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Resumo Alargado

Implication of induced Reelin-KO in the deposition of Aβ plaques in Alzheimer´s Disease mouse models

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Abstract

The aim of the present experiment was to study the implication of the absence of extracellular protein reelin in the deposition of Aβ plaques in an Alzheimer´s disease (AD) mouse model. A novel strain of inducible reelin-KO mouse models, called floxed-reelin, was crossed with J20 models to obtain the JfRLn mouse strain. Floxed-reelin mice were used so that the absence of reelin in a well-developed brain could be studied, whereas J20 mice were used as an AD model, since they carry the mutated hAPP transgene, that causes Aβ plaques to accumulate in the brain (a known trait of AD). Brain sections of every J20+ animal were stained by immunohistochemistry (IHC) against Aβ plaques and analyzed so that the percentage of hippocampus area occupied by plaques was quantified in each J20+ animal. The JfRLn mice were then divided in J20-KO and J20-controls according to their reelin genotype, and a mean of hippocampal plaque percentage was calculated for the two reelin genotypes.

Reelin is viewed as a possible target for the treatment and prevention of AD, since the downregulation of its pathway in the adult brain is involved in several neurological diseases, including AD. Reelin has been observed to decrease Aβ aggregation in vitro, and reelin overexpression in J20 mouse models decreased Aβ plaque accumulation, although reelin overexpression has also been shown to cause mispositioning of adult generated neurons in the preexisting brain circuitry. It seems that precise activity levels of the reelin pathway must be maintained in the adult brain, a dysregulation either up or down causes an imbalance in brain mechanisms and a disorganization of its structures.

The hypothesis that the absence of reelin in the J20 mouse brain increases the deposition of Aβ plaques in 1-year old mice was not supported by the obtained results. The mean of the percentage of plaque area obtained for the J20 reelin-KO (no reelin) genotype was 1.98%, while for the J20 reelin-WT (reelin control) was 1,76%. The results were submitted to a statistical t-student test, being ascertained that the difference of Aβ plaque load between genotypes was non-significant.

Keywords: Alzheimer´s disease, reelin protein, Aβ plaques, mouse models, hippocampus.

Introduction

Alzheimer´s Disease (AD) is a progressive neurodegenerative disorder characterized by memory loss and cognitive impairment. AD is the most common form of dementia, representing up to 75% of all

dementia cases. The second most common dementia worldwide is vascular dementia (related with cardiovascular disorders). In Europe, the prevalence of dementia in people aged 65 years or older is 6.4% and 4.4% for AD. Because dementia is associated with older age, the gradual increase of the age of the population will cause the number of AD cases to increase as well. Finding a way to treat and prevent dementias like AD, is at the top of current medical priorities.

Reelin is an extracellular matrix glycoprotein that plays a vital role in brain development and lamination. It continues to be expressed in the adult brain, playing an essential part in the integration of newborn neurons in the preexisting brain circuitry. Interestingly, in the adult brain, reelin participates in the control of mechanisms that are reminiscent of developmental processes. In fact, reelin controls neuronal migration, in development and throughout life, by guiding newly formed neuronal cells to their proper destination, so that normal synaptic connections may be established between neurons. The downregulation of the reelin pathway in the adult brain is involved in several neurological diseases, including AD. On the other hand, the overexpression of reelin has also been shown to cause mispositioning of adult generated neurons, in mouse models. Thus, it seems that precise levels of reelin must be maintained in the adult brain, a dysregulation either up or down causes an imbalance in the brain mechanisms and a disorganization of its structures (Pujadas et al., 2010) (Teixeira et al., 2012).

The main neuropathological hallmark of AD is the extracellular deposition of Aβ plaques, formed from the aggregation of toxic Aβ oligomers. The human APP (hAPP) mutation causes the overexpression of APP (the precursor of Aβ) and the subsequent accumulation of amyloid plaques in the brain, being known as a cause for early onset, aggressive AD in humans. Interestingly, reelin has previously been shown to reduce Aβ plaque formation in vitro, and its overexpression decreased Aβ plaque accumulation in the brains of AD mouse models (J20 mice) (although it also caused mispositioning of adult born neurons in preexisting brain circuits), creating a link between reelin and AD (Pujadas et al., 2014) (Pujadas et al., 2010).

