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**Original full article**

# Effects of Deletion of the Connexin 32 gene on pro and Anti-inflammatories Aspects in the Ethidium Bromide Toxic Demyelination Model

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**Abstract**

Gap junctions are cellular structures that allow transit of molecules between cells, allowing intercellular signaling and transportation. They are formed by proteins denominated connexins and represent key structures in highly complex and integrated tissues, such as the central nervous system (CNS). The present study evaluates the effects of connexin 32 (Cx32) deletion upon CNS inflammation and regeneration/repair after 1, 3, 7, 10 and 20 days after intracerebral injection of ethidium bromide in Cx32 Knock Out and normal mice. To accomplish so, Real Time PCR gene expression quantification was performed upon Tumour Necrosis Factor alpha (TNFα), Transforming Growth Factor beta 1 (TGFβ1), Metalloproteinase 3 (MMP3), Metalloproteinase 9 (MMP9) and Tissue Inhibitor of Metalloproteinases 1 (TIMP1) genes. Results indicate varying differences in the expression pattern, including difference in expression of all evaluated genes in the 3 days post injection period, apex of the acute inflammation mechanisms. These results suggest that Cx32 may perform important functions on molecular, inflammatory and regenerative/repair signalling in the CNS.

**Key Words:** Connexins, Mice, Encefalitis, Ethidium bromide

## Introduction

To investigate the role and function of the connexin 32 protein, a specific strain of mice was developed in which the connexin 32 gene is not expressed. Using this strain of mice, this study assesses the effects of the deletion of the connexin 32 gene in the expression of a variety of genes related to the stimulation and control of the inflammatory process (3), as well as the healing and regenerative processes occurring in the central nervous system (CNS), after a stimulation produced by an injection of an ethidium bromide (EB) solution. To achieve this purpose, real-time Polymerase Chain Reaction (real-time PCR) was used to evaluate the expression of genes for Tumor Necrosis Factor alpha (TNFα), Transforming Growth Factor beta 1 (TGFβ1), Metalloproteinase 3 (MMP3), Metalloproteinase 9 (MMP9), Tissue Inhibitor of Metalloproteinases 1 (TIMP1) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

## Connexins

Gap junctions are sites in the cell membrane with intercellular channels composed of twelve protein subunits called connexins. This type of connection is an efficient way of intercellular communication in many tissues, including brain tissue, and act as passages for exchanging ions, second messenger and metabolites between cells, permeable to molecules of up to one (1) KDa (4). Connexin expression and intercellular communication

through gap junctions are very important for the regulation of functions such as cell motility, proliferation and survival, intercellular communication and transport of substances. Gap junctions occur in most tissues; however, these structures are particularly important in the nervous system, where the tissue structure is more complex and interconnected than in other tissues (10).

**The ethidium bromide (EB) demyelinating model**

Ethidium bromide (EB) is a fluorescent purple dye, which acts by intercalation in the nucleic acids. In the experimental demyelination model it is gliotoxic, acting selectively in the CNS and peripheral nervous system (PNS), being able to promote primary demyelination, with preservation of vascular structures (6), by causing the death of oligodendrocytes and its precursors, as well as astrocytes. The EB demyelinating model was initiated in rats, and was developed to study in detail the process of degradation of the myelin sheaths and their subsequent repair, serving as a model for the study of demyelinating diseases such as multiple sclerosis (MS). Many variants of the model were developed, mostly in the CNS (5), including studies of the relationship between the process of demyelination/remyelination and connexins physiology, like this present work.

**Tumor Necrosis Factor Alpha (TNFα)**

TNFα is the main acute inflammatory response mediator, mainly produced by activated mononuclear phagocytic cells. The main TNFα activity is verified in neutrophils and monocytes stimulation and recruitment to inflammatory sites. This is accomplished through stimulation of adhesion molecules expression by vascular endothelial cells, like selectins and neutrophil integrins receptors. Additionally, it is known that this molecule is capable of increasing expression levels of the major histocompatibility complex type I molecules, increasing the cytotoxic activity of CD8+ T cells (1). In the CNS, it is known that TNFα is able to inhibit intercellular communication through gap junctions and that TNFα increases the levels of phosphorylated connexin 43 in primary astrocytes cultures (7).

