ channel subunits are expressed in the $CD4^+$ and the $CD8^+$ T cells, are upregulated in $DR^{lyp/lyp}$ $CD8^+$ T cells relative to the $DR^{lyp/lyp}$ $CD4^+$ T cells and $DR^{+/+}$ and $DR^{+/+}$ $CD8^+$ T cells, form functional channels and modulate T cell proliferation.

2. Materials and Methods.

2.1. Animals. The congenic BB rats used in this study were developed as described (Hawkins et al., 2005). All animals were housed and bred in specific pathogen free conditions. Sibling diabetes resistant (DR) lyp/+ F1 breeding pairs were used to generate F2 offspring at the expected Mendelian ratio of 25% lyp/lyp, 25% +/+ and 50% lyp/+. Development of diabetes in the diabetic prone rats $DR^{lyp/lyp}$ is gender independent with an incidence between 53 and 95 days of age. Rats were kept on a 12 h light/12 h dark cycle with food and water available ad libitum. In this study we used prediabetic (45 days old) and diabetic $DR^{lyp/lyp}$ rats that were age matched with $DR^{+/+}$ rats. The experiments were done according to guidelines of the Malmö-Lund region and Uppsala region animal care committees. All $DR^{lyp/lyp}$ rats were tested daily for glucose levels from 40 days of age until diabetes onset. Blood glucose levels above 11.1 mM defined animals as hyperglycemic.

2.2. Genotyping. Tail snips were obtained from rats at 21 days of age and DNA was extracted using a standard isopropanol/ethanol protocol. After extraction, DNA was subject to PCR using GoTaq polymerase and Well-Red Dye-labeled oligos (SigmaAldrich) (MacMurray et al., 2002; Moralejo et al., 2003) for subsequent microsatellite analyses using the CEQ 8000 system (Beckman Coulter).

2.3. Tissue collection and cell isolation procedure. Animals were sacrificed at 45 days of age or upon hyperglycemia diagnosis along with the wild-type counterpart in a CO_2 chamber. Mesenteric lymph nodes were collected from each animal and put into separate tubes containing ice-cold 1 X phosphate buffered saline (PBS) at pH 7.4. Single cell suspensions were prepared by straining the lymph nodes on a 70 μm cell strainer (BD Falcon, Belgium) with syringe plunger and then collected into a tube by draining with ice-cold 1 X PBS. The

cells were centrifuged at 300 X g for 10 minutes at 4^{o}C and then washed three times to get a clean cell suspension. The cells were counted and assessed for viability with trypan blue.

2.4. CD4^{+} and CD8^{+} T lymphocyte separation. The cell suspensions were divided in two halves, each half of the cells were magnetically labeled with MACS CD8a Micro Beads or CD4 Micro Beads (Miltenyi Biotec, Germany) and incubated for 15 minutes at 4 - 8 ^{o}C. The ratio of cells to magnetic beads was according to the protocol supplied by the company. The labeled cells were then washed with 1 X PBS buffer and centrifuged for 10 min at 300 X g at 4 ^{o}C. The cell pellets were resuspended in MACS buffer (2 mM EDTA, 0.5 % FBS, pH 7.4). The magnetically labeled cells suspended in the MACS buffer were separated by using autoMACS (Miltenyi Biotech, Germany). The positive labeled cells of CD4^{+} and CD8^{+} T cell fractions were collected. The isolated CD4^{+} and CD8^{+} T cells were assessed for viability using trypan blue and counted. The purity of the separated CD4^{+} and CD8^{+} T cells was determined by flow cytometry. Fifty percent of the positive CD4^{+} and CD8^{+} T cells were stained with FITC anti-rat CD8a, PE anti-rat αβ T cell and PE-CY5 anti-rat CD4 (BD pharmingen) in 1:100 dilution and incubated for 15 min at 4 ^{o}C . The labeled cells were then washed with a staining buffer and spun down for 2 min at 300 X g. Afterwards the stained cells were fixed with a buffer containing 2% formaldehyde and analyzed by flowcytometry (FACSCalibur, BD Biosciences). The lymphocytes were gated on the basis of forward and side scatter, analyzed using CellQuest Software and yielded more than 90% pure CD4+ and CD8+ T cells using the positive selection method**. The CD4^{+} were generally more than 97% pure but somewhat more variation was found for the CD8^{+} cells.**

2.5. RNA isolation and RT-PCR Total RNA was extracted from the magnetically separated CD4^{+} and CD8^{+} T cells using GenElute total RNA miniprep (Sigma). The RNA

concentration was measured with a spectrophotometer (Nanodrop ND-1000). Fifty nanograms of total RNA was reverse transcribed to cDNA with random hexamers, 100 μM oligoDT (TAG Copenhegen A/S, Denmark), 10 mM dNTPs (Invitrogen) and 200 U/μL Superscript III reverse trancriptase (Invitrogen). Real-time PCR was performed using 7900HT (ABI, Applied Biosystems) to amplify cDNA. A three step procedure; 95 ^{o}C for 5 min, followed by 45 cycles at 95 ^{o}C for 15 s, 60 ^{o}C for 30 s and 72 ^{o}C for 30 s. The reaction mixture contained a total volume of 10 μl with 0.5 X SYBR Green I (Invitrogen), 0.3 mM dNTPs (TAG Copenhegen A/S, Denmark), 1 X PCR buffer, 3 mM $MgCl_2$, 1 X ROX as the reference dye, 0.7 U Taq DNA polymerase (all from Sigma) , 0.4 μM of each primer and 0.25 ng/μl of cDNA per well. All the primers were verified using rat brain cDNA and the correct size of the amplified product was confirmed by gel electrophoresis on a 1.7 % agarose gels. All the samples were run in duplicates with primers against $GABA_A$ channel subunits [Table 1], associated proteins and housekeeping gene β-actin using cDNA from $CD4^+$ and $CD8^+$ T cell total RNA (TAG Copenhegen A/S, Denmark).

We used the comparative C_T method to show the relative gene expression (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). The relative gene expression presents the data of the gene of interest relative to some internal control gene. The quantitative endpoint for rt-PCR is the threshold cycle (C_T). The C_T is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrary placed threshold when the PCR is in the exponential phase of the amplification (Schmittgen and Livak, 2008). The advantage of the comparative C_T method is the ability to present the data as "fold change" in expression. For each subunit, the C_T values of target mRNA was normalized with the C_T value of the housekeeping gene β-actin using the formula $\Delta C_T = C_{T(target)} - C_{T(\beta\text{-actin})}$ and the data is

presented as $2^{-\Delta C_T}$ (Schmittgen and Livak, 2008). The method was further used to determine the relative expression of genes from two samples: $\Delta\Delta C_T = \Delta C_{T\ (sample\text{-}A)} - \Delta C_{T\ (sample\text{-}B)}$. Here sample-B was used as the control. The data is presented as $2^{-\Delta\Delta C_T}$ (Schmittgen and Livak, 2008).

2.6. Cell culture. The separated $CD4^+$ and $CD8^+$ T cells were centrifuged at 300 X g for 10 minutes. The supernatants were discarded and the cell pellets resuspended in RPMI-1640 supplemented with 2 mM L-glutamate, 25 mM HEPES, 10 % fetal bovine serum (FBS), 100 U/ml penicillin, 10 μg/ml streptomycin, 50 μg/ml gentamycin, 5 μM beta-mercaptoethanol. For electrophysiological experiments the $CD4^+$ and the $CD8^+$ T cells were cultured in a flat bottom 96 well plate (Cellstar) in an incubator with 5 % CO_2 at 37 ^oC.

