RESEARCH ARTICLE

REVISED The integration site of the *APP* transgene in the J20 mouse model of Alzheimer's disease [version 2; referees: 2 approved, 1 approved with reservations]

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Abstract

Background: Transgenic animal models are a widely used and powerful tool to investigate human disease and develop therapeutic interventions. Making a transgenic mouse involves random integration of exogenous DNA into the host genome that can have the effect of disrupting endogenous gene expression. The J20 mouse model of Alzheimer's disease (AD) is a transgenic overexpresser of human APP with familial AD mutations and has been extensively utilised in preclinical studies and our aim was to determine the genomic location of the J20 transgene insertion.
Methods: We used a combination of breeding strategy and Targeted Locus Amplification with deep sequencing to identify the insertion site of the J20 transgene array. To assess RNA and protein expression of *Zbtb20,* we used qRT-PCR and Western Blotting.
Results: We demonstrate that the J20 transgene construct has inserted within the genetic locus of endogenous mouse gene *Zbtb20* on chromosome 16 in an array, disrupting expression of mRNA from this gene in adult hippocampal tissue. Preliminary data suggests that ZBTB20 protein levels remain unchanged in this tissue, however further study is necessary. We note that the endogenous mouse *App* gene also lies on chromosome 16, although 42 Mb from the *Zbtb20* locus.
Conclusions: These data will be useful for future studies utilising this popular model of AD, particularly those investigating gene interactions between the J20 *APP* transgene and other genes present on Mmu16 in the mouse.

Keywords

Transgenic, mouse model, Alzheimer's disease, APP, Zbtb20, J20, Amyloid Precursor Protein

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REVISED **Amendments from Version 1**

1. Colour clarification of genotypes from cross in Figure 1.
2. Changed t-tests to non-parametric tests (Mann-Whitney U) in Figure 3 for qPCR and western blot experiments as normal distribution of data could not be tested or assumed due to sample sizes being too low.
3. Changed text in results and discussion to agree with reviewers that protein data is only preliminary and future studies should test for ZBTB20 protein changes in multiple tissue at multiple timepoints with negative control samples.

See referee reports

Introduction

The Tg(PDGFB-APPSwInd)20Lms (MGI:3057148, here referred to as 'J20') mouse model is a transgenic animal that overexpresses mutant human APP protein (amyloid precursor protein), and is widely used as a model of amyloid deposition and pathogenesis in the study of Alzheimer's disease (AD). J20 mice recapitulate many AD-like phenotypes, including synaptic loss, amyloid plaque deposition and cognitive impairment (Hong *et al.*, 2016; Mucke *et al.*, 2000; Palop & Mucke, 2016).

The model was developed by Mucke and colleagues using the PDGF-APPSw,Ind transgene construct described previously (Games *et al.*, 1995; Rockenstein *et al.*, 1995), which includes a human *APP* mini-gene, carrying the familial AD-linked $717_{V\text{-}F}$ (Indiana) mutation (Murrell *et al.*, 1991) and $670/671_{KM\text{-}NL}$ (Swedish) double mutation (Mullan *et al.*, 1992). The transgene construct was designed so that the *APP* mini-gene included genomic sequence for *APP* introns 6–8, allowing expression of hAPP695, hAPP751 and hAPP770 isoforms. The PDGF-APPSw,Ind transgene expression is driven in neurons throughout the brain by the human platelet-derived growth factor β chain (PDGFβ) promoter (Harris *et al.*, 2010; Sasahara *et al.*, 1991). The J20 mouse is an important model: currently, 125 articles have been catalogued in the Mouse Genome Database bibliography (Blake *et al.*, 2017), which report genotypic and/or phenotypic data from this mouse. This strain has been used for several classical genetic studies to determine the interaction of genes of interest with the *APP* transgene including a seminal report of the importance of Tau to Aß-associated neuronal dysfunction (Roberson *et al.*, 2007). Moreover, this model has been used to elucidate the role of Aß in synaptic dysfunction (Palop & Mucke, 2016; Palop *et al.*, 2007; Sanchez *et al.*, 2012).

Transgenic mice are conventionally generated by direct injection of linear foreign DNA into the pronucleus of fertilised zygotes. Once inside the cell, these linear fragments undergo circularisation and concatemer formation before integrating into the host genome as a tandem array (Bishop & Smith, 1989). In principle, transgenes insert randomly into the host genome; however, ~45% of integration sites lie within host gene regions (~13.2 exonic, 31.6% intronic), potentially as a result of increased accessibility of transcriptionally active DNA (Yan *et al.*, 2013). Integration of a transgene array into coding sequences can induce new mutations (for example, haploinsufficiency) (Haruyama *et al.*, 2009), and so it is important to know the site of integration for a transgene array in a mouse model.