The complete elimination of reelin, since the embryonic stage, has been studied with reeler mice, that display deformed brains because reelin signaling was not active in the course of brain development. The novelty of this project is the new JfRLn lineage of inducible reelin silencing mice that allows the study of the absence of reelin in a well-developed AD brain, while also allowing the control of when the reelin gene ceases to be expressed. Inducible reelin knockouts (i.e. reelin absence in a normally developed J20 adult brain), have never been thoroughly studied before. It could also be interesting to study what consequences the absence of reelin would have in a well-developed mouse brain, otherwise healthy, i.e. without Aβ plaques, although it could be difficult to identify mental illnesses in mice, if it was the case.

The role of reelin in Aβ plaque accumulation in the AD brain has been previously studied with inducible reelin overexpression mouse models (TgRln/J20 mice) (Pujadas et al., 2014). An experiment with TgRln/J20 mice, that overexpress reelin and accumulate Aβ plaques in the brain, consisted on the measurement of plaque load in the brains of these mice and of J20 reelin-WTs, to make a comparison. It was observed that the % of area occupied by plaques in the HC (and in the cortex) was lower in

TgRln/J20 mice than in J20s, at the 12 months-time point. It was also observed that reelin influences the kinetics of Aβ, in a concentration dependent way, by delaying the aggregation of Aβ42 into fibrils, in vitro.

The aim of the experiment conducted in the present project was the attainment of further understanding on the implication of protein reelin in the deposition of Aβ plaques in an Alzheimer´s disease mouse model, in order to ascertain if reelin could be a good target for the treatment and prevention of AD. A novel strain of inducible reelin-KO mouse models, called floxed-reelin, was used so that the absence of reelin in a well-developed brain could be studied. Floxed-reelin mice were used in association with J20 mice, which carry the mutated hAPP gene that causes amyloid plaques to accumulate in the brain, to obtain the JfRLn lineage. Brain sections of each J20+ animal were cut and stained by IHC against Aβ plaques, and then analyzed (with imageJ program) so that the % of hippocampus area occupied by plaques was quantified in each animal. The J20+ JfRLn mice were divided in J20-KO and J20-controls in relation to their reelin genotype. A mean of plaque % in the HC was calculated for each J20+ animal and for the two reelin genotypes, which were then compared. The initial hypothesis of the experiment was that the absence of reelin in J20 reelin-KO mice brains would lead to a more dramatic accumulation of Aβ plaques, compared to J20-WTs.

Methods

Mouse models

The experiment made use of two transgenic mouse models that were crossed with each other: an AD mouse model and a reelin-KO mouse model. The AD mouse model carried the mutated human APP gene (hAPP), that is responsible for the overexpression of APP (the Aβ precursor), leading to the accumulation of Aβ and to its deposition as part of amyloid plaques, which are a known trait of AD. The APP mutation is known to be responsible for the occurrence of early-onset AD in humans. The reelin-KO mouse model is an inducible model that, upon tamoxifen administration at the age of interest, has the reelin gene (RELN gene) silenced, and reelin ceases to be expressed, becoming ubiquitously depleted. From the crossing of the AD model (J20) with the reelin-KO model (fRLn), the mouse line JfRLn is obtained, which is the model used in the experiment. JfRLn models overexpress the mutated human APP transgene and suffer a conditional silencing of reelin upon the administration of the hormone tamoxifen. The JfRLn line is obtained through the crossing between the lineages J20 (purchased from Jackson Lab) and fRLn (this line is a crossing of two lineages itself).

The J20 mouse strain displays the AD trait of Aβ deposition like it is observed in AD patients thus being used as a model of amyloid deposition and pathogenesis in the study of AD (is used as an AD model). J20 mice present many AD-like phenotypes, such as synaptic loss, amyloid plaque deposition and cognitive impairment. J20 mice overexpress the mutated human APP (hAPP) gene, that causes the accumulation of Aβ and deposition of plaques (Mucke et al., 2000).

