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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 -aminobutyric 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 α 1, α 2, α 3, α 4, α 6, β 3, γ 1, δ , ρ 1 and ρ 2 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 lymphopenia 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 CO₂ 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°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 °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 °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 $\alpha\beta$ T cell and PE-CY5 anti-rat CD4 (BD pharmingen) in 1:100 dilution and incubated for 15 min at 4 °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 μ M oligoDT (TAG Copenhagen A/S, Denmark), 10 mM dNTPs (Invitrogen) and 200 U/ μ L Superscript III reverse transcriptase (Invitrogen). Real-time PCR was performed using 7900HT (ABI, Applied Biosystems) to amplify cDNA. A three step procedure; 95 °C for 5 min, followed by 45 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. The reaction mixture contained a total volume of 10 μ l with 0.5 X SYBR Green I (Invitrogen), 0.3 mM dNTPs (TAG Copenhagen A/S, Denmark), 1 X PCR buffer, 3 mM MgCl₂, 1 X ROX as the reference dye, 0.7 U Taq DNA polymerase (all from Sigma) , 0.4 μ M of each primer and 0.25 ng/ μ l 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 Copenhagen 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 $\Delta C_T = C_{T(\text{target})} - C_{T(\beta\text{-actin})}$ 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: $\Delta\Delta C_T = \Delta C_T (\text{sample-A}) - \Delta C_T (\text{sample-B})$. 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 % CO₂ at 37 °C.

2.7. Electrophysiology. The cells were washed with the extracellular recording solution containing in mM: 150 NaCl, 1 CsCl, 0.2 MgCl₂, 0.2 CaCl₂, 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 MgCl₂, 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 °C).

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 °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 °C in a 5 % CO₂ 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 H³- 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 $\alpha(1-6)$, $\beta(1-3)$, $\gamma(1-3)$, δ , ϵ , π , θ and $\rho(1-3)$. The channels are pentameric and normally require 2 α , 2 β 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 (Birnie 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; $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 6$, $\beta 3$, $\gamma 1$, δ , $\rho 1$ and $\rho 2$. 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 (≥ 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 $\alpha 4$ 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 ≥ 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 $\alpha 2$, $\alpha 3$, $\alpha 6$, $\beta 3$, $\gamma 1$ 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 $\alpha 2$, $\alpha 4$, $\alpha 6$, $\beta 3$ and $\gamma 1$ 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 $\alpha 4$ 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 $\alpha 3$, $\alpha 6$, $\beta 3$ and $\rho 2$ are significantly down-regulated as compared to subunits expressed in cells from the 45 days old animals and the $\alpha 4$ 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 $\alpha 3$, $\alpha 6$, $\gamma 1$, 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 $\alpha 1$ and $\alpha 2$ 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 (Birnie 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 (Loeblich 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- μ M GABA concentrations (Birnir and Korpi, 2007). Furthermore, all neuronal synaptic GABA channels contain the γ 2 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 μ M 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 μ M

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 μ M GABA and in another patch (b) where the channels were activated by 1 μ M GABA together with 100 nM THDOC. The average conductance for GABA and GABA plus THDOC activated channels was 16 ± 3 (n = 3) and 20 ± 1 (n = 6) pS, respectively. Fig. 4C. shows channels in CD8⁺ DR^{+/+} T cells activated by 1 μ M GABA. The average conductance for GABA and GABA and THDOC (50 or 100 nM) activated channels was 14 ± 1 (n = 4) and 17 ± 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- μ M 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 μ M) GABA concentrations had on cell proliferation and the results are shown in Fig. 5. Cells were stimulated with 0.5 μ g/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 (Birnie 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 (Birnie and Korpi, 2007). In CD8⁺ DR^{lyp/lyp} cells we were not able to assay the effects of GABA on proliferation due to the lymphopenia 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 GABA_A 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 $\gamma 2$ 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. $\alpha 4$ and $\alpha 6$ subunits are also associated with extrasynaptic channels and are thought to form high-affinity (nM-low μ M) 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. $\alpha_x\beta 3\delta$ or $\alpha_x\beta 3\gamma 1$, with varying functional and pharmacological properties. In addition, $\rho 1$ and $\rho 2$ 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 $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, $\gamma 3$ and δ and Bjurström et al. (2008) identified $\alpha 1$, $\alpha 4$, $\beta 2$, $\beta 3$, $\gamma 1$ and δ whereas Bath et al. (2010) did not detect the four subunits ($\alpha 1$, $\beta 1$, $\gamma 2$, 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 α 4 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. α 4-containing GABA_A receptors are thought to have one of the highest affinity for GABA and HAP-1 increases surface expression of the receptors (Birnie and Korpi, 2007). It is possible that the lack of functional α 4 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 (≥ 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., ; Bjurström 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.