2.7. Electrophysiology. The cells were washed with the extracellular recording solution containing in mM: 150 NaCl, 1 CsCl, 0.2 $MgCl_2$, 0.2 $CaCl_2$, 10 TES (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid). GABA and THDOC (3α,21-dihydroxy-5α-pregnan-20-one) were dissolved in the pipette solution which contained in mM: 75 NaCl, 75 CsCl, 1 $MgCl_2$, 1 CaCl2, 10 TES and 0.2 saclofen and used in the cell-attached recordings. The pH of the solutions was adjusted to 7.4 with NaOH. Pipettes were made from borosilicate glass and fire polished. They generally had pipette resistance between 8 and 20 MΩ when filled with the pipette solution. Currents were recorded with an Axopatch 200B amplifier (Molecular Devices,Palo Alto, CA, USA) in the cell-attached and inside-out patch-clamp configurations using conventional patch-clamp techniques. Currents were filtered at 2 kHz, digitized at 10 kHz using a DigiData 1322A analogue-to-digital converter interfaced with a computer, and analyzed by pCLAMP 9.2 software (Molecular Devices). Experiments were carried out at room temperature (20-22 ^0C).

2.8. Thymidine incorporation assay. The cells were suspended in supplemented RPMI-1640 in round bottomed 96 well culture plates (Nunc, Denmark). Wells to be used in stimulation of the cells were coated with 0,5 μg/ml of immobilized anti-rat CD3 mAbs (BD pharmingen) diluted in Tris buffer at pH 9.5 and the plate incubated at 37 ^{o}C for 5 hrs. The plates were then washed with 1 X PBS. 20,000 cells per well were added to the uncoated and anti-CD3 mAbs coated wells and incubated for 72 hrs at 37 ^{o} C in a 5 % CO_2 incubator. GABA (0, 0.1, 1.0, 100 μM) was added to the wells after 24 hrs of culturing and the plates incubated for another 48 hrs. During the last 18 hrs of the incubation period, 10 μl of 1 μCi of H^3- thymidine (Sigma) was present. The cells were then harvested onto a glassfibre filters using an automated multichannel harvester (PerkinElmer, USA). Filters loaded with the cells were dried and transferred to plastic scintillation vials containing 3 ml of scintillation fluid (PerkinElmer, USA), soaked for 3 hrs and counted in a scintillation counter. Each condition was assayed at the same time in triplicates.

2.9. Statistical analysis.

Data are presented as means $\pm$ SEM. Statistical comparisons were assessed with one way ANOVA and students t-test.

3. Results

3.1. $CD4^+$ and $CD8^+$ T cells from both $DR^{+/+}$ and $DR^{lyp/lyp}$ rats express $GABA_A$ channel subunits.

$GABA_A$ channel subunits are most highly expressed in the brain but the expression of specific subunits varies between brain regions and with development. Nineteen different $GABA_A$ receptor ($GABA_A$ channel) subunit isoforms have been cloned and they are grouped into eight sub-families α(1-6), β(1-3),γ (1-3), δ, ε, π, θ and ρ(1-3). The channels are pentameric and normally require 2α, 2β and a third subunit to form functional heteromeric channels. The exception is channels formed by the ρ subunits, they can be homomeric. The pharmacological and functional properties are defined by the subunit composition of the channels and can be influenced by intracellular accessory proteins (Birnir and Korpi, 2007; Olsen and Sieghart, 2008). Wild-type $DR^{+/+}$ rats are neither lymphopenic nor develop T1D spontaneously whereas the $DR^{lyp/lyp}$ rats are lymphopenic and develop T1D spontaneously when 53-95 days old (MacMurray et al., 2002) . We therefore examined cells from 45 days old animals when the $DR^{lyp/lyp}$ rats have not yet developed diabetes and from older (55 – 77 days) animals where the wild-type ($DR^{+/+}$) animals were age matched to the $DR^{lyp/lyp}$ animals that had developed diabetes. The results are shown in Fig. 1A. $CD4^+$ T cells and B. $CD8^+$ T cells. Nine different subunits of the possible 19 isoforms were expressed in both the $CD4^+$ and the $CD8^+$ T cells in the younger (45 days, Fig. 1Aa,c and Ba,c) and the older (≥ 55 days, Fig. 1Ab,d and Bb,d) animals. The subunits are; α1, α2, α3, α6, β3, γ1, δ, ρ1 and ρ2. Subunits that are not shown in the graph were not detected.

FIG 1 near here

3.2. Relative expression of $GABA_A$ channel subunits in $CD4^+$ and $CD8^+$ T cells.

Fig. 1 shows that the relative expression of the different $GABA_A$ channel subunit isoforms varies between the cell types and also between $DR^{lyp/lyp}$ and $DR^{+/+}$ animals. We have therefore examined how the expression levels vary depending on: **1.** Genotype of animals, $DR^{+/+}$ and $DR^{lyp/lyp}$ rats (Fig. 2 A), **2.** Age, younger (45 days old) and older ($\geq$ 55 days old) rats (Fig. 2B) and **3**. Cell-types, $CD4^+$ and $CD8^+$ T lymphocytes (Fig. 2C) using the comparative Ct method in order to calculate the "fold change" in the level of expression. Each sample is related to an internal control gene (β-actin) and then one of the two samples to be compared is defined as the calibrator (sample 1) and the second sample (sample 2) is compared to the calibrator to calculate the fold change ((Schmittgen and Livak, 2008), see also Material and Methods).

Fig 2 near here

Comparisons of $GABA_A$ channel subunit gene expression based on:

1. Genotype, $DR^{+/+}$ and $DR^{lyp/lyp}$ animals at the same age, same T cell type. The expression levels of the different genes in $CD4^+$ cells is similar in $DR^{+/+}$ and $DR^{lyp/lyp}$ animals as shown in Fig 2Aa,b. The exception is the α4 subunit that is not expressed in $CD4^+$ $DR^{lyp/lyp}$ cells from 45 days old animals. This is in contrast to the expression pattern in $CD8^+$ T cells (Fig 2Ac, d). For both the 45 and the $\geq$ 55 days old animals, the expression of many $GABA_A$ channels subunit genes is significantly higher in cells from the $DR^{lyp/lyp}$ animals as compared to the $DR^{+/+}$ animals. For α2, α3, α6, β3, γ1 and δ expression is more than 30 fold higher in $CD8^+$ $DR^{lyp/lyp}$ cells from 45 days old animals as compared to cells from $DR^{+/+}$ animals of the same age. The difference in expression is less in cells from the older group of animals but still more than 8 fold increase for the α2, α4, α6, β3 and γ1 $GABA_A$ channel subunits in $CD8^+$ $DR^{lyp/lyp}$ as compared to $CD8^+DR^{+/+}$ cells.

2. Age, cells from 45 and ≥ 55 days old BB rats, same T cell type. Fig. 2Ba and c. show that in $CD4^+$ cells that apart from the α4 subunit that is not expressed in $CD4^+ DR^{lyp/lyp}$ cells from 45 days old animals, the expression level is comparable in cells from the younger and the older animals. Similar results were obtained for the $GABA_A$ channels gene expression pattern in $CD8^+ DR^{+/+}$ cells where no difference is observed when the two age groups are compared (Fig. 2Bc). In contrast, in $CD8^+ DR^{lyp/lyp}$ T cells from the older animals, the α3, α6, β3 and ρ2 are significantly down-regulated as compared to subunits expressed in cells from the 45 days old animals and the α4 is present in the ≥ 55 days old animals.

3. Cell type, $CD4^+$ and $CD8^+$ T cells, same age animals . Fig. 2Ca and b show that there is no difference in $GABA_A$ channel gene expression in $CD4^+ DR^{+/+}$ as compared to $CD8^+ DR^{+/+}$ T cells obtained either from the 45 or the ≥ 55 days old animals. This is different in the diabetes prone $DR^{lyp/lyp}$ animals (Fig 2c and d) where in both age groups there is a significantly higher expression of genes in the $CD8^+$ T cells. In cells from the 45 days old animals α3, α6, γ1, and δ have more than 16 fold higher expression in the $CD8^+ DR^{lyp/lyp}$ cells and in the older animals, the expression level for α1 and α2 is more than 8 fold higher in $CD8^+ DR^{lyp/lyp}$ as compared to the $CD4^+ DR^{lyp/lyp}$ T cells.