The lineage fRLn (floxed-reelin) is the inducible reelin-KO mouse model. Floxed-relin lines are generated from the crossing between the lineages floxreelin and UbiCreER. The floxreelin lineage has the first exon of the RELN gene flanked by 2 loxP sequences, which are identified by the Cre recombinase. The UbiCreER lineage carries the gene encoding for Cre recombinase united with a ubiquitous promoter and with the coupling domain of HBD hormone of a mutated estrogen receptor, which retains the recombinase inactivated at the level of the endoplasmic reticulum (ER). When active, the recombinase promotes the recombination between the two loxP sequences, that are flanking the first exon of the RELN gene, resulting in the ubiquitous inactivation of RELN. The tamoxifen activates Cre by setting it free from the ER to carry out the nuclear action of recognizing the loxP sections in the genome and connecting them together, cutting out the first exon of RELN. Thus, JfRLn mice are obtained through the crossing of three different lineages (they are triple-transgenic mice), one that carries the hAPP gene (J20), one that carries the loxP sequences flanking exon 1 of RELN gene (floxed-relin), and another that carries the recombinase Cre gene (UbiCreER).

PCR genotyping

For the elimination of reelin's expression to be possible, the animal must be flox/flox (homozygous), while for J20 and for Cre it can be heterozygous. Four PCRs were carried out on tissue samples of the mice to identify each animal´s genotype. The PCRs used were Cre PCR, Flox PCR, J20 PCR, and Flox-Cre PCR. The possible genotypes for the J20 line (presence of the hAPP gene) were J20 -/- (negative) and J20 +/- (J20 positive), from the cross of a J20+/- with a J20-/-. For the presence of loxP sequences flanking the exon1 of RELN gene, the only possible genotype was flox/flox (flox positive). All mice were homozygous for flox, since only flox/flox mice were used in the crossings. For the presence of the recombinase Cre gene, the possible genotypes were Cre -/- (Cre negative) and Cre +/- (Cre positive), from the cross of Cre+/- with Cre-/- animals.

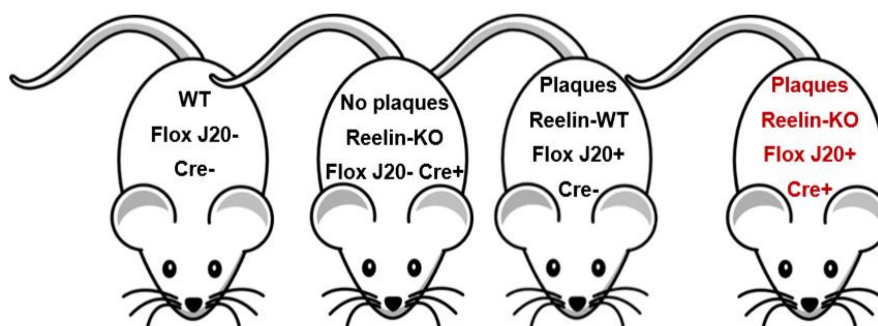

Figure 1: The 4 mutant mouse genotypes obtained. The goal genotype was the Flox J20+Cre+.

Tissue Extraction and Cutting Sections

22 mice were sacrificed at twelve months of age (between 356 and 365 days old) through an intracardiac perfusion, for their brains to be extracted and studied. The brains were then cut coronally, one by one, with a freezing microtome, or with a cryostat in some cases, in 30μM sections, which were distributed in order between ten Eppendorf tubes containing cryoprotective medium (to keep the brains from freezing). This way each tube of sections contained approximately 10% of the brain it held and was a

whole representation of it (every brain region was represented by a few sections in each tube). The tubes with the sections were kept in a freezer at -20°C.

Immunohistochemistry (IHC)

Some brain sections were then differentially stained by immunohistochemistry (IHC). IHC is an application of immunostaining used to selectively identify antigens in cells of tissue sections, using the principle of antibodies binding specifically to antigens in biological tissue. Two distinct IHCs were performed, each one with the aim of identifying a different biomarker. An IHC anti reelin is performed to selectively mark reelin protein, and two anti-Aβ IHCs are performed to selectively mark Aβ deposits.