**Transforming Growth Factor Beta (TGFβ)**

TGFβ is part of a cytokines superfamily with immunosuppressive activity, inhibiting T and B Cells (and other cells) proliferation, inhibiting macrophages and endothelial cells activation, and it antagonizes the action of the main macrophage activating factor, interferon γ. TGFβ KO mice exhibit inflammatory lesions and uncontrolled lymphoproliferation (1). Previous studies show that TGFβ is expressed at high levels in inflammatory infiltrate cells in the CNS during all phases of Experimental Autoimmune encephalomyelitis, (EAE) in a possible attempt to limit inflammation (14).

**Matrix Metalloproteinases (MMPs)**

Matrix metalloproteinases are a family of zinc-dependent proteases responsible for degradation of the proteins found in the extracellular environment, acting in a myriad of physiological processes, such as balance of production/remodeling/degradation of tissue proteins, leukocyte migration/diapedesis and tissue healing. Twenty-three MMPs have been described in mice so far (11; 16). Studies with MMP KO mice have shown that the inflammatory response in these animals by EAE stimulation tends to a Th1 response (correlated to autoimmune responses with increased synthesis of proinflammatory cytokines) with reduced Th2 response, demonstrating a protective action of MMPs in autoimmune inflammatory response (17).

**Tissue Inhibitors of Metalloproteinases (TIMPs)**

Protein homeostasis is a finely orchestrated process in a healthy body. Small imbalances in protein production/remodeling/degradation can lead to various diseases such as rheumatoid arthritis and keloids (8). In the CNS, this balance can be seen in beneficial processes like mielinogenesis and in the learning process, as well as in damaging processes such as degradation of the blood-brain barrier and oxidative stress, among many other processes (2). For this reason, control mechanisms for MMP activity are diverse and work together in a complementary way; among these mechanisms: control on gene transcription, production of MMPs in the form of pro-enzymes and the presence/activity of Tissue Inhibitors of Metalloproteinases (TIMPs), important control elements of the activity of MMPs.

Four (4) TIMPs have been described in mice so far. Its reduced number in comparison to the number of existing MMPs (23) shows that the activity of these inhibitors is relatively unspecific, each TIMP possessing the ability to control the activity of more than one MMP. It is also reported redundant activity of these TIMPs, so that some MMPs are regulated by more than one TIMP (11, 16).

**Materials and methods**

**Animals**

The lineage of KO mice used in this experiment corresponds to a tenth generation (F10) of an initial crossing of C57BL/6 x ADCx32 mice carrying the connexin 32 deletion. C57BL/6 male mice (genotype Cx32Y/+) were mated with one or two heterozygous (genotype Cx32+/-) C57Bl/6 females, carriers of a partial deletion of the connexin 32 gene. As the connexin 32 gene

is located on the X chromosome, part of the male offspring from these matings displays the genotype Cx32Y/- genotype and part displays the Cx32Y/+ one. Both genotypes were used in this study, either in the Knock Out group (KO, Cx32-) or in the Wild Type group (WT, Cx32+).

**Surgical Procedure**

Under anesthesia (a xylazin and ketamine association) and with the aid of millimetrically controled guides of a stereotactic surgery device, a 5µl 0,1% bromide solution injection was performed with a Hamilton® syringe in the basal cistern of the mice CNS. Mice were monitored until they recovered completely from anesthesia, with full postoperative analgesia and support.

**Material processing for real-time PCR**

To obtain representative samples of the different stages of the inflammatory and regenerative/healing process, the mice were euthanized and their brains were collected on days 1, 3, 7, 10 and 20 after surgical injection (Table 1). Additionally, five (5) WT and five (5) KO mice which received no treatment (Witness group) (Table 2) were euthanized, with immediate collection of its brains. The purpose of the witness group is to serve as a control of the base expression of the genes studied, thereby composing together with the experimental groups a representative picture of the expression of pro- and anti-inflammatory factors participants in the organic response in the EB demyelination model.

A tissue sample totaling 30µg was collected from the brain area corresponding to the injection point from each mouse, and the sample was preserved with RNA preserving solution and frozen at -80$^{o}$ C. Later, extraction of total RNA was carried out, with its quantification, reverse transcription, verification of contamination by genomic DNA, validation of the specificity of the assays, comparative CT efficiency validation and subsequent real time PCR of the molecules of interest, all accordingly to standard procedures of the Laboratory of Molecular Biology, Department of Veterinary Preventive Medicine and Epidemiology of FMVZ-USP (São Paulo, Brazil).