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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: α 1, α 2, α 3, α 4, α 6, β 3, γ 1, δ 1, ρ 1 and ρ 2 whereas no expression was detected for: α 5, β 1, β 2, γ 2, γ 3, ϵ , θ , π and ρ 3. 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 \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 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 (0.5 μg/ml) in the presence or absence of GABA (0.1, 100 μM) and cell proliferation assayed. The data is the mean ± SEM from experiments on cells obtained from 4 or 5 animals aged 45 or ≥ 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
α 1	CTC CTA CAG CAA CCA GCT ATA CCC	GCG GTT TTG TCT CAG GCT TGA C	113 bp
α 2	AAG AGA AAG GCT CCG TCA TG	GCT TCT TGT TTG GTT CTG GAG TAG	134 bp
α 3	ACA AGC ACC ACC TTC AAC ATA G	AGG TCT TGG TCT CAG CAG GA	174 bp
α 4	GAT GTC AAC AGC AGA ACT GAG GTG	TTG TGC CAG ATC CAG AAG GTG GTG	345 bp
α 5	GCC TTG GAA GCA GCT AAA ATC	GAA GTC TTC TCC TCA GAT GCT CT	178 bp
α 6	CAC TCT GAC TCC AAG TAC CAT CTG	GTA CAC AAG GTT GAA TCC TG	221 bp
β 1	CCC TCA GAA AAA AGG AGC GA	TCA CGG CTG CTC AGT GGT TT	231 bp
β 2	GCC TGG ATG TCA ACA AGA TGG ACC	CTA GGC AAC CCA GCT TTC CGA TAC	169 bp
β 3	CCT ACT AGC ACC GAT GGA TGT T	GAT GCT TCT GTC TCC CAT GTA C	163 bp
γ 1	TGA CAC GTT CTT CAG GAA CTC AA	AAC CCT TCC ATC ACT CCA TAT CC	91 bp
γ 2	CGG AAA CCA AGC AAG GAT	TCT CTT GAA GGT GGG TGG CA	134 bp
γ 3	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
ρ 1	TGG ACA GCA GCT ACA GTC ACG G	AAG CAG CTG GGA AAA TGA TC	209 bp
ρ 2	CAA GAA GCC ACA TTC TTC CA	TTC TGG AAG ATA TAG AGT CC	133 bp
ρ 3	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

References.