Reelin IHC (G10 ab)

The reelin IHC was performed in all the animals, using the primary antibody G10 that binds to reelin (ab78540), 2019), to confirm the reelin genotype of each mouse, and to verify if the silencing of the RELN gene in FloxCre+ animals was successful, upon the tamoxifen injection. 2 sections from each animal were selected, one containing the ventricles (more anterior region of the brain) and another containing the hippocampus (more posterior region of the brain). The FloxCre- animals are expected to show high levels of reelin signal since they do not possess the Cre recombinase. On the other hand, FloxCre+ animals are not expected to present any reelin signal, since they do carry the Cre recombinase. After this IHC the animals were divided in reelin wild types (WT) and reelin knockouts (KO).

Anti-Aβ (α-3D6 ab) IHC

A first round of anti-Aβ IHC was performed to visualize amyloid β plaques in sections containing the regions of the hippocampus, cortex, and corpus callosum (white matter), since these are the zones of the brain where the accumulation of plaques is more evident. The primary antibody used to stain Aβ was α-3D6 (raised in mouse), which recognizes amino acids 1-5 of the Aβ peptide (without recognizing the APP molecule) (Alzforum.org, 2019). This HIC was first performed to all animals, in 2 sections of each animal, one containing the ventricles (anterior) and another containing the hippocampus (posterior), to confirm the J20 genotypes. The animals were then divided in genotypes, according to the results of the Aβ IHC differential staining, in J20+ (with plaques) and J20- (without plaques).

The mice that presented signal for Aβ plaques in the first round of anti-Aβ IHC were selected for a second round of anti-Aβ IHC. Because the final tissue analysis is carried out to determine the % of hippocampal area, of each animal, that has a positive signal for Aβ peptide (i.e. the % of HC area that is occupied by plaques), only the sections of animals of the genotypes FloxJ20Cre- and FloxJ20Cre+ were selected for the second round of anti-Aβ IHC (since these are the ones that presented plaques). One tube of sections of each J20+ animal was used for this IHC. Upon this IHC, differentially stained sections against Aβ, representing the whole brain (from anterior to posterior), are obtained for every animal with plaques, and the plaque load of each one can be observed throughout brain regions.

Image Taking

Images of each brain section that resulted from the second anti-Aβ IHC were taken in a NIKON microscope, model ECLIPE-600, at a 10x magnification. The focus of these images was the HC, since that was the zone to be further analyzed.

Quantification of Plaque Area

The pictures were then used for the quantification of the percentage of HC area containing Aβ, for each animal. The goal was to compare hippocampal plaque loads between reelin genotypes and to ascertain if the difference in the presence of reelin had a visible effect on the amount of plaques that accumulated in the HC of these mice.

The images were processed and analyzed with the software *Fiji (Fiji Is Just ImageJ)*, based on the program *ImageJ*, which is an open source image processing program designed for scientific images. In each image, the area of interest was cropped around, in this case the hippocampus. After the HC areas were cropped, the *ImageJ* macro (quantifier) was trained, using the option *trainable weka segmentation*. The training of the quantifier was done with the same images that were to be quantified, to maximize accuracy. The program used for the Aβ plaques quantification presented four different categories - tissue, plaques, vessels and outside - in which the different regions of the images were differentiated and classified, based on color and texture. The obtained result from the quantification was an excel file with a table containing the name of every picture analyzed and the quantification, for each picture, of the fractions of area occupied by tissue, plaques, vessels and outside.

Results

Flox+Cre+ animals were reelin-KO

It was observed that sections which did not display reelin signal, belonged to mice previously genotyped by PCR as Flox+Cre+. Mice that did not express reelin were considered reelin-KOs. Moreover, it was ascertained that the sections from mice that had been previously genotyped as Flox+Cre- by PCR, were differentially stained by the IHC, displaying reelin signals. If reelin signal is observed in a brain section of a Flox+Cre- mouse, then the Cre- genotype is confirmed. All Cre- mice expressed reelin and were therefore classified as reelin-WTs. Two mouse models that were expected to be reelin knockouts, because they had been previously genotyped by PCR as Flox+Cre+, displayed reelin signal and were classified as failed or partial inactivations, being excluded from further calculations. Upon observation of the sections resulting from the reelin IHC, the animals were grouped: five mice presented no reelin signal and were classified as reelin-KOs (complete reelin inactivations upon tamoxifen administration), whereas fifteen displayed reelin signal (and were Cre-), being classified as reelin-WTs. All animals were Flox+.