**Results**

The samples were processed and analysed in a StepOne real-time PCR Thermocycler (Applied Biosystems INC). Data collection and analysis was performed with the aid of the StepOne program SDS 1.2 (Applied Biosystems Inc.). The ΔCT data of the TNFα, TGFβ1, MMP3, MMP9 and TIMP1 genes from both WT and KO groups were compared using the Wilcoxon-Mann-Whitney nonparametric hypothesis testing (18) by Minitab ® 15.1.1.0, due to the fact that in real-time PCR experiments using realistically small sample sizes, a distribution-free Wilcoxon test provides be a more robust and appropriate alternative for analysis. ΔΔCT between KO and WT groups was established for each gene and the relative variation was calculated.

The data analysis indicates the existence of differences in the patterns of expression of part of the genes studied and in part of the groups, comparatively between the Cx32-KO and Cx32-WT groups (P <0.05) (Figures 2 to 6 and Tables 3-7). These differences are

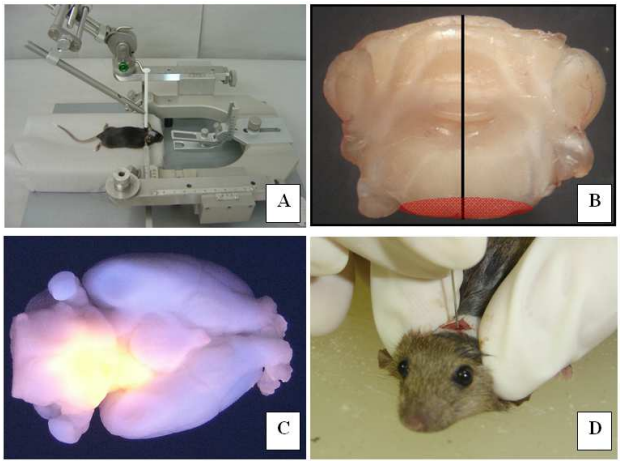


**Figure 1 –** Details of the surgical procedure of ethidium bromide solution inoculation. **A)** Shaved mouse positioned over heated platform in the stereotaxic surgery equipment. **B)** Indicative scheme of the needle pathway and the ethidium bromide inoculation spot. **C)** Ultraviolet light photography of the brain showing the ethidium bromide inoculation spot. **D)** Demonstration of the Hamilton needle insertion in the aenesthesied mouse.

**Table 1 –** Number of mice present in each Experimental group, at 1, 3, 7, 10 and 20 days post-injection

| | Experimental Group | |
|---|---|---|
| **Days** | **F1 Cx32-KO** | **C57BL/6** |
| **1** | 5 mice | 5 mice |
| **3** | 5 mice | 5 mice |
| **7** | 5 mice | 5 mice |
| **10** | 5 mice | 5 mice |
| **20** | 5 mice | 5 mice |
| **Total** | **50 mice** | |

**Table 2 –** Number of mice present in each Witness group.

| | Witness Group | |
|---|---|---|
| **Days** | **F1 Cx32-*KO*** | **C57BL/6** |
| **-** | 5 mice | 5 mice |
| **Total** | **10 mice** | |

reflected as increases in the levels of expression (up-regulation) or reductions in the levels of expression (down-regulation) of the genes studied, namely:

- The witness Cx32-KO group showed variation of TGFβ1 gene expression (120% when compared to Cx32-WT control group); MMP3 (32% compared to control); MMP9 (48% compared to control) and TIMP1 (20% compared to control);
- The three days post-inoculation Cx32-KO group showed upregulation of all genes studied: TNFα (161% compared to control), TGFβ1 (161% compared to control), MMP3 (255% compared to control), MMP9 (139% compared to control) and TIMP1 (194% compared to control);
- The 20 days post-inoculation Cx32-KO group showed upregulation in expression of the TNFα gene (215% compared to control);
- The expression levels of genes in the other groups showed no statistically significant differences (P> 0.05).

