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Research Report

Decreased expression of multidrug efflux transporters in the brains of GSK-3 β transgenic mice

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ABSTRACT

Multidrug efflux transporters protect cells in the brain from potentially harmful substances but also from therapeutically useful drugs. Thus any condition that causes changes in their expression is of some importance with regard to drug access. In this study, changes in efflux transporter expression are investigated in mice containing a mutant constitutively active glycogen synthase kinase-3 (GSK-3 β) transgene, driven by the Thy-1 promoter so limiting its localization predominantly to neurons and some glial cells. As expected, decreases in β -catenin were evident via Western blot analyses of cortical homogenates prepared from brains of these transgenic mice. As assessed by real time qRT-PCR, decreased transcript levels of the mdr1b isoform of P-glycoprotein, Mrp1 and Mrp4, (transporters associated with neurons and/or glial cells) were observed in the cortex but not the subventricular zone or hippocampus of the transgenic compared to wild type mouse brains. By contrast, no such decreases were evident with the mdr1a isoform of P-glycoprotein and Bcrp, transporters predominantly found in brain endothelium. Such transporter expression changes could not be accounted for by alterations in blood vessel density or neuronal to glial cell ratios as analyzed both from immunocytochemical staining and from RT-PCR. These observations support previous *in vitro* data showing that manipulations to GSK-3 β activity that alter signaling via β -catenin can influence the expression of efflux transporters. Implications from this are that drug distribution into cells within the brain of these transgenic mice could be enhanced, hence warranting further investigation.

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1. Introduction

It is known that efflux transporters of the ATP-binding cassette (ABC) family such as P-glycoprotein (ABCB1), breast cancer related protein (BCRP) (ABCG2) and multidrug resistance associated proteins (ABCCs) (MRPs) present in the brain are important

for protecting cells from the damaging effects of xenobiotics. Because of their broad substrate profiles, these transporters influence the pharmacokinetics and availability of many different types of molecules. It is still not yet known how levels and location of these transporters are regulated in the brain (Dallas et al., 2006) but clearly any condition in which regulatory

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pathways are altered are likely to result in changes in expression levels and hence alterations to entry of substances to the brain.

Recent evidence shows that signaling via the Wnt/ β -catenin pathway plays a role in transcriptional regulation of P-glycoprotein and other ABC transporters. The Wnt/ β -catenin, otherwise termed 'canonical,' signaling pathway regulates diverse biological processes which includes developmental patterning, cellular proliferation, cell fate specification, and differentiation (Logan and Nusse, 2004). In this pathway, the beta isoform of the multifunctional serine/threonine kinase, glycogen synthase kinase-3 (GSK-3 β) in its active form is responsible for the phosphorylation of β -catenin, leading to its subsequent degradation via the ubiquitin-proteasomal system. Interaction of Wnt ligands with their cell surface receptors leads to inactivation of GSK-3 β . As a result, free β -catenin is allowed to accumulate and be translocated to the nucleus where it binds to the transcription factor Tcf/Lef promoting the transcription of Wnt target genes.

Amongst the many transcription factor binding sites identified on the promoter of the MDR1 gene that encodes P-glycoprotein (Scotto, 2003), multiple Tcf4-binding sequences have been seen. Furthermore the MDR1 gene has been found to be transcriptionally down-regulated after inactivation of Tcf4 in a human colorectal carcinoma cell line, suggesting that MDR1 is a direct target gene of the Tcf4/ β -catenin transcriptional complex (Yamada et al., 2000). It has been shown in another cell type, i.e. in 3T3-L1 cells that ectopic expression of Wnt-1 induces upregulation of MDR1 (Longo et al., 2002) as detected via microarray analysis. More recently, results of an *in vitro* study have shown that the perturbation to steps in the Wnt/ β -catenin pathway affects expression of P-glycoprotein, BCRP and MRPs in human brain endothelial cells (Lim et al., 2008). Whether perturbations to this pathway *in vivo* affect efflux transporter expression has not yet been established.