- Alam, S., Laughton, D.L., Walding, A., Wolstenholme, A.J., 2006. Human peripheral blood mononuclear cells express GABAA receptor subunits. *Mol Immunol* 43, 1432-1442.
- Bhat, R., Axtell, R., Mitra, A., Miranda, M., Lock, C., Tsien, R.W., Steinman, L., Inhibitory role for GABA in autoimmune inflammation. *Proc Natl Acad Sci U S A* 107, 2580-2585.
- Bhat, R., Axtell, R., Mitra, A., Miranda, M., Lock, C., Tsien, R.W., Steinman, L., 2010. Inhibitory role for GABA in autoimmune inflammation. *Proc Natl Acad Sci U S A* 107, 2580-2585.
- Birnir, B., Korpi, E.R., 2007. The impact of sub-cellular location and intracellular neuronal proteins on properties of GABA(A) receptors. *Curr Pharm Des* 13, 3169-3177.
- Bjurstom, H., Wang, J., Ericsson, I., Bengtsson, M., Liu, Y., Kumar-Mendu, S., Issazadeh-Navikas, S., Birnir, B., 2008. GABA, a natural immunomodulator of T lymphocytes. *J Neuroimmunol* 205, 44-50.
- Braun, M., Wendt, A., Birnir, B., Broman, J., Eliasson, L., Galvanovskis, J., Gromada, J., Mulder, H., Rorsman, P., 2004. Regulated exocytosis of GABA-containing synaptic-like microvesicles in pancreatic beta-cells. *J Gen Physiol* 123, 191-204.
- Chen, Z.W., Olsen, R.W., 2007. GABAA receptor associated proteins: a key factor regulating GABAA receptor function. *J Neurochem* 100, 279-294.
- Elder, M.E., Maclaren, N.K., 1983. Identification of profound peripheral T lymphocyte immunodeficiencies in the spontaneously diabetic BB rat. *J Immunol* 130, 1723-1731.
- Gladkevich, A., Korf, J., Hakobyan, V.P., Melkonyan, K.V., 2006. The peripheral GABAergic system as a target in endocrine disorders. *Auton Neurosci* 124, 1-8.
- Hawkins, T., Fuller, J., Olson, K., Speros, S., Lernmark, A., 2005. DR.lyp/lyp bone marrow maintains lymphopenia and promotes diabetes in lyp/lyp but not in +/+ recipient DR.lyp BB rats. *J Autoimmun* 25, 251-257.
- Kneussel, M., Brandstatter, J.H., Laube, B., Stahl, S., Muller, U., Betz, H., 1999. Loss of postsynaptic GABA(A) receptor clustering in gephyrin-deficient mice. *J Neurosci* 19, 9289-9297.
- Korpi, E.R., Sinkkonen, S.T., 2006. GABA(A) receptor subtypes as targets for neuropsychiatric drug development. *Pharmacol Ther* 109, 12-32.
- Lernmark, A., Freedman, Z.R., Hofmann, C., Rubenstein, A.H., Steiner, D.F., Jackson, R.L., Winter, R.J., Traisman, H.S., 1978. Islet-cell-surface antibodies in juvenile diabetes mellitus. *N Engl J Med* 299, 375-380.
- Levite, M., 2008. Neurotransmitters activate T-cells and elicit crucial functions via neurotransmitter receptors. *Curr Opin Pharmacol* 8, 460-471.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402-408.
- Loeblich, S., Bähring, R., Katsuno, T., Tsukita, S., Kneussel, M., 2006. Activated radixin is essential for GABAA receptor alpha5 subunit anchoring at the actin cytoskeleton. *EMBO J* 25, 987-999.
- MacMurray, A.J., Moralejo, D.H., Kwitek, A.E., Rutledge, E.A., Van Yserloo, B., Gohlke, P., Speros, S.J., Snyder, B., Schaefer, J., Bieg, S., Jiang, J., Ettinger, R.A., Fuller, J., Daniels, T.L., Pettersson, A., Orlebeke, K., Birren, B., Jacob, H.J., Lander, E.S.,

- Lernmark, A., 2002. Lymphopenia in the BB rat model of type 1 diabetes is due to a mutation in a novel immune-associated nucleotide (Ian)-related gene. *Genome Res* 12, 1029-1039.
- Markholst, H., Eastman, S., Wilson, D., Andreasen, B.E., Lernmark, A., 1991. Diabetes segregates as a single locus in crosses between inbred BB rats prone or resistant to diabetes. *J Exp Med* 174, 297-300.
- Michalkiewicz, M., Michalkiewicz, T., Ettinger, R.A., Rutledge, E.A., Fuller, J.M., Moralejo, D.H., Van Yserloo, B., MacMurray, A.J., Kwitek, A.E., Jacob, H.J., Lander, E.S., Lernmark, A., 2004. Transgenic rescue demonstrates involvement of the Ian5 gene in T cell development in the rat. *Physiol Genomics* 19, 228-232.
- Moralejo, D.H., Park, H.A., Speros, S.J., MacMurray, A.J., Kwitek, A.E., Jacob, H.J., Lander, E.S., Lernmark, A., 2003. Genetic dissection of lymphopenia from autoimmunity by introgression of mutated Ian5 gene onto the F344 rat. *J Autoimmun* 21, 315-324.
- Olsen, R.W., Sieghart, W., 2008. International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. *Pharmacol Rev* 60, 243-260.
- Ramanathan, S., Poussier, P., 2001. BB rat lyp mutation and Type 1 diabetes. *Immunol Rev* 184, 161-171.
- Reyes-Garcia, M.G., Hernandez-Hernandez, F., Hernandez-Tellez, B., Garcia-Tamayo, F., 2007. GABA (A) receptor subunits RNA expression in mice peritoneal macrophages modulate their IL-6/IL-12 production. *J Neuroimmunol* 188, 64-68.
- Rorsman, P., Berggren, P.O., Bokvist, K., Ericson, H., Mohler, H., Ostenson, C.G., Smith, P.A., 1989. Glucose-inhibition of glucagon secretion involves activation of GABAA-receptor chloride channels. *Nature* 341, 233-236.
- Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3, 1101-1108.
- Sheng, G., Chang, G.Q., Lin, J.Y., Yu, Z.X., Fang, Z.H., Rong, J., Lipton, S.A., Li, S.H., Tong, G., Leibowitz, S.F., Li, X.J., 2006. Hypothalamic huntingtin-associated protein 1 as a mediator of feeding behavior. *Nat Med* 12, 526-533.
- Tian, J., Lu, Y., Zhang, H., Chau, C.H., Dang, H.N., Kaufman, D.L., 2004. Gamma-aminobutyric acid inhibits T cell autoimmunity and the development of inflammatory responses in a mouse type 1 diabetes model. *J Immunol* 173, 5298-5304.