J20+ animals presented plaque deposits

It was observed that all the sections belonging to mouse models previously genotyped by PCR as J20+ displayed positive signal for Aβ plaques, obtained from the Aβ IHC, as it was expected. Because

differentially stained Aβ plaques were observed in these tissue sections, the J20+ genotype was confirmed (the phenotype matched the genotype). In a similar way, the sections belonging to mouse models previously genotyped by PCR as J20- presented no signal for Aβ plaques, as expected. Because no Aβ signal was observed in the sections of J20- mice (i.e. no differential staining for Aβ was encountered in these tissues), the J20- genotypes were confirmed. Thus, all the J20 genotypes were confirmed in this first step of Aβ IHC. Twelve mice were classified as J20+ (Aβ plaques), whereas ten were classified as J20- (no Aβ plaques). Only the J20+ animals were studied further, since the aim was to compare the plaque loads of the two reelin genotypes.

The novelty of this experiment was the study of animals with the genotype Flox+J20+Cre+, since those mice have brains with Aβ plaques and no reelin, simultaneously (i.e. these are models of AD pathology combined with reelin absence). Only two mice were classified both as reelin-KO and J20+.

Second anti-Aβ IHC of J20+ sections for further plaque quantification

The J20+ animals were selected for a second round of anti-Aβ IHC. This time, one entire tube of brain sections of each animal was used. This anti-Aβ IHC was done to differentially stain Aβ plaques in the tissue sections, making them visible under the microscope, for further quantification. Photos of every brain section that was submitted to the second round of Aβ IHC and contained the hippocampus were taken in the microscope, so that the quantifier could then analyze them and quantify the plaque area.

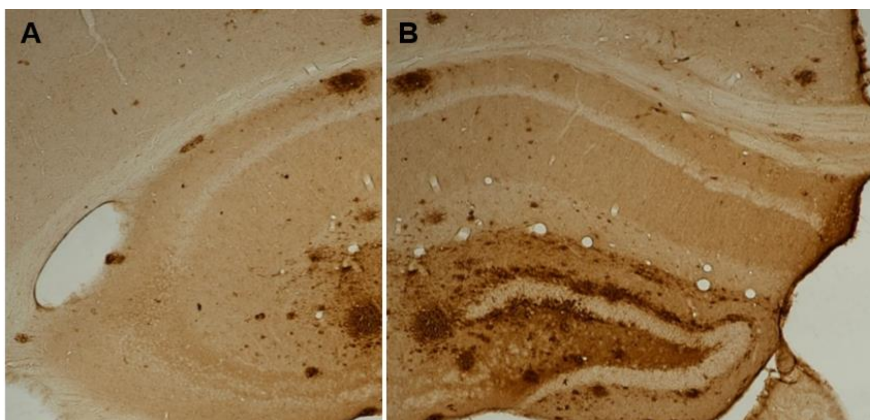

Figure 2: Pictures A and B correspond to one hippocampus section (10x magnification) of a J20+ mouse model. An IHC against Aβ was performed in the section, being the Aβ plaques the darker stains.

Aβ plaque load in J20-KOs was not statistically higher than in J20-WTs

After the HC areas were cropped around, and the *ImageJ* quantifier was trained, the quantifier was run on all the images. With the information obtained from the analysis with *ImageJ*, of the fraction of area corresponding to tissue, plaques, vessels and outside, for each image, the fraction of total area was calculated by the sum of the fractions of tissue, plaques and vessels (only excluding the fraction of outside area). The fractions of area occupied by plaques (plaque area) obtained from cropped pictures that corresponded to the same HC section were added and the same was done for the fractions of total hippocampal area that corresponded to the same HC section. Thus, the fraction values of total plaque area and of total hippocampal area were obtained for each HC section. These values were then used

to calculate the % of plaque area in each HC section, through the division of the fraction of total plaque area in a HC times 100 by the fraction of total area of that same HC. Thus, the values of the % of plaque area in the HC were obtained for every HC section.