The Wnt-GSK-3 β signaling pathway has been implicated in major psychiatric diseases, such as schizophrenia (Lovestone et al., 2007) and bipolar disorder (Mathew et al., 2008). Several of the antipsychotics used in treating these conditions have been shown to influence P-gp activity and to be substrates for the transporter (Wang et al., 2006; Zhu et al., 2007). Therefore, an interaction between P-gp and the Wnt-GSK-3 β signaling pathway could be of considerable interest in the efficacy of the pharmacotherapy of schizophrenia and bipolar disorder.

A suitable model for studying the effects of perturbing the Wnt signaling pathway *in vivo* is the transgenic mouse model expressing a constitutively active GSK-3 β mutant, i.e. GSK-3 β [S9A], which resists de-activation by the upstream enzymes p90 ribosomal S6 kinase, p70 ribosomal S6 kinase and protein kinase B (Stambolic and Woodgett, 1994). Such a model has been developed for use in behavioral studies for analyzing the role of GSK-3 β in bipolar disorders and for observing the effectiveness of various drugs potentially useful as therapeutic agents for alleviating the symptoms (Prickaerts et al., 2006). The possibility exists however that access and availability of such agents may be altered in the transgenic mice as a result of changes to multidrug efflux transporters brought about by the overactive GSK-3 β enzyme.

The present study was undertaken to determine if expression of these multidrug efflux transporters might be altered in

the brains of the transgenic mice expressing the constitutively active GSK-3 β mutant, i.e. GSK-3 β [S9A] so having a bearing on transporter expression and hence activity with regard to drug access. For this purpose, expression of ABC efflux transporters was analyzed in the brains of these GSK-3 β transgenic mice and compared with that in the wild type littermates. Results showed that those ABC transporters that are located in brain cells in which the constitutively active GSK-3 β was likely to be expressed in the transgenic mice, i.e. Mdr1b, Mrp1 and Mrp4 were expressed at transcript level in lower than normal amounts. By contrast, those transporters that are predominantly located in blood vessels i.e. Mdr1a and Bcrp where it is unlikely that the constitutively active GSK-3 β is overexpressed showed no differences from normal in the expression of their transcripts. These observations suggest that whether or not drug access across the blood-brain barrier is affected in the transgenic animals, drug entry into cells within the brain might be enhanced, with obvious consequences for drug distribution.

2. Results

2.1. Comparison between transgenic and wild type mice brain: β -catenin levels

To obtain evidence of downregulation of the Wnt/ β -catenin signaling in the brains of the GSK-3 β transgenic mice, β -catenin protein levels in brain tissue homogenates were determined by Western blot analysis. Overexpression of GSK-3 β would be expected to lead to increased degradation of β -catenin in the transgenic mice. As a consequence, less β -catenin would be available for translocation to the nucleus to activate the transcription of Wnt target genes. As revealed from Western blot analyses, decreases in the expression of β -catenin per mg protein were indeed noted in the brains of transgenic mice (band intensity of 0.68 ± 0.22 , $n=5$, $*p < 0.05$ compared to that in the wild type mice after normalization to β -actin bands). An example of a typical Western blot is shown in Fig. 1A.

2.2. Comparison between transgenic and wild type mice brains: ABC efflux transporter expression

To estimate expression levels of the various ABC transporters of interest in the brains of the transgenic and wild type mice, total RNA was extracted from the cerebral cortex, subventricular zone (SVZ) and hippocampus of GSK-3 β transgenic and wild type mice and analyzed by real time qRT-PCR. By this means, transcript levels compared to housekeeper gene, β -actin for the genes (*mdr1a*, *mdr1b*) encoding the two isoforms of P-glycoprotein in mice, i.e. Mdr1a and Mdr1b, for genes (*mrp1*, *mrp4*) encoding the multidrug resistance associated proteins, Mrp1 and Mrp4 and for the gene (*bcrp*) encoding the murine Bcrp could be measured.