Fig 1 A

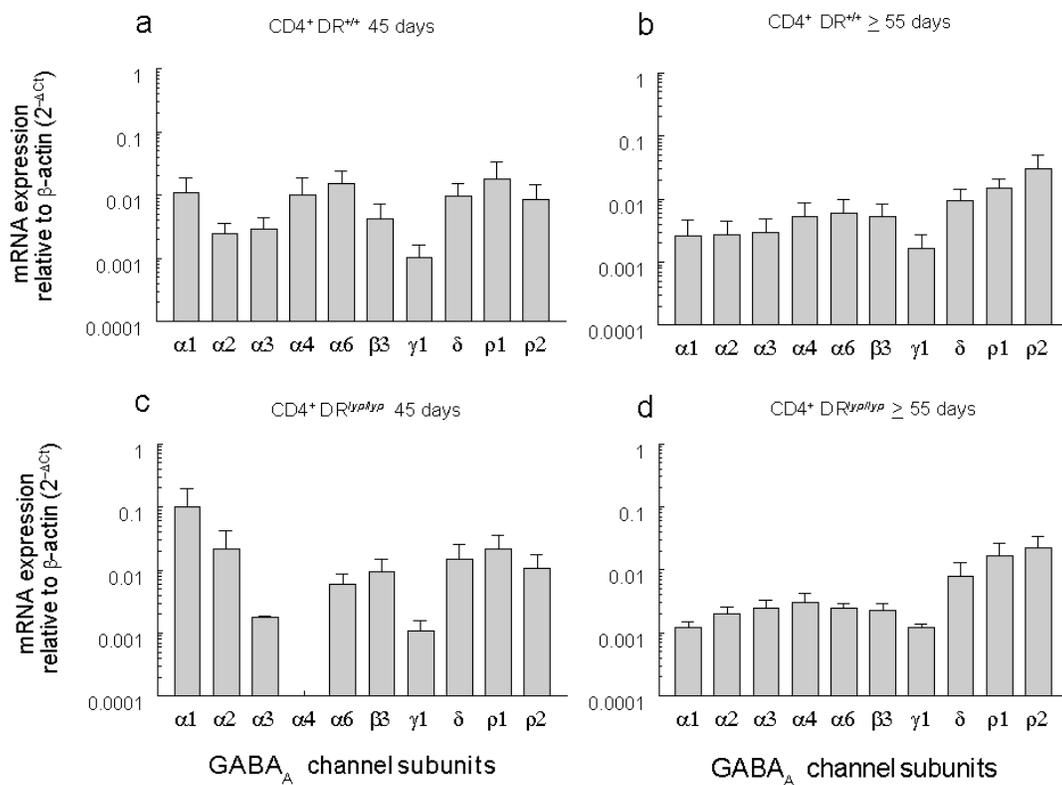


Fig 1 B

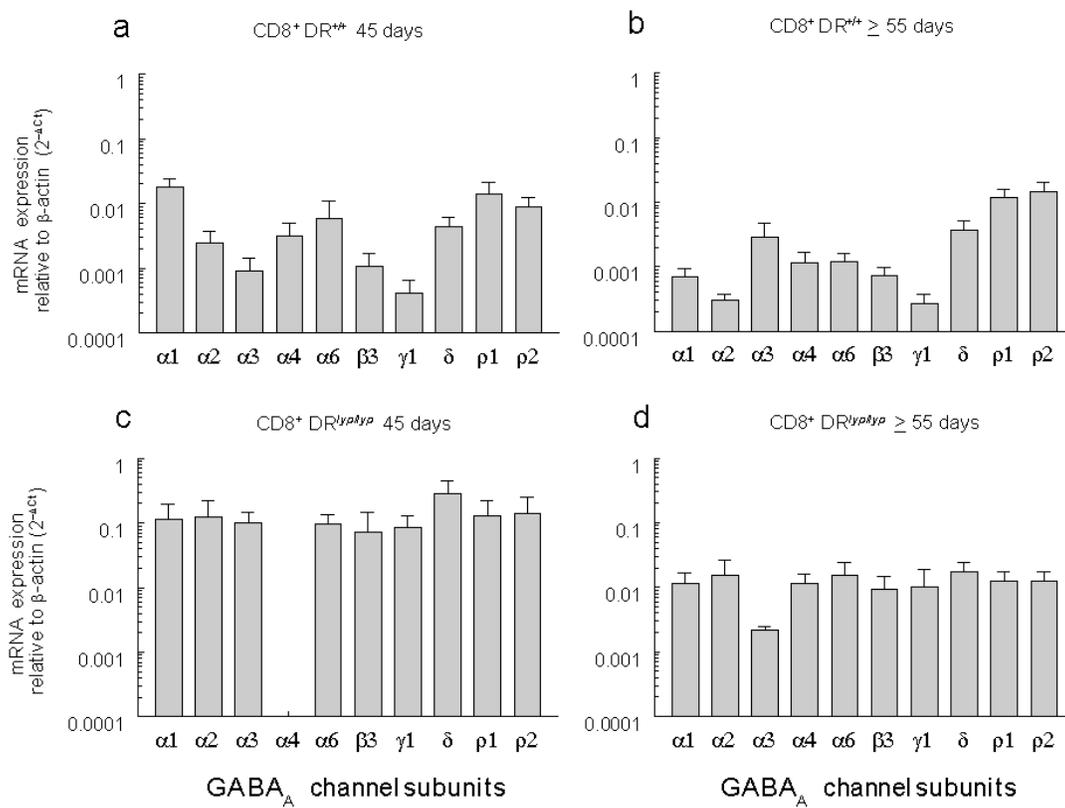