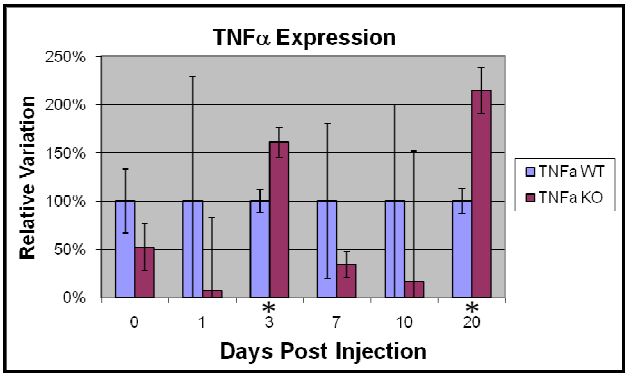


**Figure 2** – Comparative graphic of the medium relative variation values and respective standard deviation values of expression of TNFα in the wild type (WT) and Knock Out (KO) mice at 1, 3, 7, 10 and 20 days post injection, and witness group (0). *: Sample statistically different, with P<0,05.

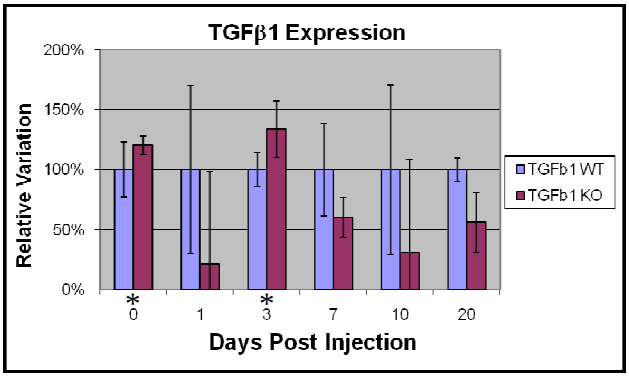


**Figure 3 –** Comparative graphic of the medium relative variation values and respective standard deviation values of expression of TGFβ in the wild type (WT) and Knock Out (KO) mice at 1, 3, 7, 10 and 20 days post injection, and witness group (0) *: Sample statistically different, with P<0,05.

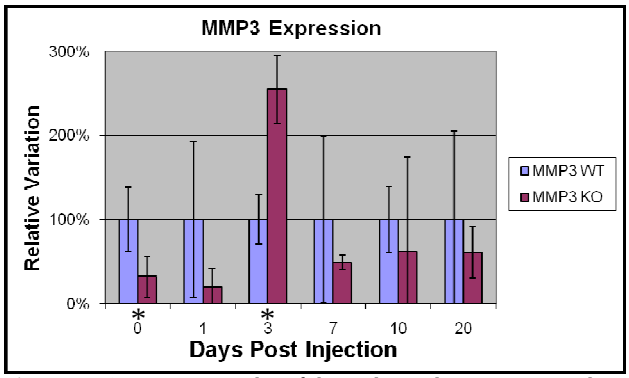


**Figure 4 –** Comparative graphic of the medium relative variation values and respective standard deviation values of expression of MMP3 in the wild type (WT) and Knock Out (KO) mice at 1, 3, 7, 10 and 20 days post injection, and witness group (0) *: Sample statistically different, with P<0,05.

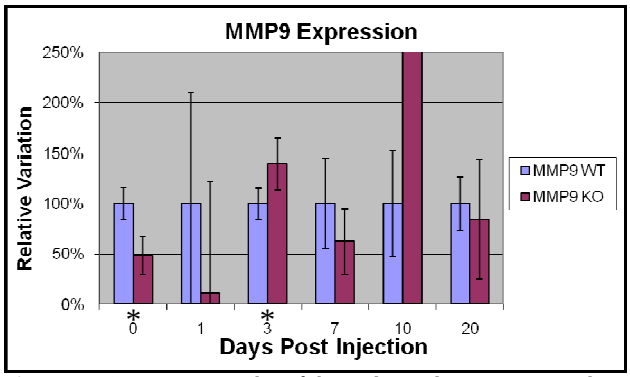


**Figure 5 –** Comparative graphic of the medium relative variation values and respective standard deviation values of expression of MMP9 in the wild type (WT) and Knock Out (KO) mice at 1, 3, 7, 10 and 20 days post injection, and witness group (0). KO 10 days sample marked 696% and was not marked in the graphic since it was not statistically different (P>0,05). *: Sample statistically different, with P<0,05.