A recent study suggested an association of a heterozygous deletion of the *CHMP2B* gene (charged multivesicular body protein 2B) with early-onset Alzheimer's disease (Hooli *et al.*, 2014). Interestingly, mutations in *CHMP2B* are a rare genetic cause of Frontotemporal dementia (Skibinski *et al.*, 2005). We have previously reported generation of a *Chmp2b* knockout mouse (Ghazi-Noori *et al.*, 2012). To determine if *Chmp2b* deletion modulates APP/Aß biology *in vivo*, we attempted to cross our *Chmp2b* knockout with the J20 mouse, to study potential double mutant progeny. The *Chmp2b* locus lies on mouse chromosome 16.

Here, we present the outcome of these genetic cross experiments and the resulting mapping of the J20 transgene array integration site by Targeted Locus Amplification (TLA) with deep sequencing. We discuss how integration of the transgene affects expression of the flanking loci.

Methods

Animal welfare

Mice were housed in controlled conditions in accordance with guidelines from the UK Medical Research Council in Responsibility in Use of Animals for Medical Research (1993). Two female J20 positive animals were killed at 6 months of age to provide splenic material for the TLA study. Furthermore, 3 month hippocampal tissue was collected from J20 animals: N=5, 3 male, 2 female. C57BL/6J controls: N=5, 3 male, 2 female. All used for qRT-PCR and western blotting. All experiments were conducted under license from the UK Home Office and with Local Ethical Review approval. Tg(PDGFB-APPSwInd)20Lms/2Mmjax animals (J20) were obtained from The Jackson laboratory (stock no. 034836) and maintained on a C57BL/6J background in our animal facility. Chmp2b knockout animals were already available in our animal facility (Ghazi-Noori *et al.*, 2012). Mice had access to a mouse house with bedding material and wood chips. All animals had continual access to water and RM1 (Special Diet Services) (stock animals) or RM3 (Special Diet Services) (breeding animals) chow *ad libitum*. Mice were housed in individually ventilated cages in a specific pathogen free facility with a 12 hour light/dark cycle.

Genotyping of J20 and *Chmp2b* knockout mice

DNA was extracted from tail tip or ear biopsy by the HOTSHOT method (Truett *et al.*, 2000).

The presence of the J20 human APP transgene was tested by PCR using primers (Eurofins) *APP*-F: 5'-GTGAGTTTGTAAGTGATGCC-3' *APP*-R: 5'-TCTTCTTCTTCCACCTCAGC-3', control primers ContF: 5'-CAAATGTTGCTTGTCTGGTG-3' ContR: 5'-GTCAGTCGAGTGCACAGTTT-3). Copy number of the human APP transgene was validated by quantitative PCR using a Taqman Fast machine (Applied Biosystems) with the following primers and probes: h*APP*F: 5'-TGGGTTCAAACAAAGGTGCAA-3' h*APP*R: 5'-GATGAAGATCACTGTCGCTATGAC-3' hAPP-probe: FAM-CATTGGACTCATGGTGGGCGGTG-3' qContF:

5'-CACGTGGGCTCCAGCATT-3' qContR: 5'-TCACCAGTCATTTCTGCCTTTG-3' qContProbe: VIC-CCAATGGTCGGGCACTGCTCAA-3'.

The *Chmp2b* knockout locus was detected by PCR with the following primers: Int2_F: 5'-CCATTGCCACTTGGATGTAA-3' Int2_R: 5'-GACGCACTTTAAGGTCACAGC-3' KO_R: 5'- TCTCTGTGCAAGAAGCATGAA-3'. PCR products were separated by agarose gel electrophoresis and visualised using a BioRad Gel Doc XR UV transilluminator.

Extraction of spleen cells from J20 animals

Mice were sacrificed by rising concentration of CO_2 and confirmed by dislocation of the neck and the spleen dissected and kept on ice. Splenocytes were then dissociated through a 40µm mesh filter and the cells collected by centrifugation at 4°C at 500 × *g* for 5 minutes. The supernatant was discarded and the pellet re-suspended in 1 ml red blood cell lysis buffer (4.13g NH_4Cl, 0.5g $KHCO_3$, 193.5µl 0.5M EDTA dissolved in 500ml H_2O) for three minutes at room temperature to lyse splenic erythrocytes. To terminate the lysis reaction, 0.5ml phosphate buffered saline (PBS) was added and the splenocytes were collected again by centrifugation at 500 × g for 5 minutes. The supernatant was discarded and the pellet re-suspended in 0.5ml PBS before a final centrifugation step for 2 minutes. The supernatant was discarded and the pellet was re-suspended in 1ml freezing buffer (PBS with 10% fetal calf serum and 10% dimethyl sulphoxide). Samples were stored at -80°C before preparation for TLA processing.