Fig 2 A

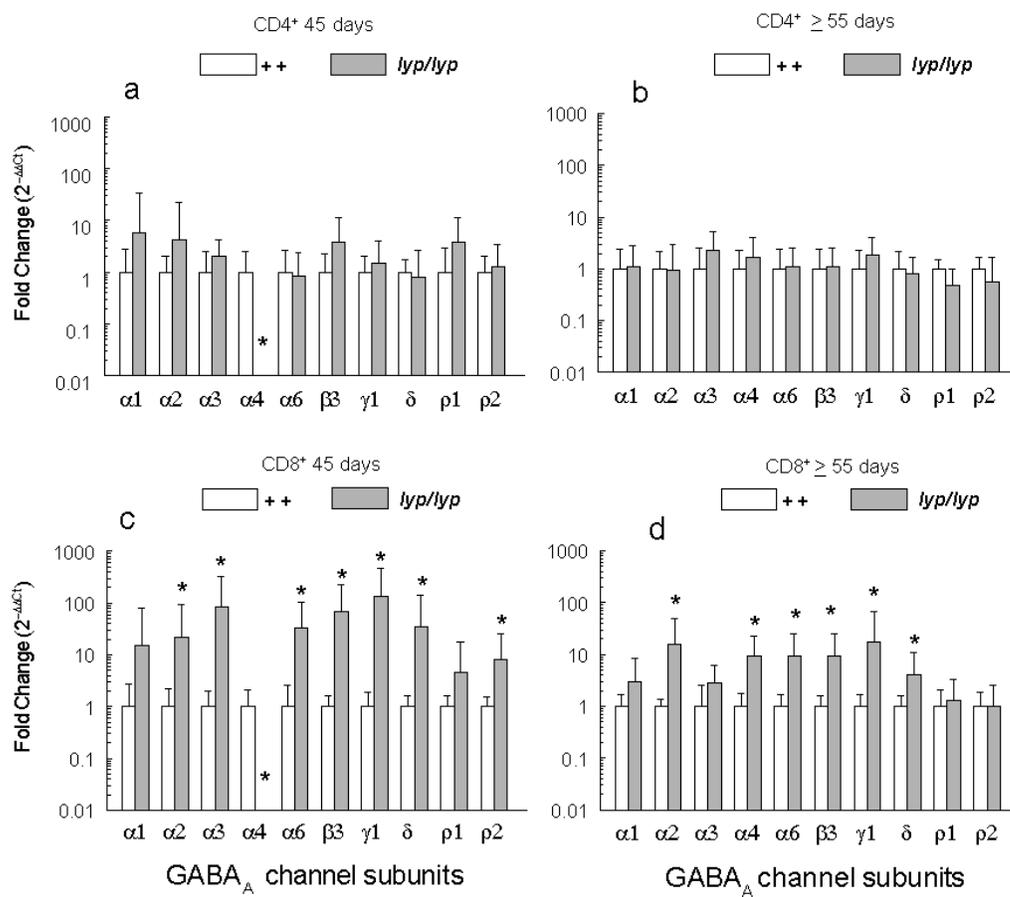