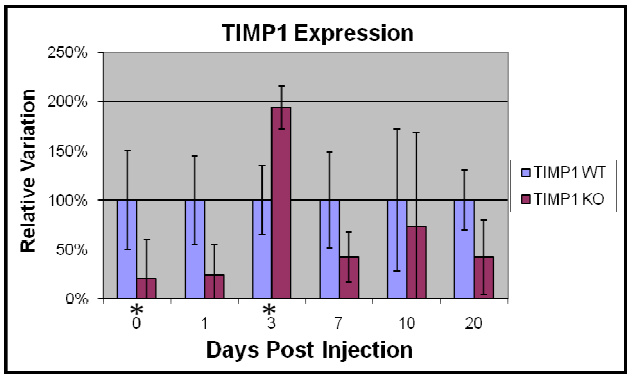


**Figure 6** - Comparative graphic of the medium relative variation values and respective standard deviation values of expression of TIMP1 in the wild type (WT) and Knock Out (KO) mice at 1, 3, 7, 10 and 20 days post injection, and witness group (0) *: Sample statistically different, with P<0,05.

**Table 3 –** Results of the Wilcoxon-Mann-Whitney test, comparing the ΔCT values from the WT and KO groups for the TNFα assay, indicating the existence or absence of difference between the groups, the respective significance level (P), values of ΔCT e ΔΔCT and respective standard deviation and relative variation of the expression between the groups. Values above one (1) indicate upregulation, while values below one indicate downregulation.

| TNFα | | | | | | |
|---|---|---|---|---|---|---|
| Day | P | Conclusion | $\Delta C_T$ TIMP1 WT | $\Delta C_T$ TIMP1 KO | $\Delta\Delta C_T$ KO-WT | Relative Variation |
| 0 | 0,2963 | Equal | 13,97±0,66 | 14,37±0,49 | 0,40±0,55 | 0,52 |
| 1 | 0,1437 | Equal | 13,34±2,57 | 14,98±1,51 | 1,64±2,19 | 0,07 |
| 3 | 0,0122 | Different | 15,11±0,23 | 14,04±0,31 | -1,07±0,38 | 1,61 |
| 7 | 0,5309 | Equal | 12,31±1,60 | 13,47±0,27 | 1,16±0,38 | 0,34 |
| 10 | 0,5309 | Equal | 14,96±1,15 | 15,51±2,70 | 0,54±2,10 | 0,16 |
| 20 | 0,0122 | Different | 15,87±0,27 | 14,11±0,48 | -1,75±0,65 | 2,15 |

**Table 4 –** Results of the Wilcoxon-Mann-Whitney test, comparing the ΔCT values from the WT and KO groups for the TGFβ1 assay, indicating the existence or absence of difference between the groups, the respective significance level (P), values of ΔCT e ΔΔCT and respective standard deviation and relative variation of the expression between the groups. Values above one (1) indicate upregulation, while values below one indicate downregulation.

| TGFβ1 | | | | | | |
|---|---|---|---|---|---|---|
| Day | P | Conclusion | $\Delta C_T$ TIMP1 WT | $\Delta C_T$ TIMP1 KO | $\Delta\Delta C_T$ KO-WT | Relative Variation |
| 0 | 0,0216 | Different | 9,50±0,45 | 8,94±0,15 | -0,56±0,29 | 1,2 |
| 1 | 0,5309 | Equal | 9,86±1,39 | 10,25±1,55 | 0,39±1,86 | 0,21 |
| 3 | 0,0216 | Different | 9,47±0,28 | 8,64±0,47 | -0,83±0,41 | 1,34 |
| 7 | 0,6761 | Equal | 9,18±0,77 | 9,49±0,33 | 0,31±0,43 | 0,6 |
| 10 | 0,8345 | Equal | 11,90±1,41 | 12,01±1,55 | 0,11±1,56 | 0,31 |
| 20 | 0,2101 | Equal | 9,43±0,20 | 9,65±0,49 | 0,21±0,62 | 0,56 |

**Table 5 –** Results of the Wilcoxon-Mann-Whitney test, comparing the ΔCT values from the WT and KO groups for the MMP3 assay, indicating the existence or absence of difference between the groups, the respective significance level (P), values of ΔCT e ΔΔCT and respective standard deviation and relative variation of the expression between the groups. Values above one (1) indicate upregulation, while values below one indicate downregulation.