Targeted Locus Amplification

Processing of samples for TLA was performed by Cergentis B.V. (Utrecht, The Netherlands), as previously described (de Vree *et al.*, 2014). A primer pair targeted to the *APP* transgene sequence was used to perform the TLA. Sequences of the PCR primers are (5' to 3'): 1917_APP_F GAAACTCATCTTCACTGGCA; 1698_APP_R GGGTAGACTTCTTGGCAATA. PCR products were purified and library prepped using the Illumina NexteraXT protocol and sequenced on an Illumina Miniseq sequencer.

Sequence alignment and analysis of TLA

TLA reads were mapped using BWA-SW, which is a Smith-Waterman alignment tool. This allows for partial mapping, which is optimally suited for identifying break-spanning reads. The mouse Mm10 genome assembly version was used for mapping. Visualisation and interpretation of the data were performed using the Integrative Genomics Viewer (IGV) from the Broad Institute (Robinson *et al.*, 2011).

RNA extraction and quantitative reverse transcription PCR

RNA was extracted from whole hippocampus from J20 animals and age and litter matched controls. Total RNA was extracted using the Qiagen miRNeasy kit and reverse transcribed using the Applied Biosystems High-Capacity RNA-to-cDNA™ Kit.

Quantitative RT-PCR was carried out on the *Zbtb20* gene transcript using a predesigned PrimeTime® probe-based qPCR assay (assay ID: Mm.PT.58.41805451, Integrated DNA Technologies [IDT]) targeted to exons 8–9 (RefSeq transcript NM_181058) with a FAM probe. TaqMan reactions were run with Taqman Universal Master Mix 2 on a 7500 Fast machine (Applied Biosystems) using standard cycling conditions. Transcript levels were normalised against Applied Biosystems mouse *Actb* (Assay ID: 4352933E) and Integrated DNA Technologies *B2m* (assay ID: Mm.PT.39a.22214835) endogenous controls in independent experiments and the results averaged geometrically. Both controls contained VIC probes.

Tissue preparation and western blotting for ZBTB20

For analysis of ZBTB20 in hippocampus, J20 and age/sex-matched wildtype littermate controls were dissected under ice-cold PBS before homogenisation in radioimmunoprecipitation buffer (150mM sodium chloride, 50mM Tris, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate) with Protease Inhibitor Cocktail Set 1 (Merck). Total protein concentration was determined using Bradford assay (Bio-Rad). Samples from individual animals were run separately and not pooled.

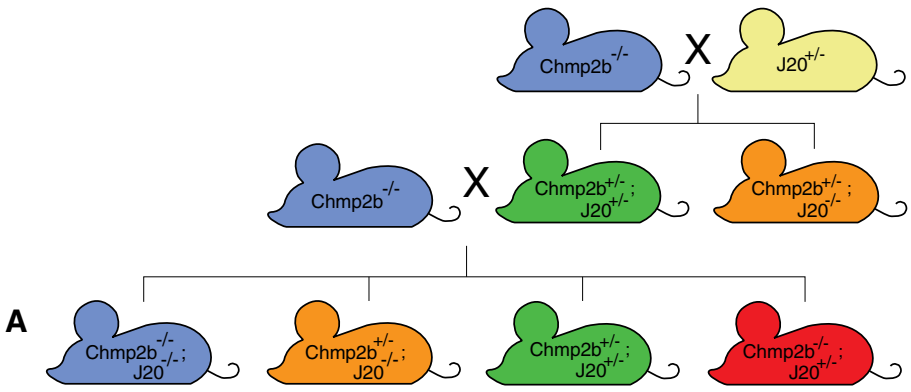
Equal amounts of hippocampal brain proteins were denatured in LDS sample buffer (ThermoFisher) and β-Mercaptoethanol for 10 minutes at 100°C, prior to separation by SDS polyacrylamide gel electrophoresis in 4–12% pre-cast gels (ThermoFisher). Separated proteins were transferred to 0.2µm nitrocellulose membrane and blocked in 5% milk/phosphate buffered saline (with 0.05% Tween 20, PBST) for one hour at room temperature. The membrane was then cut horizontally at the 49KDa band (SeeBlue Plus II protein ladder, Invitrogen) and the lower half was incubated in mouse monoclonal antibody to β-Actin (A5441, Sigma-Aldrich) diluted 1:200,000 in 1% bovine serum albumin (BSA)/PBST overnight at 4°C. The upper half was incubated overnight with rabbit polyclonal primary antibody against ZBTB20 (23987-1-AP, ProteinTech) diluted 1:1000 in 1% BSA/PBST. After washing with PBST the upper and lower membranes were incubated with HRP-conjugated secondary rabbit and mouse antibodies, respectively, diluted in 1% BSA/PBST for 1 hour at room temperature. SuperSignal™ West Pico Chemiluminescent Substrate and X-ray film was used to visualise bands, Image J 1.49c software (NIH) was used to analyse band intensity. Graphpad prism 5 (Graphpad Software, Inc.) was used to plot graphs and SPSS 25 (IBMCorp) was used to perform statistical analyses.