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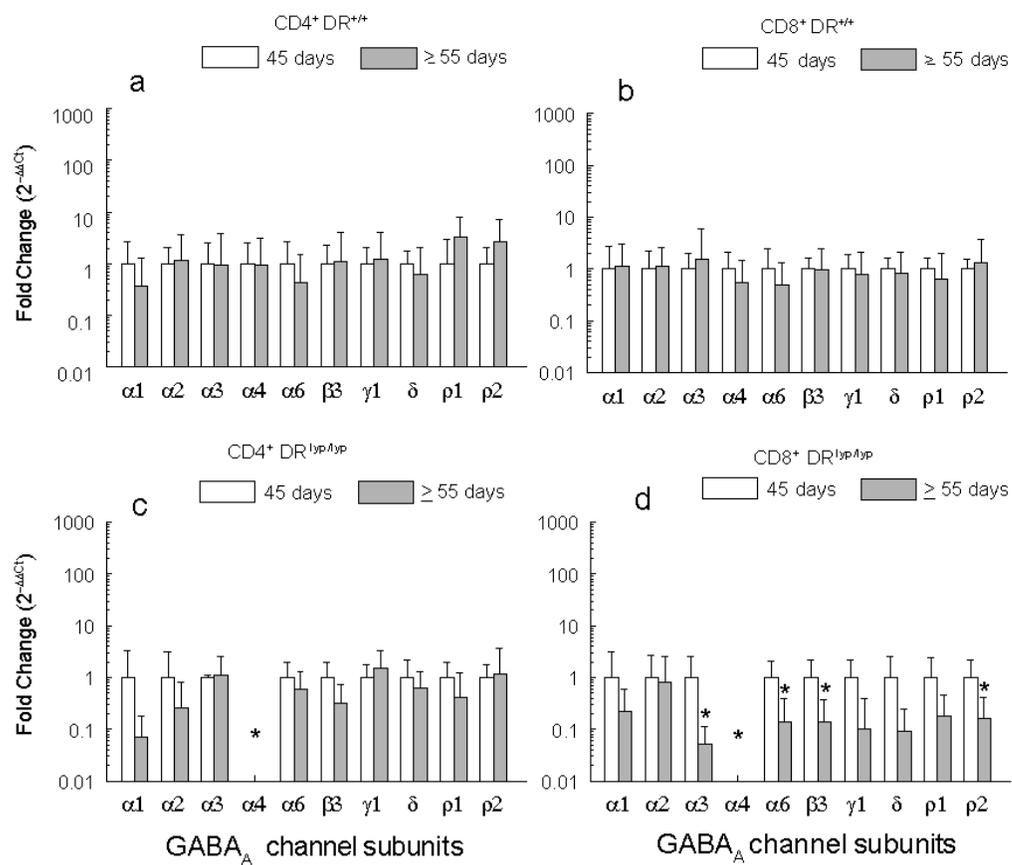