| MMP3 | | | | | | |
|---|---|---|---|---|---|---|
| Day | P | Conclusion | $\Delta C_T$ TIMP1 WT | $\Delta C_T$ TIMP1 KO | $\Delta\Delta C_T$ KO-WT | Relative Variation |
| 0 | 0,0367 | Different | 15,25±0,76 | 16,36±0,48 | 1,11±0,54 | 0,32 |
| 1 | 0,2963 | Equal | 12,82±1,86 | 13,47±0,43 | 0,65±1,64 | 0,2 |
| 3 | 0,0122 | Different | 15,81±0,58 | 13,80±0,81 | -2,01±0,65 | 2,55 |
| 7 | 0,6761 | Equal | 13,29±1,96 | 14,01±0,17 | 0,72±0,32 | 0,49 |
| 10 | 0,5403 | Equal | 16,27±0,77 | 16,08±2,25 | -0,18±0,86 | 0,62 |
| 20 | 1 | Equal | 15,14±2,09 | 15,02±0,60 | -0,12±0,84 | 0,61 |

**Table 6 –** Results of the Wilcoxon-Mann-Whitney test, comparing the ΔCT values from the WT and KO groups for the MMP9 assay, indicating the existence or absence of difference between the groups, the respective significance level (P), values of ΔCT e ΔΔCT and respective standard deviation and relative variation of the expression between the groups. Values above one (1) indicate upregulation, while values below one indicate downregulation.

| MMP9 | | | | | | |
|---|---|---|---|---|---|---|
| Day | P | Conclusion | $\Delta C_T$ TIMP1 WT | $\Delta C_T$ TIMP1 KO | $\Delta\Delta C_T$ KO-WT | Relative Variation |
| 0 | 0,0367 | Different | 11,65±0,32 | 12,31±0,37 | 0,66±0,41 | 0,48 |
| 1 | 0,2963 | Equal | 10,96±2,19 | 12,37±2,20 | 1,42±1,74 | 0,11 |
| 3 | 0,0367 | Different | 12,23±0,31 | 11,32±0,51 | -0,91±0,43 | 1,39 |
| 7 | 1 | Equal | 10,91±0,89 | 10,89±0,51 | -0,02±0,70 | 0,62 |
| 10 | 0,0601 | Equal | 14,37±1,06 | 10,78±0,65 | -3,60±0,79 | 6,96 |
| 20 | 0,3913 | Equal | 11,62±0,53 | 11,09±3,48 | -0,54±0,79 | 0,84 |

**Table 7 –** Results of the Wilcoxon-Mann-Whitney test, comparing the ΔCT values from the WT and KO groups for the TIMP1 assay, indicating the existence or absence of difference between the groups, the respective significance level (P), values of ΔCT e ΔΔCT and respective standard deviation and relative variation of the expression between the groups. Values above one (1) indicate upregulation, while values below one indicate downregulation.

| TIMP1 | | | | | | |
|---|---|---|---|---|---|---|
| Day | P | Conclusion | $\Delta C_T$ TIMP1 WT | $\Delta C_T$ TIMP1 KO | $\Delta\Delta C_T$ KO-WT | Relative Variation |
| 0 | 0,0367 | Different | 12,87±1,010 | 14,39±0,791 | 1,52±0,831 | 0,2 |
| 1 | 0,6761 | Equal | 10,53±0,898 | 10,90±0,610 | 0,37±1,694 | 0,24 |
| 3 | 0,0216 | Different | 12,01±0,699 | 10,54±0,426 | -1,48±0,522 | 1,94 |
| 7 | 0,0601 | Equal | 11,27±0,983 | 12,24±0,515 | 0,96±0,300 | 0,42 |
| 10 | 0,8345 | Equal | 14,83±1,446 | 14,60±1,922 | -0,23±0,694 | 0,73 |
| 20 | 0,2963 | Equal | 12,51±0,621 | 13,12±0,751 | 0,61±0,654 | 0,42 |

## Discussion

After four decades and numerous publications, there remains little doubt that connexins, in particular Cx32, Cx43 and Cx26, are extremely important structures in cell communication, inflammation, growth regulation and other cellular processes. Nevertheless the mechanism or mechanisms by which connexins and/or GJIC regulate these cellular processes is far from clear (9).