Results

Mouse breeding strategy

To investigate the role of *Chmp2b* in the pathogenesis of AD, we attempted to generate a homozygous knockout of *Chmp2b* in the Tg(PDGFB-APPSwInd)20Lms (J20) *APP* transgenic mouse strain. To accomplish this we set up matings over two generations (Figure 1a), firstly between *Chmp2b*$^{-/-}$ and hemizygous J20 mice (referred for simplicity as TgAPP$^{J20/-}$, where 'J20' denotes the presence of the transgene). This cross produced

A



B

Generation 2 Genotypes	Number of Animals	Observed Ratio	Expected Ratio
Chmp2b$^{-/-}$; J20$^{-/-}$	18	0.237	0.250
Chmp2b$^{+/-}$; J20$^{-/-}$	5	0.066	0.250
Chmp2b$^{+/-}$; J20$^{-/-}$	53	0.697	0.250
Chmp2b$^{-/-}$; J20$^{-/-}$	0	0.000	0.250

Figure 1. Breeding scheme and observed ratios of offspring in a two-generation cross between *Chmp2b*$^{-/-}$ and J20$^{+/-}$ animals. (**A**) Two-generation breeding scheme to generate Chmp2b$^{-/-}$; J20$^{+/-}$ animals. (**B**) Observed ratios of offspring genotype differed significantly from the expected ratio $\chi 2(3, N = 90.21) = 0.58$, $p < 0.0001$.

Chmp2b$^{+/-}$;Tg*APP*$^{J20/-}$ and *Chmp2b*$^{+/-}$;Tg*APP*$^{-/-}$ progeny. In the second stage, the *Chmp2b*$^{+/-}$;Tg*APP*$^{J20/-}$ offspring were crossed to *Chmp2b*$^{-/-}$ null animals. This cross was predicted to produce four progeny genotypes at equal Mendelian ratios (0.25):

(i) homozygous for *Chmp2b* knockout, without the *APP* transgene (*Chmp2b*$^{-/-}$;Tg*APP*$^{-/-}$),

(ii) homozygous for *Chmp2b* knockout, hemizygous for *APP* transgene (*Chmp2b*$^{-/-}$;Tg*APP*$^{J20/-}$),

(iii) hemizygous for *Chmp2b* knockout, hemizygous for *APP* transgene (*Chmp2b*$^{+/-}$;Tg*APP*$^{J20/-}$),

(iv) hemizygous for *Chmp2b* knockout, without the *APP* transgene (*Chmp2b*$^{+/-}$;Tg*APP*$^{-/-}$).

The second stage cross resulted in 76 progeny, but the observed ratio of genotypes significantly differed from the expected ratio (Figure 1b). Strikingly no *Chmp2b*$^{-/-}$;Tg*APP*$^{J20/-}$ progeny were produced. This suggests that either *Chmp2b*$^{-/-}$;Tg*APP*^{J20} may not be viable or the Tg*APP* allele might be on the same chromosome as *Chmp2b* - mouse chromosome 16 (Mmu16), preventing typical Mendelian segregation.

Targeted locus Amplification analysis of J20 transgene insertion

To determine the cause of the absence of *Chmp2b*$^{-/-}$;Tg*APP*^{J20} offspring, we mapped the site of the J20 Tg*APP* transgene insertion, and so sequenced flanking regions around the insertion site by TLA. Sequence analysis (Figure 2a) showed that the Tg*APP* insertion site lies on Mmu16. Targeted reads mapped the integration breakpoints to genomic co-ordinates Mmu16: 43,127,050 (3' end of the transgene array) and Mmu16: 43,127,512 (5' end of the transgene array). In addition, sequencing around the insertion site revealed a 41.17kb deletion in the mouse genome between chr16:43,085,979 and chr16:43,127,149 (Figure 2b). Thus the lack of *Chmp2b*$^{-/-}$;Tg*APP*^{J20} offspring is the result of the insertion of TgAPP^{J20} on Mmu16, explaining the absence of *Chmp2b*$^{-/-}$;Tg*APP*^{J20} progeny from the stage 2 cross.