3.3. Relative expression of specific intracellular proteins in $CD4^+$ and $CD8^+$ T cells. $GABA_A$ channels are known to interact with a number of intracellular proteins that may affect their function and pharmacology in neurons (Birnir and Korpi, 2007). We examined if proteins known to cluster the channels at synaptic and extrasynaptic sites like gephyrin (Kneussel et al., 1999) and radixin (Loebrich et al., 2006), respectively, were expressed in the lymphocytes. We further examined if the $GABA_A$ receptor associated protein (GABARAP, (Chen and Olsen, 2007)) that is involved in intracellular transport of the channels and affects

their pharmacology is present and whether HAP-1 (Huntington associated protein -1, (Sheng et al., 2006)) that may stabilize the channels in intracellular vesicles and aid their recycling to the plasma membrane is expressed in the T cells. The results are shown in Fig. 3 A. $CD4^+$ T cells and B. $CD8^+$ T cells. All four proteins are expressed in T cells from the 45 and $\geq$ 55 days $DR^{+/+}$ and $DR^{lyp/lyp}$ animals with the exception of HAP-1 which was not expressed in $CD8^+$ $DR^{lyp/lyp}$ cells from 45 days old animals. Radixin is expressed 8 times the level of gephyrin in both $DR^{+/+}$ and $DR^{lyp/lyp}$ cells, supporting the notion of extrasynaptic-like channels in the lymphocytes. GABARAP had the highest mRNAs expression level of the four intracellular proteins examined whereas HAP-1 had the lowest or was similar to the level of the gephyrin mRNAs.

3.4. Physiological concentrations of GABA activate channels.

In the brain GABA channels are located at synapses, where they are activated by mM concentrations of GABA, and outside of synapses (extrasynaptic) where the channels can be activated sub-μM GABA concentrations (Birnir and Korpi, 2007). Furthermore, all neuronal synaptic GABA channels contain the γ2 subunit which is absent in the lymphocytes and thus by default, the $GABA_A$ channels in lymphocytes are of the extrasynaptic kind whether they are located at the immunological synapse or elsewhere on the cell surface. We examined if we could activate channels in the $CD4^+$ and $CD8^+$ T cells from the $DR^{+/+}$ rats with 1 μM of GABA. The results are shown in Fig. 4. The currents were recorded in intact cells at depolarized 60 mV (cell-attached patch-clamp configuration, the pipette potential was -60 mV). The channels in Fig. 4A ($CD8^+$ $DR^{+/+}$) show the typical behavior of extrasynaptic-like GABA channels; they are activated after a delay from the time of drug application and they increase in conductance with time (a, b). In this patch the channels were activated by 1 μM

GABA + 100 nM THDOC. THDOC (tetrahydrodeoxy-corticosterone) is a steroid known to modulate high-affinity GABA channels. Fig 4B. shows channels activated in $CD4^+$ $DR^{+/+}$cells by (a) 1 μM GABA and in another patch (b) where the channels were activated by 1 μM GABA together with 100 nM THDOC. The average conductance for GABA and GABA plus THDOC activated channels was 16 $\pm$ 3 (n = 3) and 20 $\pm$ 1 (n = 6) pS, respectively. Fig. 4C. shows channels in $CD8^+$ $DR^{+/+}$ T cells activated by 1 μM GABA. The average conductance for GABA and GABA and THDOC (50 or 100 nM) activated channels was 14 $\pm$ 1 (n = 4) and 17 $\pm$ 2 (n = 6) pS, respectively. The results show that the $GABA_A$ subunits expressed in the T cells do form functional extrasynaptic-like channels.

Figure 4 near here

3.5. $CD4^+$ and $CD8^+$ T cell proliferation is decreased by sub-μM GABA concentration.

If GABA has immunomodulatory effects, physiological concentrations of GABA can be expected to modulate proliferation of activated T cells. We therefore examined what effects low physiological (100 nM) and a high (100 μM) GABA concentrations had on cell proliferation and the results are shown in Fig. 5. Cells were stimulated with 0.5 μg/ml anti-CD3 antibody and the GABA concentration varied. Control cells were grown in the absence of the anti-CD3 antibody. Each bar represents data from four or five animals and each experiment was done in triplicate. Because the levels of $GABA_A$ channel subunits were similar in the different age groups in $DR^{+/+}$ $CD4^+$ and $DR^{+/+}$ $CD8^+$ T cells and the $DR^{lyp/lyp}$ $CD4^+$ T cells, we grouped the data for the different ages. Adding GABA to the extracellular media of stimulated $DR^{+/+}$ $CD4^+$ (Fig 5a) and $DR^{lyp/lyp}$ $CD4^+$ (Fig 5b) cells inhibited cell proliferation by 30 to 40 % and no significant difference was seen between the low and high concentrations. The result show that 100 nM GABA is saturating and increasing the GABA

concentration 1000 fold does not increase the inhibitory effect on proliferation. The inhibition of the $DR^{+/+}$ $CD8^{+}$ T cell proliferation (Fig 5) in 100 nM GABA was about 30%. The proliferation assay was not done for $CD8^{+}$ T cells from $DR^{lyp/lyp}$ animals as the cell number was lower than required for the assay to be done. The results are consistent with an immunomodulatory role of GABA where GABA imposes a break or slows down cell proliferation.

Fig 5 near here

4. Discussion.

The aim of this study was to examine if T cells from congenic BB rats, a model of type 1 diabetes, expressed $GABA_A$ channel subunits, if functional channels are formed and then, if any correlation could be found between $GABA_A$ channel expression and the onset of diabetes in the rats. We found that surprisingly many $GABA_A$ channels subunits are expressed and that functional $GABA_A$ channels are formed in both the $CD4^+$ and the $CD8^+$ T cells. The channels were activated by submicromolar GABA concentrations in line with formation of high-affinity extrasynaptic-like $GABA_A$ channels (Birnir and Korpi, 2007). The $CD8^+ DR^{lyp/lyp}$ T cells from the 45 days old animals had the highest expression of the $GABA_A$ channel subunits (> 30 fold increase) and although the expression decreased with age, the expression remained high in $CD8^+ DR^{lyp/lyp}$ T cells from older animals (> 8 fold increase) that had developed diabetes as compared to cells from the $DR^{+/+}$ animals that do not develop diabetes. Thus animals that do develop type-1 diabetes do express higher levels of $GABA_A$ channel subunits in CD8+ T cells than their wild-type counterparts. The expression of $GABA_A$ channels subunits in $CD4^+$ T cells were not significantly altered between strains or animals at different ages. GABA decreased cell proliferation between 30 to 40 % in $CD4^+ DR^{+/+}$ and $DR^{lyp/lyp}$ cells and in $CD8^+ DR^{+/+}$ cells. That maximal inhibition was reached already with 100 nM GABA is in accordance with properties of high-affinity extrasynaptic-like $GABA_A$ channels (Birnir and Korpi, 2007). In $CD8^+ DR^{lyp/lyp}$ cells we were not able to assay the effects of GABA on proliferation due to the lympophenia of the animals but considering the high level of the $GABA_A$ channel subunit expression it is likely that even in these cells GABA would inhibit cell proliferation.