Many studies have been conducted in the past decade analyzing the effects of several stimuli on the expression and function of connexins (e.g. effects of citokines on Cx expression) (15), but it was only more recently, with the advent of genetically modified organisms (GMOs), that it became possible to study the opposite: the effects of Cx deletion on the expression and function of the stimuli. The present work is one of the few dedicated to this line of research, thanks to a very specific line of Cx32 Knock Out mice.

The present work indicates the existence of differences in the patterns of expression of part of the genes studied and in part of the groups, comparatively between the Cx32-KO and Cx32-WT groups. In particular, we verified that 3 days after the inflammatory/demyelinating stimuli of EB injection, in the Cx32 KO mice the expression of all genes studied (TNFα, TGFβ1, MMP3, MMP9 and TIMP1) were upregulated. The true question lies in why does this happen.

It is becoming increasingly clear that connexins have profound effects on gene expression. Cx-deficient and Cx mutant mice present a range of debilitating phenotypes as in hereditary diseases of the lens (zonular pulverulent cataract), nervous system (X-linked Charcot-Marie-Tooth disease, hereditary non-syndromic deafness), skin (palmoplantar keratoderma), and bone and teeth (occulodigitoldental dysplasia—ODDD). Deficiencies in these mouse phenotypes and human diseases have generally been interpreted in the context of altered intercellular communication within tissues in which each connexins are expressed. However, gene array analyses of transcription profiles of tissues and cells from specific connexin-deficient mice have indicated that there are large-scale alterations in these connexin null transcriptomes, from which it is inferred that gap junctions may be ‘‘hubs’’ in gene expression networks. Therefore, in addition to the role of gap junctional communication in the maintenance of homeostasis, morphogenesis, cell differentiation, and growth control in multicellular organisms, altered phenotype in connexin-deficient mice and in disease-causing mutants could also arise from altered linkage of gene expression modules through absent or aberrant ‘‘hubs’’ (9).

In order to explicitly delineate the roles of Cx32 and Cx43 in the expression of growth–development–maintenance genes in brain and heart, Kardami et al have reanalyzed previously published cDNA array data deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo, platform GPL1698, series GSE1954 (samples GSM34855, 34858–34864, 34869–34872) and GSE1961 (samples: GSM35025–35032). They have compared the expression regulation of these genes in the brain of Cx32 and Cx43 null mice with respect to their wild-type counterparts (9).

This review demonstrated that twenty-one growth genes were significantly regulated in both Cx43 null and Cx32 null brains. Interestingly, more than four times as many of these genes were commonly regulated in brains of Cx43 null and Cx32 null mice as in Cx43 null brain versus Cx43 null heart, suggesting an overlapping of Cx43- and Cx32-dependent transcriptomic modules in the brain (9).

Among these genes, Transforming growth factor beta 2 (TGFβ2) showed an upregulation of 1,66x between Cx32 KO and WT mice. This data corroborates the finding in our work regarding the TGFβ1 gene (a closely related gene of the TGFβ superfamily), which showed an upregulation in the order of 1,20x between KO and WT mice. Our work demonstrated that this upregulation is even higher when comparing KO and WT mice 3 days post-injection, with an increase in the order of 1,34x.

These microarray data thus demonstrate that Cx32 plays a significant role in regulating the expression of genes involved in growth. The robustness of the transcriptomic effect of Cx32 removal on growth genes in both brain of $Cx32^{-/-}$ mice indicate that Cx32 may act as a central node in the transcriptomic control of growth. Moreover, comparison of genes regulated in Cx43 and Cx32 null brains indicate both a remarkable degree of overlap (which may be due either to channel-mediated effects or to common binding of regulatory molecules to both connexins) and also connexin-dependent transcriptomic effects (that could either be due to permeability differences between these connexins or to different binding partners) (9).

Brain inflammation is a widespread process associated with neurodegenerative diseases. This pathophysiological response is characterized by astrocyte reactivity and microglia activation, resulting in production of proinflammatory cytokines, mainly from activated microglia. Soluble factors secreted by activated microglia were identified as responsible for a strong inhibition of astrocyte–astrocyte gap junction communication; tumor necrosis factor-α and interleukin-1β were shown to be specifically involved in this inhibition.