TLA also allowed us to assess transgene sequence integrity. We found three SNPs (hAPP transgene sequence: 624 G > A, 979 G > A, 10649 G > A) and four indels (TG: 185 G > +1T, TG: 1168 G > +8GGCGGGAC, TG: 1423 C > +1G, TG: 5932 C > -1T) within the integrated transgene construct; however, all were silent mutations or found within intronic sequence. Furthermore, TLA analysis showed at least one transgene copy is truncated at the 3' end (TG: 12088), ablating the ampicillin cassette, and is fused to the 5' of another transgene copy to form a concatemer (TG:12088 fused to TG:3).

Assessment of ZBTB20 expression

The integration site co-ordinates localize the J20 transgene insertion and deletion entirely within intron 1 of the gene

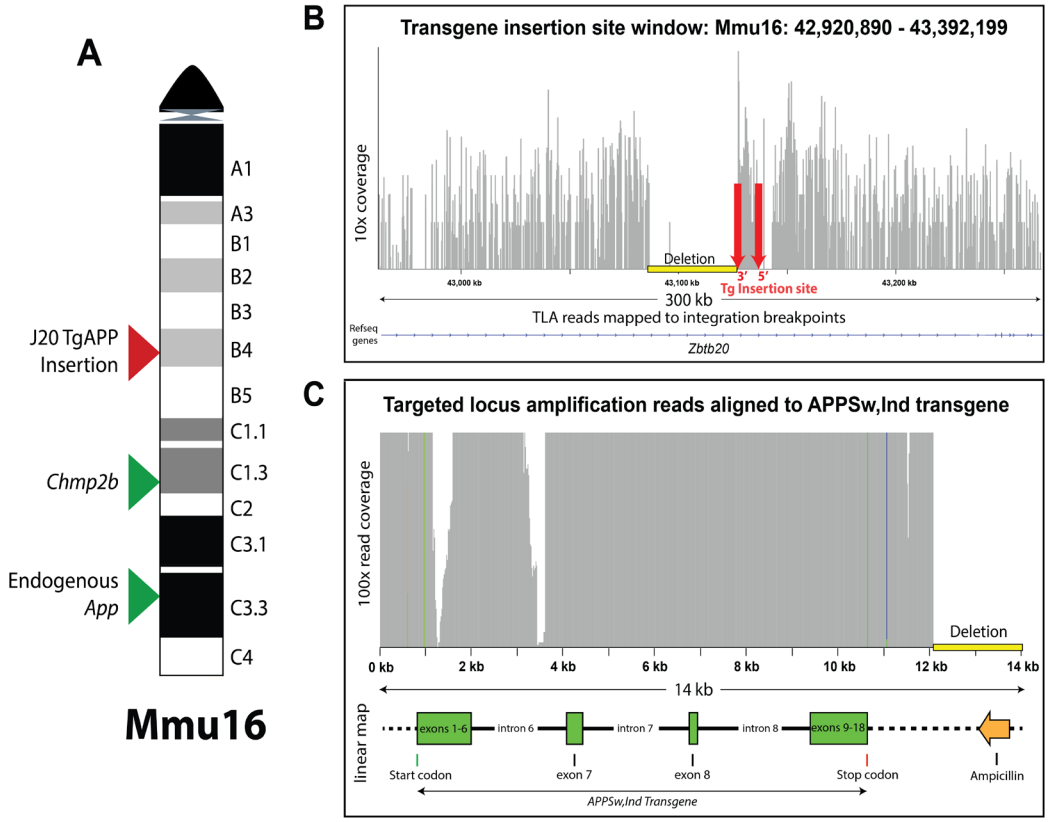


Figure 2. Insertion site of the J20 APP transgene (Tg) on mouse chromosome 16. (**A**) Representation of Mmu16 showing insertion site of the J20 Tg array. Also shown are the positions of endogenous mouse *App* and *Chmp2b*. (**B**) TLA applied to the APPSwInd transgene in the J20 mouse. Read mapping to the mouse (mm10) genome assembly shows the exact Tg insertion site (red arrows) with associated genomic deletion (yellow bar). Gene annotation is shown below labelling a small portion of intron 1 of the *Zbtb20* gene. (**C**) TLA reads mapped to the APP Tg sequence. Complete coverage was achieved, showing that head to tail concatemerization of the Tg has occurred at position 12088 ablating the ampicillin cassette of the plasmid construct. Coloured lines represent SNPs found in the Tg sequence compared to the published sequence. A linearised map of the plasmid construct is shown below.