The large number of different $GABA_A$ channels subunits are expressed in the rat $CD4^+$ and $CD8^+$ T lymphocytes allows for formation of channels varying in their functional and pharmacological profile. To-date the composition of no native GABAA channel is known. It is possible to partially address the channel composition by examining what drugs can modulate functional properties of the channels. Synaptic $GABA_A$ channels in the brain normally contain the γ2 subunit which is absent in the lymphocytes but the δ subunit is present. Delta is a prominent subunit in neural extrasynaptic-type $GABA_A$ channels and makes the channels highly sensitive to the steroid THDOC. α4 and α6 subunits are also associated with extrasynaptic channels and are thought to form high-affinity (nM-low μM) $GABA_A$ channels (Olsen and Sieghart, 2008). The other $GABA_A$ channel subunits present can be either associated with extrasynaptic or synaptic-like channels. Therefore, a number of different $GABA_A$ channels may be formed in the T cells i.e. $\alpha_X\beta3\delta$ or $\alpha_X\beta3\gamma1$, with varying functional and pharmacological properties. In addition, ρ1 and ρ2 may form homomeric channels and are potentially associated with naïve T cells (Bjurstom et al., 2008). In the lymphocytes the $GABA_A$ channels may be located at or outside the immunological synapse. It will be very interesting to examine whether a subsets of $GABA_A$ channels are specifically located at the immunological synapse similar to what is observed for inhibitory neuronal synapses.

A limited number of studies have examined the expression of $GABA_A$ subunits in T cells. In cells from mice Tian et al. (2004) detected transcripts for α1, α2, β1, β2, γ3 and δ and Bjurström et al. (2008) identified α1, α4, β2, β3, γ1 and δ whereas Bath et al. (2010) did not detect the four subunits (α1, β1,γ2, ε) they tested for. Intracellular proteins associated with channel clustering (radixin, gephyrin), intracellular transport of subunits (GABARap)

and recycling of channels (HAP-1) were similarly expressed in cells from the different stains with the exception of HAP-1 that was expressed in all but cells from 45 days $DR^{lyp/lyp}$ animals. Thus at least a part of the normal neuronal intracellular machinery required for full channel function is present in the lymphocytes. It is interesting to note that in animals that develop diabetes the $GABA_A$ subunit α4 is not expressed in $CD4^+DR^{lyp/lyp}$ or $CD8^+DR^{lyp/lyp}$ T cells from the 45 days old animals and, in addition, in $CD8^+DR^{lyp/lyp}$ T cells, the recycling protein HAP-1 is not detected. α4-containing $GABA_A$ receptors are thought to have one of the highest affinity for GABA and HAP-1 increases surface expression of the receptors (Birnir and Korpi, 2007). It is possible that the lack of functional α4 $GABA_A$ and / or decreased surfaced expression of $GABA_A$ channels in the younger rats contributes to predisposing the animals for diabetes.

$GABA_A$ channel subunit expression is highest in $CD8^+ DR^{lyp/lyp}$ cells. This is neither a property of the $^{lyp/lyp}$ genotype as the $CD4^+ DR^{lyp/lyp}$ have lower expression levels nor the $CD8^+$ phenotype, as the $CD8^+ DR^{+/+}$ cells also had lower $GABA_A$ channel subunit expression than the $CD8^+ DR^{lyp/lyp}$ cells. Furthermore, only in $CD8^+ DR^{lyp/lyp}$ cells from diabetic ($\geq$ 55 days) animals is the expression level of some of the $GABA_A$ channels subunits down-regulated as compared to the younger animals. Whether this is just a coincidence or is related to the onset of the disease cannot be determined from our studies. But, it is possible that stimulated T cells might down regulate mechanisms that oppose T cell activation and proliferation and then one such target might be the $GABA_A$ channels.

In the pancreas GABA is produced and released from the insulin producing β cells (Braun et al., 2004). In the islet, GABA may be involved in paracrine mechanisms where GABA secreted by the β cells activates $GABA_A$ channels on the glucagon secreting α cells

(Rorsman et al., 1989). Another potential role of GABA is inhibition of lymphocytes (Bhat et al., ; Bjurstom et al., 2008; Tian et al., 2004). The high-affinity $GABA_A$ channels on the lymphocytes are activated by GABA concentrations present around the β cells and thus activated lymphocytes will be inhibited when within the islets. GABA might be considered to be a part of the islet´s immunomodulatory environment and thereby contributing to mechanisms in place for increasing β cells survival. With decreasing β cell mass, the extracellular concentration of GABA in the islet is expected to decrease and at some stage reach a critical point where it is simply too low to activate channels. Lymphocytes now entering the islet will no longer be inhibited and destruction of the β cells at a faster rate may follow. Subtypes of the channels expressed in lymphocytes may potentially be targeted with drugs already available making the channels more sensitive to GABA and/or more effective inhibiting the cells. There are many classes of drugs in clinical use aimed at modulating $GABA_A$ channels in the brain (Korpi and Sinkkonen, 2006). If different sub-populations of lymphocytes express different sub-types of $GABA_A$ channels they might be specifically targeted and potentially β cell survival and hence β cell mass may be enhanced in critical circumstances. Our results raise the question of whether an enhanced $GABA_A$ channel activity might delay or possibly prevent the development of type-1 diabetes.

Acknowledgements:

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Figure Legends

Figure 1.

$GABA_A$ channel subunits are expressed in T cells. A. $GABA_A$ channel subunits ($GABA_A$ receptor) are expressed in $CD4^+$ (A) and $CD8^+$ (B) T cells from the congenic $DR^{+/+}$ and $DR^{lyp/lyp}$ rats. The subunits expressed are: α1, α2, α3, α4, α6, β3, γ1, δ1, ρ1 and ρ2 whereas no expression was detected for: α5, β1, β2, γ2, γ3, ε, θ, π and ρ3. The relative expression of each $GABA_A$ channel subunit was calculated using $2^{-\Delta Ct}$. All individual experiments were run in duplicates and the data is the mean ± SEM of experiments from 3 to 6 animals. **A**. $CD4^+$ T cells were isolated from **a**. $DR^{+/+}$ 45 days **b**. $DR^{+/+}$ 55-77 days **c.** $DR^{lyp/lyp}$ 45 days **d.** diabetic $DR^{lyp/lyp}$ 55-77 days old rats. **B.** $CD8^+$ T cells were isolated from **a.** $DR^{+/+}$ 45 days **b.** $DR^{+/+}$ 55-77 days **c.** $DR^{lyp/lyp}$ 45 days **d.** diabetic $DR^{lyp/lyp}$ 55-77 days old rats.

Figure 2.

Comparisons of $GABA_A$ channel subunit gene expression based on:

A. Genotype. Relative expression of $GABA_A$ channel subunits in diabetes prone $DR^{lyp/lyp}$ rats compared with age-matched $DR^{+/+}$ rats. $CD8^+$ $DR^{lyp/lyp}$ T cells but not $CD4^+$ $DR^{lyp/lyp}$ T cells express higher level of $GABA_A$ channel subunits than $CD4^+$ $DR^{+/+}$ and $CD8^+$ $DR^{+/+}$ T cells. The "fold change" of each $GABA_A$ channel subunit was calculated using $2^{-\Delta\Delta Ct}$. **a.** $CD4^+$ T cells, 45 days **b.** $CD4^+$ T cells, 55-77 days **c**. $CD8^+$ T cells, 45 days **d.** $CD8^+$ T cells, 55-77 days old BB rats. Star represents subunits expression in cells from $DR^{lyp/lyp}$ animals that is significantly different ($*P < 0.05$) from expression in cells from $DR^{+/+}$ animals.

B. Age. Relative expression of $GABA_A$ channel subunits level in cells from 55-77 days old rats compared with expression levels in cells from 45 days old rats of the same strain. Only $CD8^+ DR^{lyp/lyp}$ T cells show differential expression of $GABA_A$ channel subunits with age. The "fold change" of each $GABA_A$ channel subunit was calculated using $2^{-\Delta\Delta Ct}$. **a.** $CD4^+ DR^{+/+}$ **b.** $CD8^+ DR^{+/+}$ **c.** $CD4^+ DR^{lyp/lyp}$ **d.** $CD8^+ DR^{lyp/lyp}$. Star represents subunits expression level in cells from the 55-77-days old animals that is significantly different (*$P < 0.05$) from expression level in cells from the 45 days old animals.