Fig 2 C

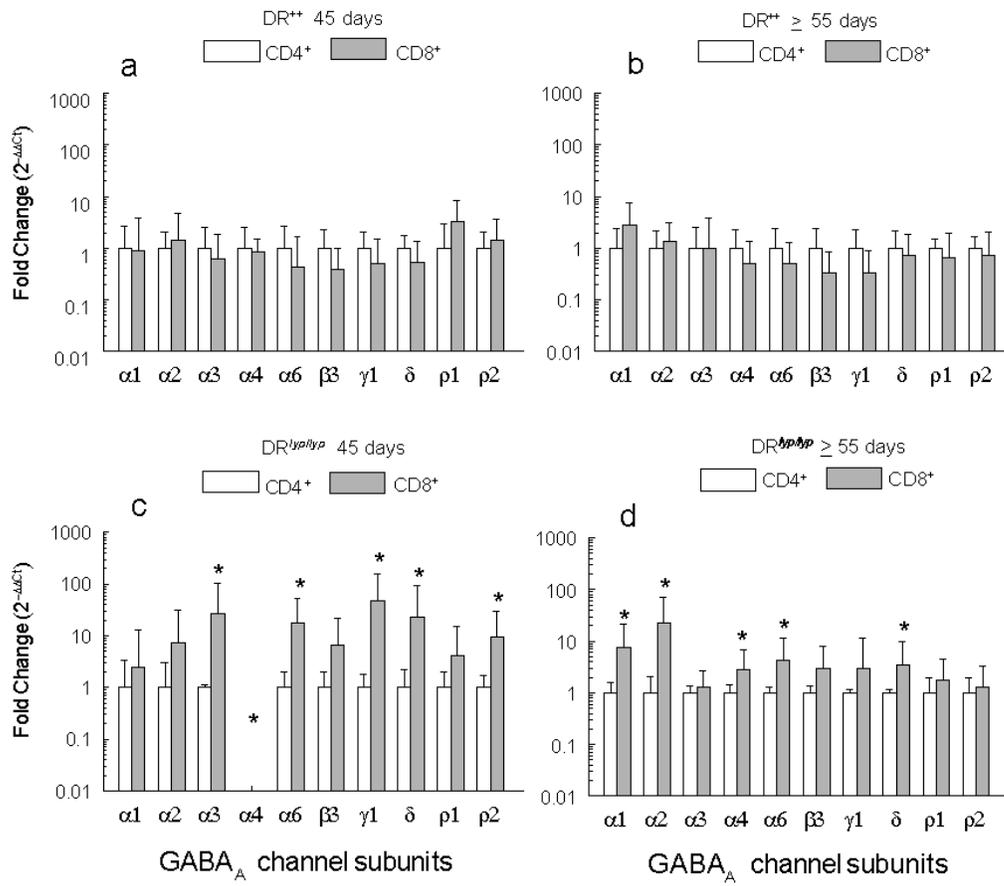
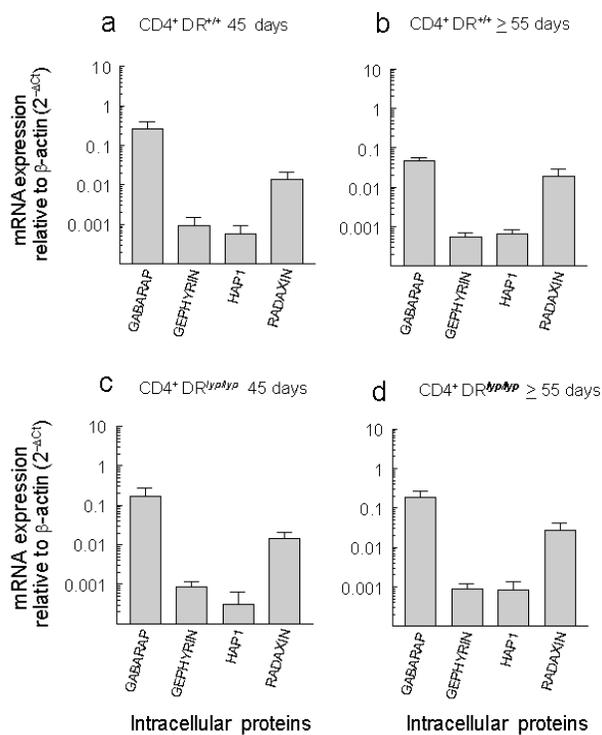