Moreover, anterior and posterior means of the % of plaque area in the HC were calculated for each animal. The anterior mean was obtained by the calculation of the average of the values of plaque area % from HC sections considered from anterior brain regions, whereas the posterior mean was obtained by the calculation of the average of the values of hippocampal plaque area % from HC sections considered from posterior brain regions. These means were calculated to check how the plaques distributed throughout the mouse brains, to ascertain if the deposition was more intense in anterior or posterior regions. The criteria used to separate the HC in anterior and posterior was based on bregma criteria, using the Paxinos Atlas of the mouse brain (Paxinos, Charles Watson and Evan Calabrese, 2015).

Furthermore, the total mean of the plaque area % in the HC was calculated for each animal. The mean of the fractions of total area of HC was also calculated for each animal. After the mean hippocampal plaque % was calculated for every animal, these were divided according to their genotypes: J20 reelin-KO (no reelin) and J20-controls (reelin) and the same 4 means were calculated for each genotype. 9 animals were of the genotype J20 WT and 2 were of the genotype J20 KO. The anterior and posterior plaque % means were also calculated for both groups, to check if the different reelin genotypes influence the distribution of plaques in the brain, in terms of their deposition being more anterior or posterior. Then, the mean of the % of area occupied by Aβ plaques in the HC was calculated for the two genotypes, to find out if the intensity of plaque deposition in the mouse brains was affected by the absence of reelin.

The mean of the total HC area was also calculated for both genotypes, to verify if the two means of plaque % are comparable between them, i.e. to check if the areas of the HC are similar in both genotypes, because if the areas were not comparable, similar plaque loads would originate distinct plaque percentages, or vice versa. Four bar graphs representing Mean +/- SEM were created, one for each of the means, comparing the two genotypes (J20 controls and J20 reelin-KO). A t-student statistical analysis was completed to compare the results of each mean, between the two genotypes.

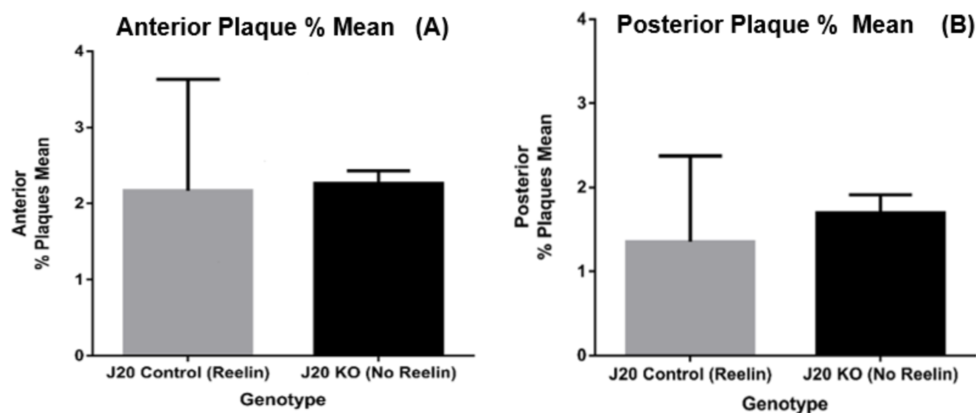

Figure 3: A) Anterior mean of plaque % in the HC for J20-controls and J20 reelin-KO. Results of the unpaired t test: P value = 0,9319 (N.S.), t=0,08788 df=9. **B)** Posterior mean of plaque % in the HC for J20-controls and J20 reelin-KO. Results of the unpaired t test: P value = 0,6603 (N.S.), t=0,4544 df=9.

The outcome of the t-student analysis done both on the results of the anterior plaque % mean and of the posterior plaque % mean were that the difference between the 2 genotypes was not significant (N.S.). This implies that the difference, between genotypes, in the % of HC area occupied by Aβ plaques, in the anterior brain regions, was not significant, and the same is true for posterior brain regions.