C. Cell type. Relative expression of $GABA_A$ channel subunits level in $CD8^+$ T cells and compared with $CD4^+$ T cells from the same BB rat strain. Subunits expression level is similar in $CD4^+ DR^{+/+}$ and $CD8^+ DR^{+/+}$ T cells but higher for several subunits in $CD8^+ DR^{lyp/lyp}$ T cells as compared with $CD4^+ DR^{lyp/lyp}$ T cells . The "fold change" of each $GABA_A$ channel subunits was calculated using $2^{-\Delta\Delta Ct}$. a. $DR^{+/+}$ 45 days old b. $DR^{+/+}$ 55-77 days old c. $DR^{lyp/lyp}$ 45 days old d. $DR^{lyp/lyp}$ 55-77 days old. Star represents subunits expression level in $CD8^+$ T cells that is significantly different (*$P < 0.05$) from the expression level in $CD4^+$ T cells.

Figure 3.

$GABA_A$ channels associated proteins are expressed in T cells. The proteins are: GABARAP ($GABA_A$ Receptor Associated Protein), gephyrin, HAP-1 (Huntingtin Associated Protein 1) and radixin. The relative expression of each protein was calculated using $2^{-\Delta Ct}$. All individual experiments were run in duplicates and the data is the mean $\pm$ SEM of experiments from 3 to 6 animals. **A**. $CD4^+$ T cells were isolated from **a**. $DR^{+/+}$ 45

days **b**. $DR^{+/+}$ 55-77 days **c.** $DR^{lyp/lyp}$ 45 days **d.** diabetic $DR^{lyp/lyp}$ 55-77 days old rats. **B.** $CD8^+$ T cells were isolated from **a.** $DR^{+/+}$ 45 days **b.** $DR^{+/+}$ 55-77 days **c.** $DR^{lyp/lyp}$ 45 days **d.** diabetic $DR^{lyp/lyp}$ 55-77 days old rats.

Figure 4.

Functional $GABA_A$ channels are formed in the T lymphocytes.

A. GABA channels are activated with 1 μM GABA + 100 nM THDOC in a cell-attached configuration on CD8+$DR^{+/+}$ T cells. Channels opened after a delay and channel conductance increased with time; a 0.38 pA, b 1.7 pA**.** Single-channel currents activated by 1 μM GABA (**Ba** $CD4^+$ $DR^{+/+}$**, C** $CD8^+$ $DR^{+/+}$) or 1 μM GABA plus 100 nM THDOC (**Bb**) in the cell-attached configuration at depolarized 60 mV (pipette potential -60 mV). Stars identify openings of channels in the current record.

Figure 5.

Physiological, low GABA concentrations decrease proliferation of $CD4^+$ and $CD8^+$ T cells. Cells were stimulated with anti-CD3 antibody (0.5μg/ml) in the presence or absence of GABA (0.1, 100 μM) and cell proliferation assayed. The data is the mean $\pm$ SEM from experiments on cells obtained from 4 or 5 animals aged 45 or $\geq$ 55-77 days. Proliferation assays from animals of the same genotype were grouped together. Each experiment was done in triplicate and normalized to the value obtained for stimulated cells in the absence of GABA (stimulation index equal to 1). One way ANOVA ** < 0.01, * < 0.05, > 0.05 no significance. A. $CD4^+$ $DR^{+/+}$ B. $CD4^+$ $DR^{lyp/lyp}$ C. $CD8^+$ $DR^{+/+}$.

Table1 Primers used to amplify $GABA_A$ channel subunits and associated proteins.

	Forward(5'-3')	Reverse(5'- 3')	size of amplicon
α1	CTC CTA CAG CAA CCA GCT ATA CCC	GCG GTT TTG TCT CAG GCT TGA C	113 bp
α2	AAG AGA AAG GCT CCG TCA TG	GCT TCT TGT TTG GTT CTG GAG TAG	134 bp
α3	ACA AGC ACC ACC TTC AAC ATA G	AGG TCT TGG TCT CAG CAG GA	174 bp
α4	GAT GTC AAC AGC AGA ACT GAG GTG	TTG TGC CAG ATC CAG AAG GTG GTG	345 bp
α5	GCC TTG GAA GCA GCT AAA ATC	GAA GTC TTC TCC TCA GAT GCT CT	178 bp
α6	CAC TCT GAC TCC AAG TAC CAT CTG	GTA CAC AAG GTT GAA TCC TG	221 bp
β1	CCC TCA GAA AAA AGG AGC GA	TCA CGG CTG CTC AGT GGT TT	231 bp
β2	GCC TGG ATG TCA ACA AGA TGG ACC	CTA GGC AAC CCA GCT TTC CGA TAC	169 bp
β3	CCT ACT AGC ACC GAT GGA TGT T	GAT GCT TCT GTC TCC CAT GTA C	163 bp
γ1	TGA CAC GTT CTT CAG GAA CTC AA	AAC CCT TCC ATC ACT CCA TAT CC	91 bp
γ2	CGG AAA CCA AGC AAG GAT	TCT CTT GAA GGT GGG TGG CA	134 bp
γ3	GCT GTC GAA AGC CAA CCA TCA G	AAC TCC TGC CAG TAC ATG GAA TTG	174 bp
δ	CCC ACT TCA ATG CTG ACT ACA GG	TTG AGT CTG GAA CGG ATG CCT C	258 bp
ε	CAG ATG GCT CTC ATC CAT AAG GA	GCT AGA GAA AGA CAG AGG GCA AGA	129 bp
θ	TAG GAC TTG GCT GGC AGA GAG TA	ATG CTG GAG GAG AGC TCG AA	72 bp
π	TGA CAA CAG TGT TGT CCA TGA CA	TGG CCT TTA TGA AGC AGT TGG T	81 bp
ρ1	TGG ACA GCA GCT ACA GTC ACG G	AAG CAG CTG GGA AAA TGA TC	209 bp
ρ2	CAA GAA GCC ACA TTC TTC CA	TTC TGG AAG ATA TAG AGT CC	133 bp
ρ3	GGT GTG AGC GCC TCT ATG C	GGG AGC TGA CCC ACA TGT ACA	70 bp
GABARAP	AAG CTC CCA AAG CTC GGA TA	AAT TCG CTT CCG GAT CAA GA	101 bp
Gephyrin	AAA CCA CGA CCA TCA AAT CCG	CCA ACA AAG AAG GAT CCT GGA C	122 bp
HAP-1	CGC GCG GCG TTT ATT CGA GA	GGC TGT GTT CAG GTC CCG TTC T	132 bp
Radixin	TGA CTG TTG TAC AGT GGT TGT GC	AGT GGT AAG GCT GAC CGT CTC T	92 bp
β-Actin	GGG AAA TCG TGC GTC ACA TT	GCG GCA GTG GCC ATC TC	76 bp