Zinc-finger and BTB domain containing 20 *(Zbtb20,* transcript NM_001285805.1) on Mmu16. *Zbtb20* is a member of the BTB/POZ family of transcriptional repressors and functions primarily as a transcriptional repressor (Xie *et al.*, 2008); it is important for hippocampal development and function, the site of greatest Aβ deposition in aging J20 animals (Mucke *et al.*, 2000). Moreover, missense mutations in this gene are associated with Primrose syndrome, a cause of intellectual disability with autism (Cordeddu *et al.*, 2014; Mattioli *et al.*, 2016). Additionally, haploinsufficiency of the gene has been suggested as an important factor in del3q13.31 syndrome, a cause of developmental delay and intellectual disability (Rasmussen *et al.*, 2014). To determine whether transgene insertion has affected *Zbtb20* transcription in J20 animals in hippocampal tissue, we investigated mRNA and protein levels of ZBTB20 in wildtype and J20 animals.

Firstly, a predesigned RT-qPCR assay was chosen from IDT to overlap the exon 8–9 boundary within the protein coding region of the *Zbtb20* transcript, downstream of the transgene insertion site. Importantly this junction is present in all predicted RefSeq protein coding transcript isoforms. We detected significantly less transcript in J20 animals compared to wildtype using this assay (Figure 3a). To determine whether reduction in *Zbtb20* transcript results in a reduction of ZBTB20 protein in J20 animals, we assayed total hippocampal protein by western blot for ZBTB20 with an affinity purified polyclonal antibody, and observed that no change in ZBTB20 protein level in adult J20 hippocampal tissue could be determined by this method. We aimed to validate this result with another antibody (ab48889, Abcam) however, we were unable to determine a specific band at the correct molecular weight (data not shown). Because of the postnatal lethality demonstrated in *Zbtb20*$^{-/-}$ mice (Sutherland *et al.*, 2009) and the complexity in producing control adult Zbtb20$^{-/-}$ hippocampal tissue, we were not able to validate this result using an appropriate negative control.

Discussion

Using TLA we have located the insertion site of the J20 *APP* transgene on Mmu16 within intron 1 of the *Zbtb20* gene. We have also found a 41kb deletion of intronic sequence flanking the insertion site. Due to the key role of *Zbtb20* in hippocampal

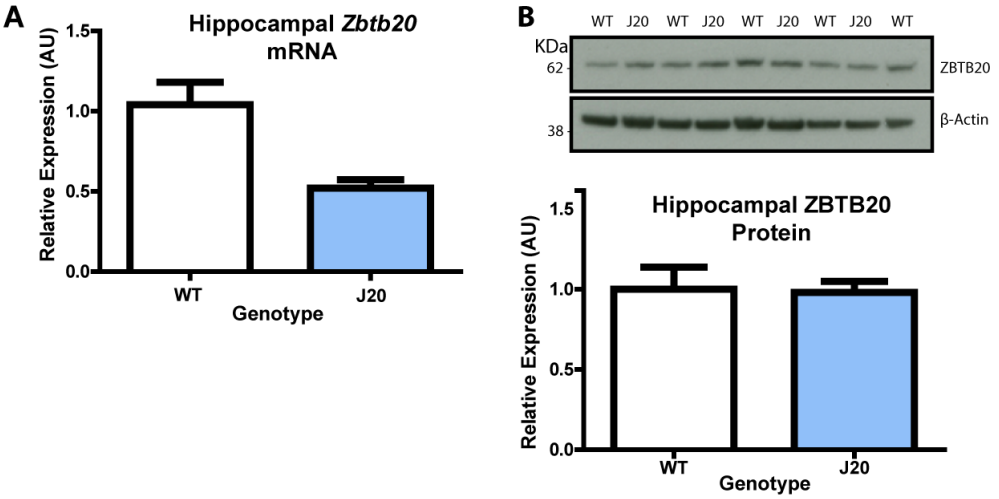


Figure 3. Expression of *Zbtb20* mRNA and protein in 3 month old wildtype and J20$^{+/-}$ hippocampus. (**A**) *Zbtb20* mRNA expression is significantly reduced in J20 hippocampus compared to wildtype (WT), detected by quantitative RT-PCR using primers spanning exons 8–9 (Mann Whitney U = 1, n = 5 wildtype/6 J20. P = 0.009). (**B**) ZBTB20 protein levels are unchanged in J20 hippocampus compared to wildtype animals (Mann-Whitney U = 10, n = 5 wildtype/4 J20, P = 1). Sample sizes were too low to test for normality so non-parametric testing was conducted. Error bars show standard error of the mean; uncropped blots can be viewed at http://doi.org/10.17605/OSF.IO/4UGZF.