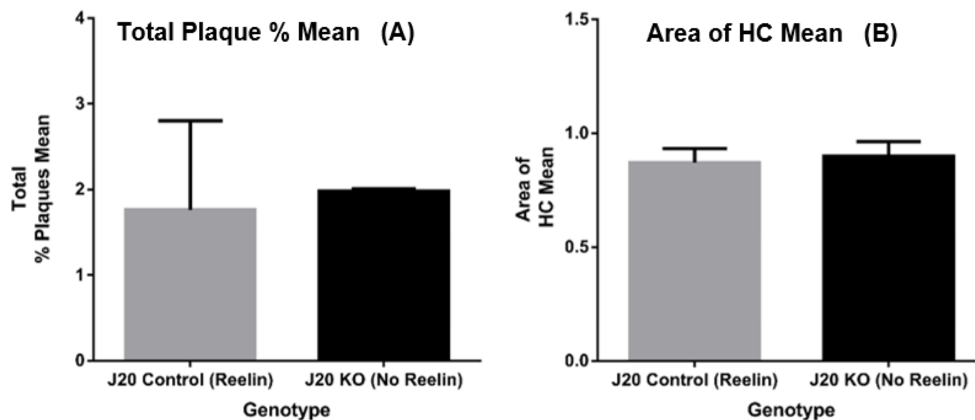

Figure 4: A) Total mean of plaque % for J20-controls and J20 reelin-KO. Results of the unpaired t test: P value = 0,7816 (N.S.), t=0,2857 df=9. **B)** Mean of HC Area for J20-controls and J20 reelin-KO. Results of the unpaired t test: P value = 0,5640 (N.S.), t=0,5989 df=9.

The outcome of the t-student analysis done on the results of the total means of Aβ plaque % in the HC, of the 2 genotypes, was that the difference between the 2 genotypes was not significant (N.S.), indicating no significant difference in the % of area occupied by plaques in the HC, between genotypes. The outcome of the t-student statistical test done on the HC area means was also non-significant (N.S.), meaning that the areas of HC are similar in both genotypes, and that the values of plaque area % in the HC are comparable.

Discussion

The initial hypothesis of the experiment was that the absence of reelin would lead to the increase of amyloid beta deposition in the hippocampus and throughout the brain of J20+ mouse models, sacrificed at 12 months. The results obtained do not support the initial prediction. In fact, although the mean of HC area occupied by plaques is higher in the case of animals that were reelin-KOs than in the case of reelin-WTs, this difference is not statistically significant. The results also show that a bigger % of HC area is occupied by Aβ plaques in the more anterior regions of the brain versus the posterior ones, which is explained by the fact that the hippocampus considered posterior are bigger than the anterior ones (the amount of plaques was not smaller).

It is important to consider that unfortunately only two reelin knockouts were J20+, which represents a very small group sample (n=2). Because the sample is small it cannot be representative of the whole reelin-KO population (i.e. it cannot be easily generalized to bigger groups). Moreover, the results of the means of HC plaque area % in J20 reelin-control animals displayed a high variation between themselves

(elevated SEM). In fact, most animals expressing reelin had plaque % results around 1% (i.e. 2/3 of the J20-WTs had significantly less plaque load than the 2 J20-KO animals studied), while two mice presented plaque means above 3% and another mouse presented a 2,2% plaque mean %. Thus, only 2/3 of the reelin-WT animals displayed the expected results of lower Aβ plaque load than reelin-KOs.

This variation in the influence of reelin on plaque deposition, seems to suggest that reelin does not cause the same effect of slowing down the deposition of plaques in every organism. It might have a more positive impact in some individuals than in others, in which case its results would be difficult to predict with certainty. The variation in plaque load between animals can also be because some animals carrying the hAPP mutation accumulate Aβ more dramatically than others, maybe because some are more susceptible to the mechanisms that lead to plaque development, induced by the mutation. The variation can also be due to the small population number, i.e. it might be rare and specific of this population of analyzed mice, in which case a bigger population of animals would cause this variance to lose significance.

It is also possible that some of the J20-WT mice presented bigger Aβ plaque percentages because the reelin activity was lower in these animals. In fact, although these mice were all WT for reelin expression, reelin activity was not measured and compared. In the brains of some animals the reelin pathway might have become less active in the course of plaque accumulation, which can be an explanation for the higher plaque load observed in some J20-WT mice. Nonetheless, it was expected that the J20-KO mice would present significantly higher amounts of Aβ plaques than J20-WTs, and it was not the case.

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