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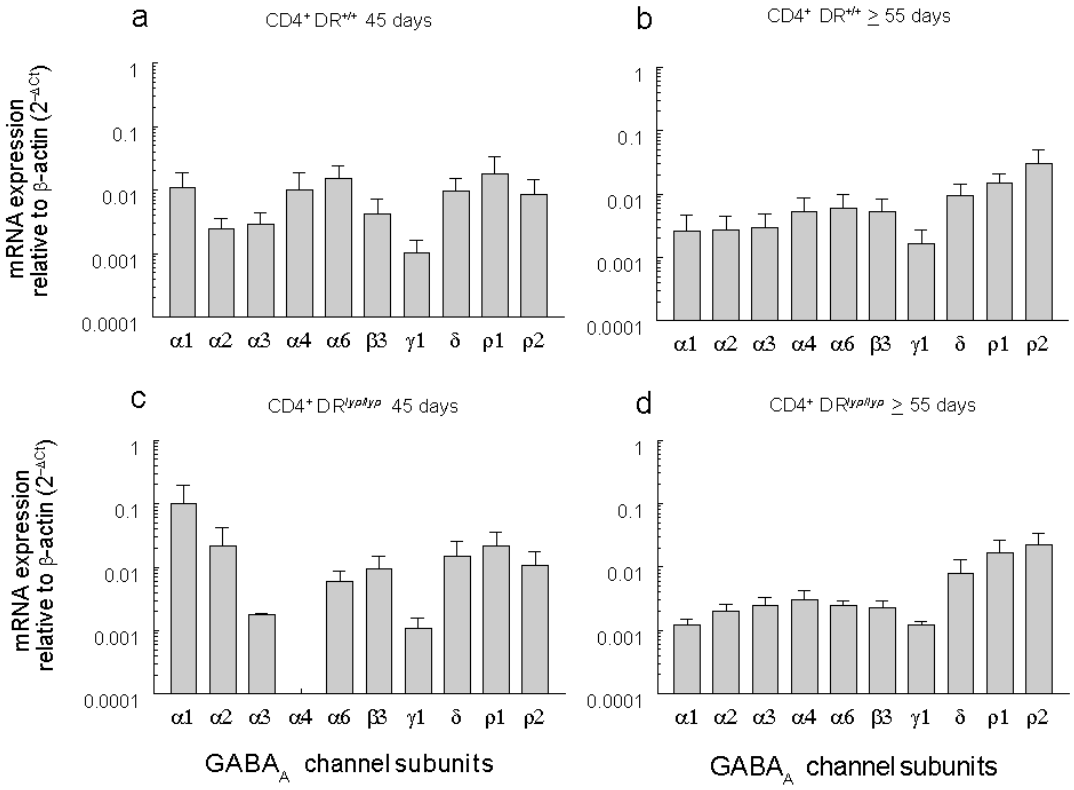
Fig 1 A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b
CD4+ DR+/+ ≥ 55 days
c
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d
CD4+ DRlyp/lyp ≥ 55 days
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0.001
0.0001
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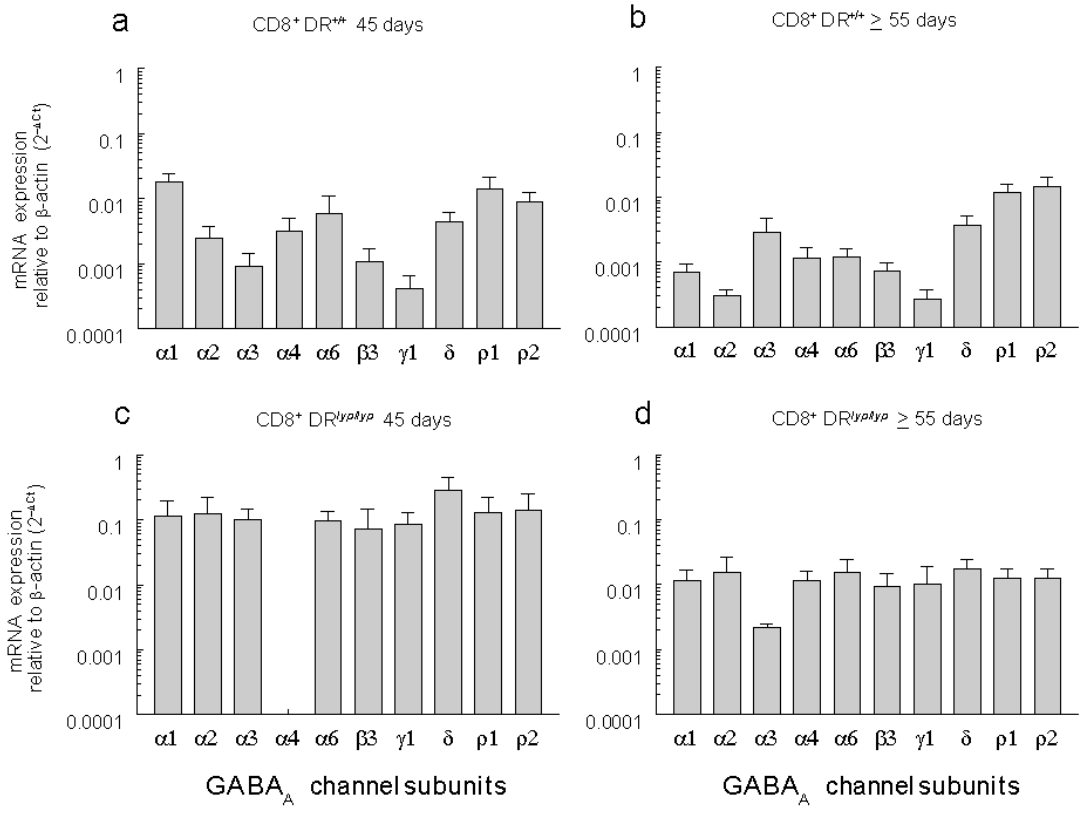
GABAA channel subunits

Fig 1 B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b
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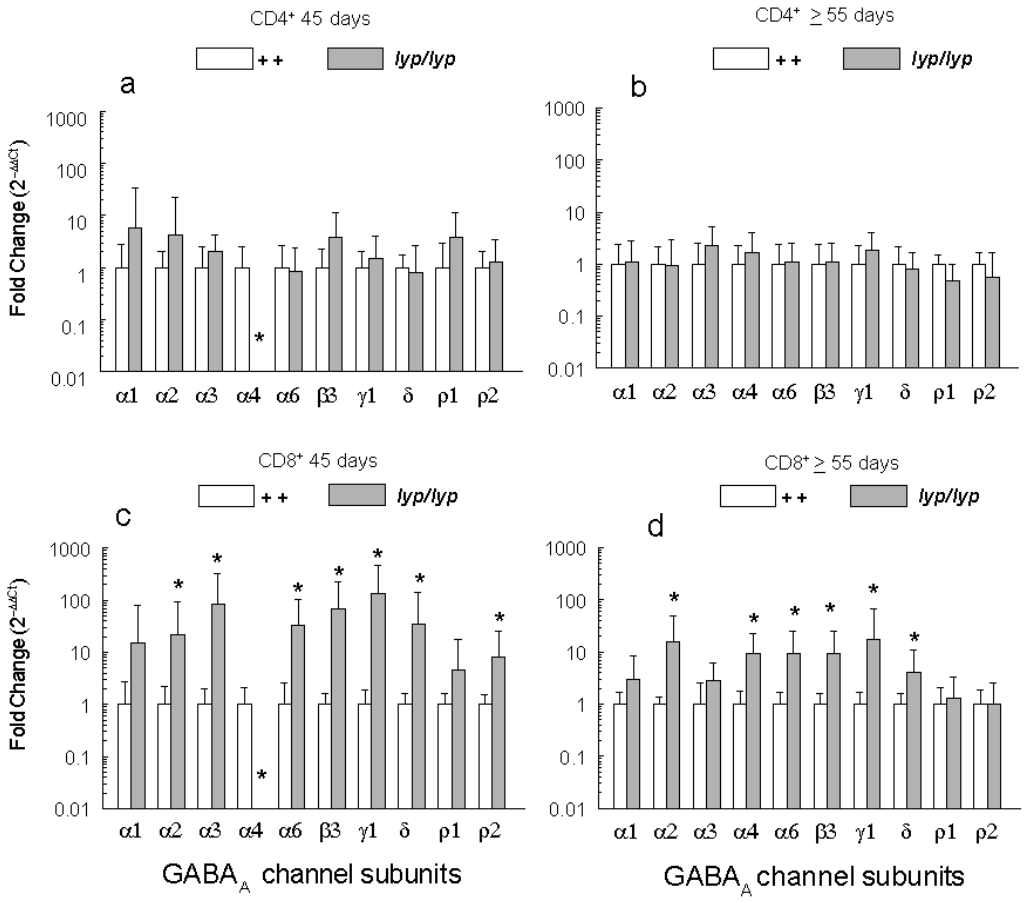
α1 α2 α3 α4 α6 β3 γ1 δ ρ1 ρ2
GABAA channel subunits