There are at least two distinct mechanisms by which expression of other genes may be modified by the expression of connexins (9):

- **Channel-dependent mechanisms** - In this model, signaling molecules are directly exchanged between cell cytoplasms thereby coordinately regulating gene expression patterns in the nucleus
- **Connexin-dependent but channel-independent mechanisms -** In this model, connexins that may or may not be at the junctional membrane either bind a molecule with transcriptional activity or can cleave such a portion of the carboxyl terminus to signal to the nucleus.

Regarding Cx43, previous works demonstrated that hemichannels and gap junction channels in astrocytes are regulated oppositely by tumor necrosis factor-α and interleukin-1β released from activated microglia. This cytokines reduce intercellular communication via gap junctions. Treatment with these two cytokines reduced the total and cell surface Cx43 levels, suggesting that the increase in membrane permeability was attributable to an increase in hemichannels activity (12).

Similarly, the closure of gap junction channels by uncoupling agents was reported to increase neuronal vulnerability when co-cultures of astrocytes and neurons were exposed to oxidative stress or to glutamate. In agreement with this hypothesis, in Cx43 heterozygote knock-out mice, ischemia induced by the occlusion of the middle cerebral artery resulted in a larger infarct volume as

compared to that observed in wild type mice, suggesting that gap junctions in astrocytes plays a neuroprotective role (13).

Many cellular and molecular changes are also accompanied by modifications in astrocyte GJIC. Indeed, complex changes in astrocyte gap junctions, such as increase or loss of Cx32 and Cx43 expression and GJIC, have been observed after brain injuries and pathologies associated to reactive gliosis. These observations suggest that during local inflammation in the brain, pro-inflammatory cytokines, endothelins and purines may regulate the expression of connexin-43 and GJIC astrocytes. Presently, the functional consequences of GJIC inhibition in astrocytes during inflammation are not understood fully.

As mentioned above, inhibition of GJIC may restrict the passage of active molecules to neighboring astrocytes, reduce the spread of apoptotic signals within astrocytic networks, and isolate intact tissues from primary lesion sites. Alternatively, increased GJIC will support the propagation of cell damage, contribute to the bystander effect, and decrease neuronal vulnerability to oxidative stress. This data suggest a plausible explanation as to why in the present work many of the genes studied appear upregulated, since a larger quantity of molecules and second messengers would be necessary to achieve a similar degree of cell communication in Cx32 KO mice. This would be especially important in the 3 days post injection, in which we observed an upregulation of all the genes studied (TNFα, TGFβ1, MMP3, MMP9 and TIMP1).

Available data related to Gap junction changes during and after brain pathologies and lesions are still sparse, and appear somewhat contradictory. These differences may result from the spatiotemporal complexity of the connexin changes, differences in the types of brain damages, insult time, and differences in species and model systems. The answer to these questions is an exciting challenge for the next coming years, which should be addressed by using transgenic animals (13), like in the present work.

## Conclusion

In this study it was found that the witness KO group, which received no manipulation or procedure that could elicit an inflammatory response, showed significant reduction in the expression (downregulations) of the MMP3, MMP9 and TIMP1 genes and significant increase in the expression of the TGFβ1 gene when compared to the witness WT group. Additionally, there was a significant increase in expression (upregulation) of all the genes studied (TNFα, TGFβ1, MMP3, TIMP1 and MMP9) in the 3 days post-inoculation Cx32-KO group. Important to note that this period corresponds to the peak of the acute inflammatory response mechanisms. In the group of 20 days post-injection, there was a significant upregulation of the TNFα gene in the Cx32-KO group.

Based on the previously stated, we conclude that there are differences in the patterns of gene expression of the Tumor Necrosis Factor Alpha (TNFα), Transforming Growth Factor Beta 1, (TGFβ1), Matrix Metalloproteinase 3 (MMP3), Matrix Metalloproteinase 9 (MMP9) And Tissue Inhibitor of Metalloproteinases 1 (TIMP1) genes between Cx32-KO and Cx32-WT mice, either under normal conditions and in response to inflammatory and regenerative/healing stimuli promoted by intracerebral injection of a solution of ethidium bromide.

These differences suggest that Cx32 may perform important functions on molecular inflammatory and regenerative/repair signalling in the CNS. However, further studies are necessary to assess the biological consequences of such differences in the context of the CNS physiology and pathology.

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