cell differentiation and function, we determined its expression in hippocampal tissue, and found that while *Zbtb20* transcript expression is reduced in J20 hippocampus, we could find no evidence that protein expression is altered, although we note the current technical limitations of our study because of the lack of an appropriate negative control. In a similar study investigating the transgene insertion site of the R6/2 Huntingdon's disease model mouse, Jacobsen *et al.* discovered that the *HTT* exon 1 transgene inserted within intron 7 of the *Gm12695* gene, causing an almost 30 fold increase of expression of this gene compared to wildtype in cortical tissue irrespective of CAG-repeat length polymorphisms, showing that the genomic aberration caused by foreign DNA insertion within intronic sequence can alter gene regulation (Jacobsen *et al.*, 2017). Notably, disruption of the expression of nearby genes may also occur in gene-targeted systems. For example, two of the four lines of mice that are null for the Prion protein gene *Prnp*, exhibited late onset ataxia and neurodegeneration -- this was subsequently found to be the result of aberrant upregulation of a gene (*Prnd*) downstream of *Prnp*, which occurred as a result of induced exon skipping (Moore *et al.*, 1999). In the J20 model, hippocampal *Zbtb20* transcription is perturbed without concomitant protein reduction. This is perhaps not as surprising as it seems; recent data indicate mRNA levels explain only around 40% of variability in protein levels (Wilhelm *et al.*, 2014), with protein abundance being primarily dependent on translational control (Schwanhäusser *et al.*, 2011; Schwanhäusser *et al.*, 2013).

It may be important to assess expression in multiple neuronal cell types and other tissues throughout development in the J20 model, considering that ectopic expression of *Zbtb20* in the subiculum and post-subiculum results in aberrant CA1 type development in those regions with associated CA1-specific markers (Nielsen *et al.*, 2010) and the gene has been shown to have a role in liver function (Sutherland *et al.*, 2009). The transgene sequence itself is intact in the J20, excluding several single nucleotide mutations; however, these are either silent or found within the transgene construct's three intronic regions and are unlikely to affect the J20 transgene. TLA analysis and in-house copy number qPCR (data not shown) indicate that this transgene has integrated multiple times in an array on Mmu16. Multiple transgene insertion may be liable to recombination events, causing loss of some copies of the transgene, resulting in delayed onset of phenotype in affected animals. This potential confound can be allayed by undertaking a genomic copy-number qPCR to confirm copy-number consistency between individuals. The Jackson Laboratory have published their assay for general use.

Genetic engineering can result in unintended disruption of the genome, which can confound interpretation of phenotype if the genomic alterations cause changes in protein expression. Transgenic models have been and continue to be powerful tools for biomedical research, but knowledge of the insertion site and the local effects on gene expression will inform phenotyping studies.

Data availability

Aligned BAM files from the TLA, uncropped western blot for Figure 3B, hippocampal qPCR data and western blot data for Zbtb20 are available on OSF http://doi.org/10.17605/OSF.IO/4UGZF (Tosh, 2017).

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Current Referee Status: ✔ ? ✔

Version 2

Referee Report 28 November 2018

https://doi.org/10.21956/wellcomeopenres.16188.r34050

✔ **Guillaume Pavlovic** [1,2]
[1] Institut Clinique de la Souris (ICS), CNRS, INSERM, University of Strasbourg, Illkirch, France
[2] PHENOMIN-ICS, Strasbourg, France

In this second version, the authors have responded very clearly and precisely to the three main remarks made by the reviewers.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Referee Report 16 October 2017

https://doi.org/10.21956/wellcomeopenres.13248.r26484

? **Guillaume Pavlovic** [1,2]
[1] Institut Clinique de la Souris (ICS), CNRS, INSERM, University of Strasbourg, Illkirch, France
[2] PHENOMIN-ICS, Strasbourg, France

The authors have found non mendalian ratio in cross between Chrmp2b-/- and J20+/- animals. They found that J20 transgene is integrated ~22 Mb of the Chrmp2b gene in the Zbtb20 gene.
The report is well written, easy to understand and relevant. However, the Western Blot data does not allow to conclude that *Zbtb20* protein expression remains unchanged.

Comments:
- Figure 3
Unpaired t-test: your statistical analysis hypothesize normal distribution of the data. Using a non parametric test will be more correct if you do not provide the normality test results.
Please indicate what are the error bars (SEM, SD or 95% CI)?