Fig 2 A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++
lyp/lyp
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1
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0.01
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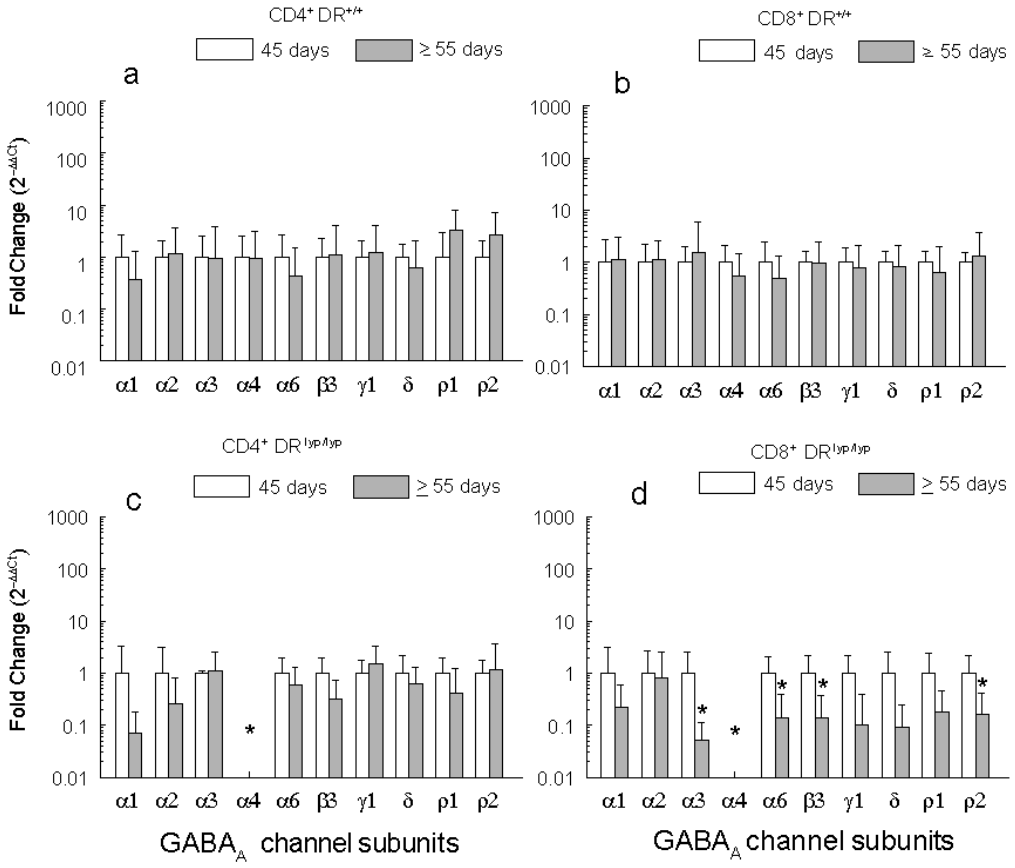
d
GABAA channel subunits

Fig 2 B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≥ 55 days
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d
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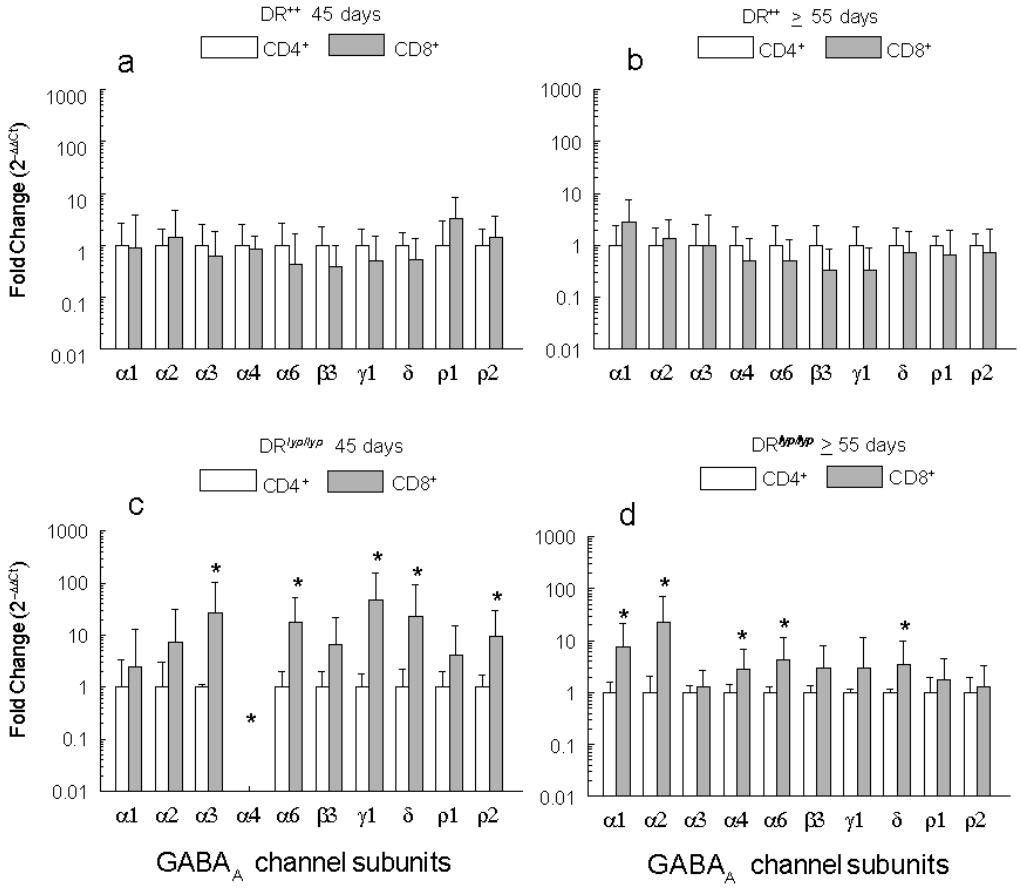
*
GABAA channel subunits

Fig 2 C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d
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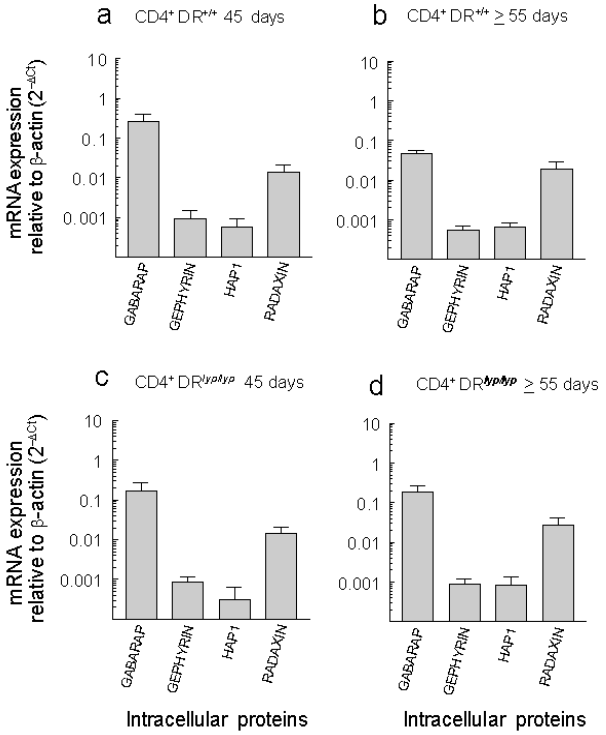
GABAA channel subunits