Fig 3 A



B

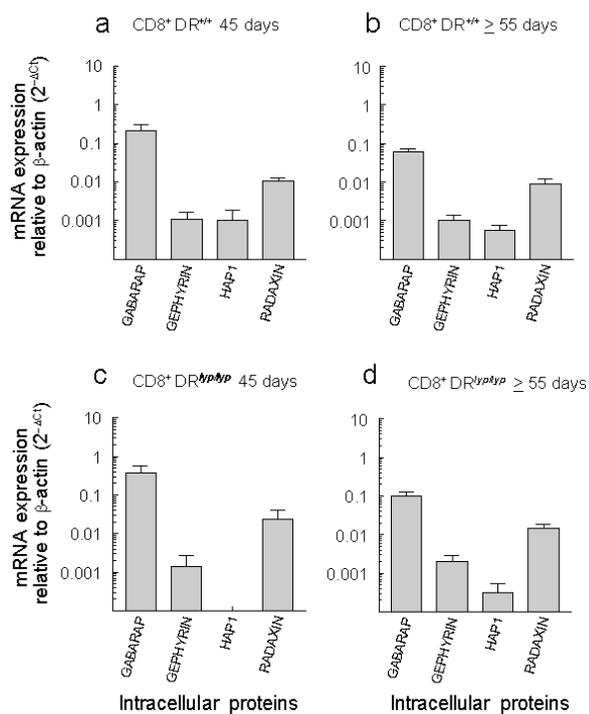


Fig 4

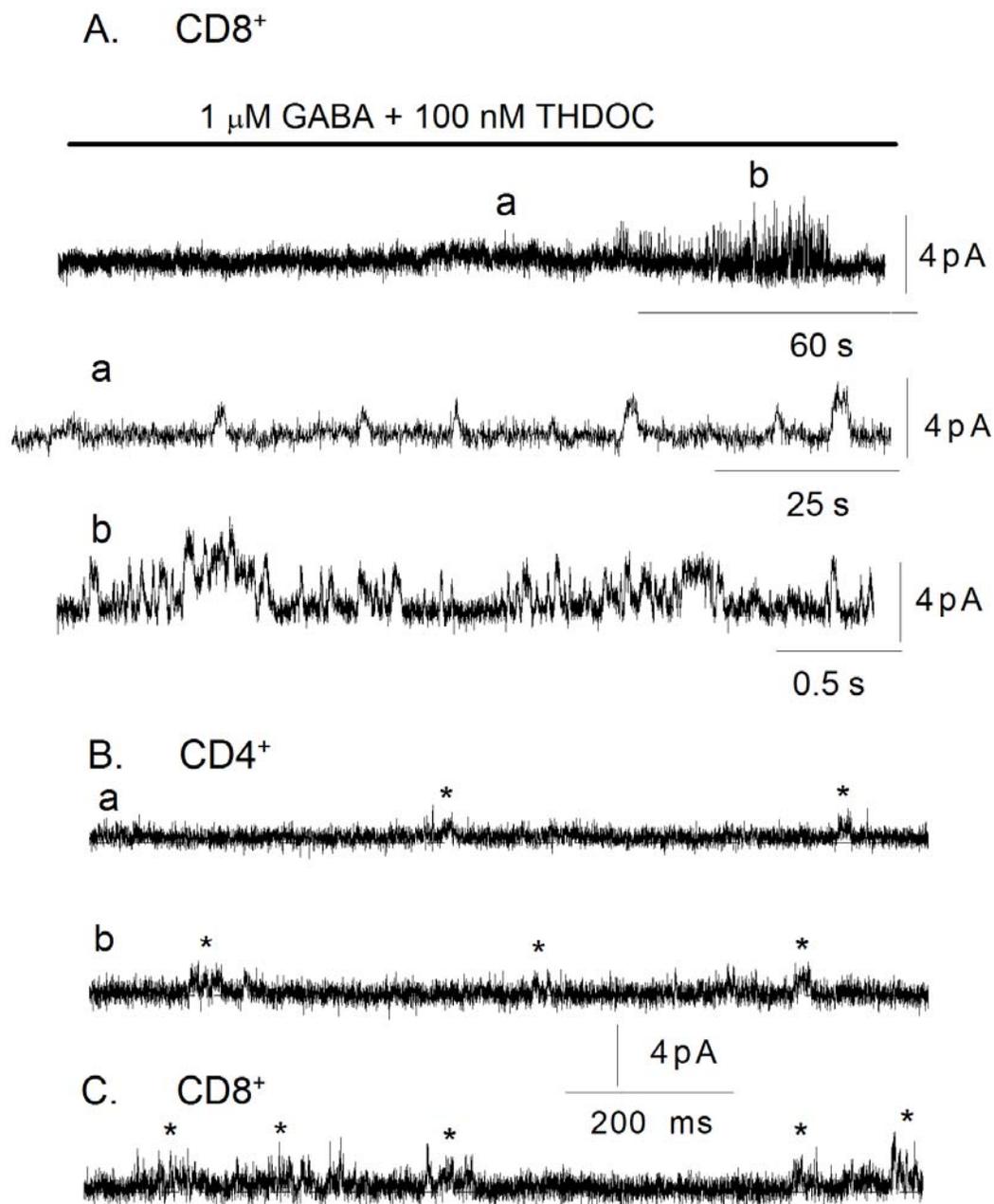


Fig 5