- Assessment of ZBTB20 expression: Western Blot analysis
The western blot results provided in the paper do not prove that the “ZBTB20 protein is unaltered in adult

J20 hippocampal tissue". Knock-out controls and molecular size markers are missing to prove that the showed band is not an unspecific signal.
Moreover, the lack of appropriate controls (i.e. different quantity of protein extract) do not allow to validate the use of Figure 2B as a quantitative Western Blot. See why in the publications 1 to 3
Finally, as recommended in PLOS ONE for example (http://journals.plos.org/plosone/s/submission-guidelines), the author should provide:

- Original uncropped and unadjusted blots and gels, including molecular size markers, should be provided in either the figures or the supplementary files.
- The image should include all relevant controls, and controls should be run on the same blot or gel as the samples.
- All blots and gels that support results reported in the manuscript should be provided. Please provide ZBTB20 additional antibody results (which are data not shown in the paper) as additional files. Add reference of this additional antibody in material and methods.

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 23 Sep 2018

Justin Tosh, University College London, Institute of Neurology, UK

The authors thank Dr Pavlovic for his insightful comments.

We have updated the figure legend to reflect the reviewers comments (error bars are SEM) and have also sign-posted the link to the supplementary data in the legend for Figure 3; where all primary data including uncropped blots can be viewed, and note that the analysis of our western blots complied with best practice (http://journals.plos.org/plosone/s/submission-guidelines).

In our qPCR and western blot experiments we could not test for, or assume normality of data distribution, so an alternative non-parametric test was used instead (Mann-Whitney U).

Competing Interests: No competing interests were disclosed.

Referee Report 25 September 2017

https://doi.org/10.21956/wellcomeopenres.13248.r25970

Takaomi C. Saido
Laboratory for Proteolytic Neuroscience, RIKEN Brain Science Institute, Saitama, Japan

Gene expression is regulated by promoters, enhancers, silencers, etc. in a tissue-specific manner. For instance, Zbtb20 plays an essential role in hepatic *de novo* lipogenesis, so it is necessary to examine the mRNA and protein levels of Zbtb20 in all the tissues in addition to hippocampus including the liver because the transgene is inserted into every corresponding chromosome. In particular, it is surprising that the authors avoided examining developed and developing cortex because Zbtb20 modulates the sequential generation of neuronal layers in developing cortex. It would also be quite convincing if there was a negative control in Figure 3B (and in additional Western blot data) using tissues from Zbtb20-KO mice. One comment on the Western blot data, the quantities of protein are not sufficently normalized.

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Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: Neuroscience

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 23 Sep 2018

Justin Tosh, University College London, Institute of Neurology, UK

The authors thank Dr Saido for his insightful comments.

We agree the transgene is inserted into *Zbtb20* in every cell of the body, and therefore may affect multiple organs. However it is beyond the scope of this initial characterization of the genomic location of the *APP* transgene in the J20 animal model to study transcription of *Zbtb20* in every organ of the mouse. We wished to communicate the issue of disruption of the *Zbtb20* locus to the scientific community immediately so that further detailed studies can be undertaken.

We note that have not been able to source tissue from either $Zbtb20^{-/-}$ or $Zbtb20^{-/+}$ mice, and that $Zbtb20^{-/-}$ mice have poor postnatal survival (Sutherland 2009). Thus it is not possible at this time to verify the results presented in Figure 3B using an appropriate negative control (adult hippocampus from a $Zbtb20^{-/-}$). Therefore we agree that until we can undertake this important validation of the experiment, we cannot state categorically that the ZBTB20 protein level is unchanged in the J20 model system and have edited our paper accordingly.

Competing Interests: No competing interests were disclosed.

Referee Report 19 September 2017

https://doi.org/10.21956/wellcomeopenres.13248.r25971

Karen H. Ashe
Department of Neurology, University of Minnesota, Minneapolis, MN, 55455, USA

The authors have shown that an unexpected ratio of genotypes that occurred when J20 mice were bred to Chmp2b KO mice was due to the insertion of the J20 transgene array near the Chmp2b gene, resultig in linkage disequilibrium. The report is interesting, relevant and well-done. The only correction I recommend

is to change Figure 1 so that the colors in the rectangles match the colors of the mice (e.g., make the orange rectangle red and the red rectangle orange).

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: Neurobiology of Alzheimer's disease

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 23 Sep 2018

Justin Tosh, University College London, Institute of Neurology, UK

The authors thank Professor Ashe for her insightful comments. In Version 2 of the article we have altered Figure 1 to improve colour clarity as suggested.

Competing Interests: No competing interests were disclosed.