Fig 3 A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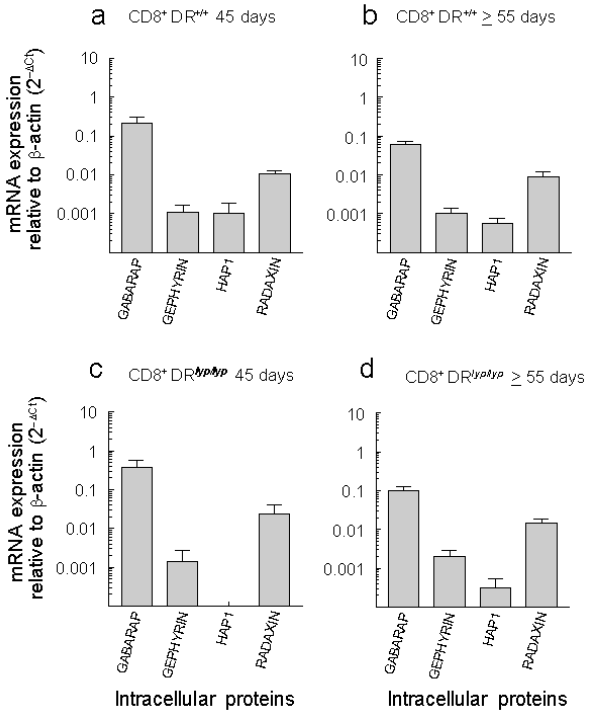
GABARAP
GEPHYRIN
HAP1
RADAXIN
Intracellular proteins

B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mRNA expression relative to β-actin (2-ΔCt)
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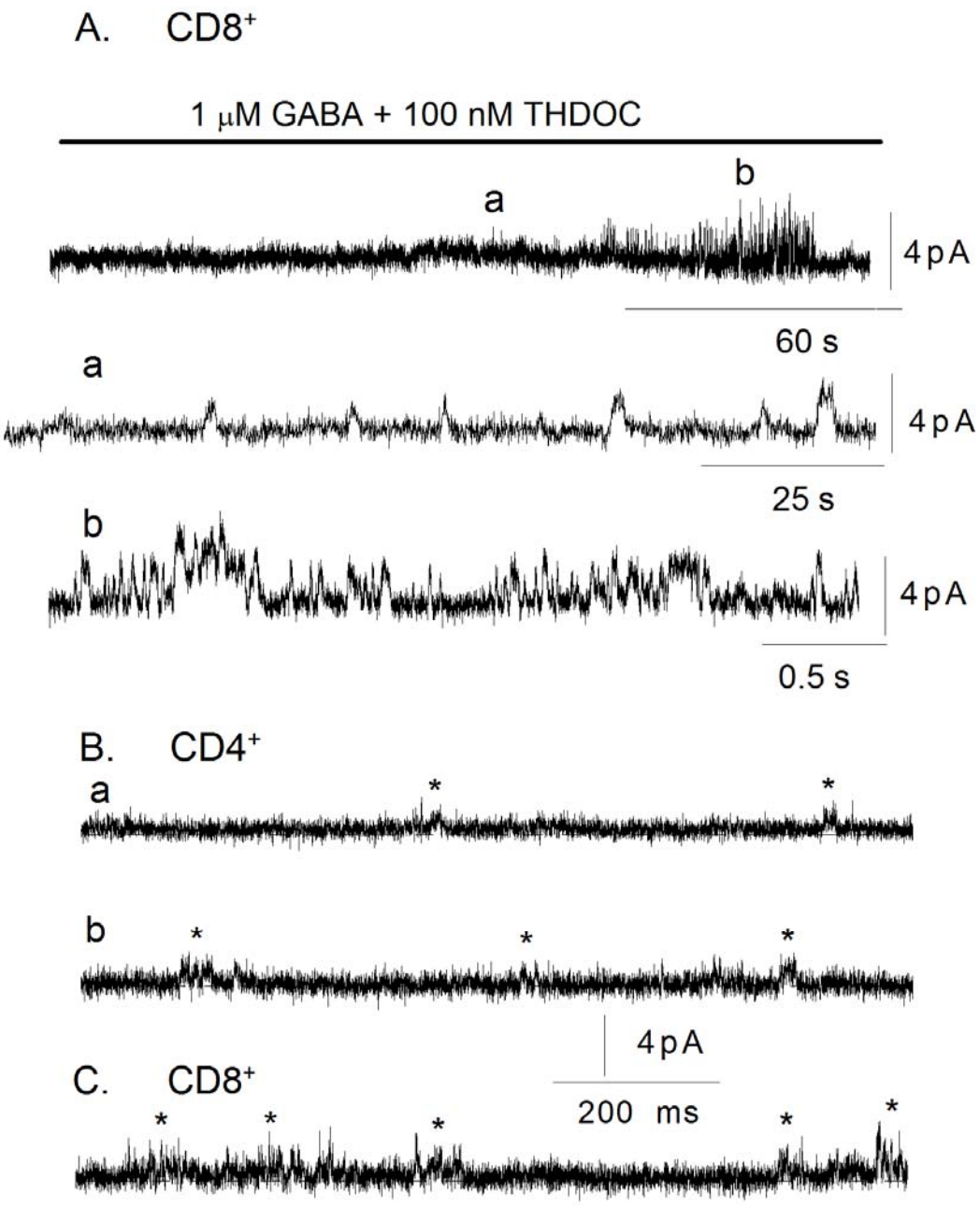
GABARAP
GEPHYRIN
HAP1
RADAXIN
Intracellular proteins

Fig 4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1 μM GABA + 100 nM THDOC
a
b
4 pA
60 s
a
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b
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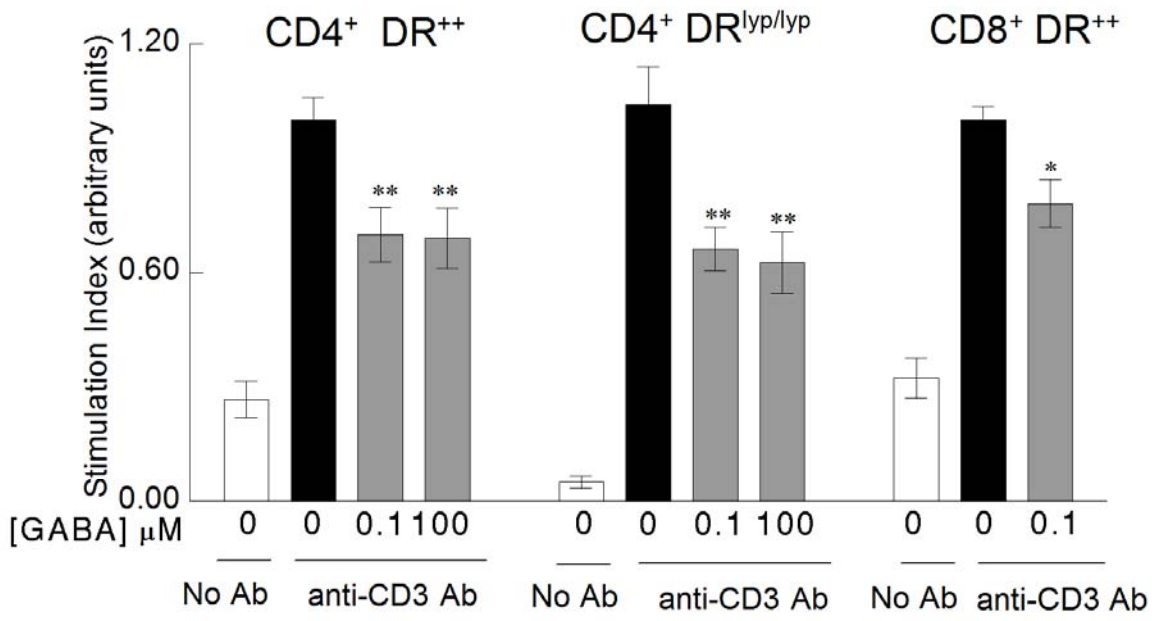
B. CD4+
a
b
4 pA
200 ms
C. CD8+

Fig 5


CD4+ DR++
CD4+ DRlyp/lyp
CD8+ DR++
Stimulation Index (arbitrary units)
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0.60
0.00
**
**
**
**
*
[GABA] μM
0
0
0.1
100
0
0
0.1
100
0
0
0.1
No Ab
anti-CD3 Ab
No Ab
anti-CD3 Ab
No Ab
anti-CD3 Ab