As shown in Table 1, differences in expression were seen but only in the cortex and not in either SVZ or hippocampus. Significant decreases were evident with *mdr1b* (0.80 ± 0.03 compared to that in the wild type), *mrp1* (0.80 ± 0.06 compared to that in the wild type) and *mrp4* (0.84 ± 0.03 compared to that

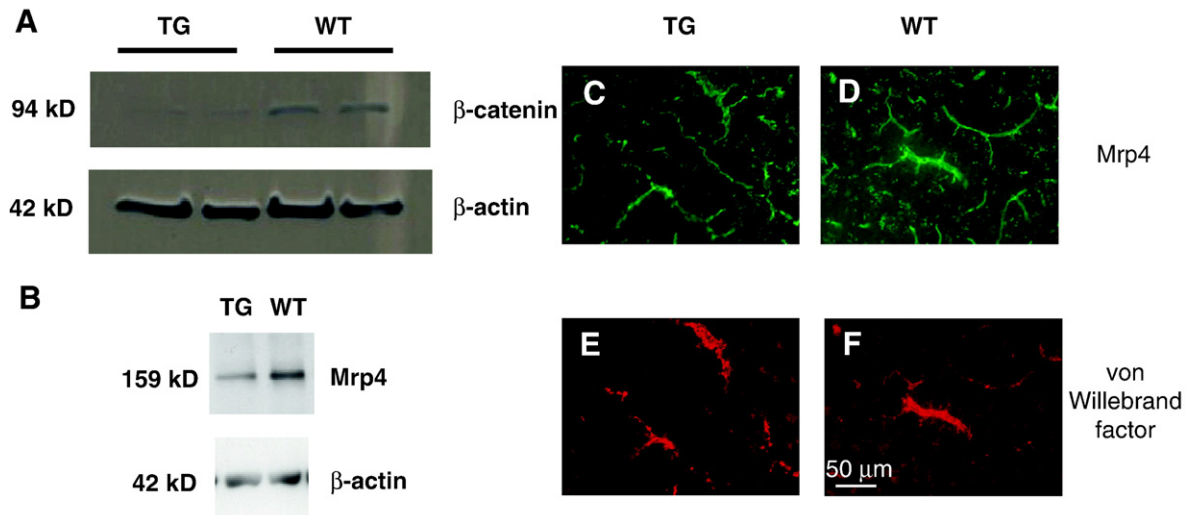


Fig. 1 – Comparisons of β -catenin and Mrp4 expression in transgenic and wild type mice. (A and B) Western blots showing (A) β -catenin and (B) Mrp4 in tissue lysates from the cerebral cortices of GSK-3 β transgenic (TG) and wild type (WT) mice. Equal protein loading was verified by probing with anti- β -actin antibody (as shown) and Ponceau staining (not shown). These images are representative of those obtained from 5–6 independent experiments showing significantly lower levels of both β -catenin ($p < 0.05$, $n = 5$) and of Mrp4 ($p < 0.01$, $n = 6$) in the transgenic mice compared to their wild type brethren. (C–F) dual immunofluorescent staining for (C and D) Mrp4 and (E and F) endothelial cell marker, von Willebrand factor in the cerebral cortex of (C and E) GSK-3 β transgenic mice and (D and F) wild type mice.

in the wild type). The results for *mrp4* were corroborated at the protein level by data from Western blot analysis, as shown in Fig. 1B, (band intensity of 0.57 ± 0.06 compared to that in the wild type, $n = 6$, $**p < 0.01$). By contrast, no decreases were evident at the transcript level with either *mdr1a* or *bcrp* in the transgenic mice.

Comparing transporter expression in the three separate brain regions, no differences between these regions were noted in expression of either *mdr1a* or *mdr1b* in the brains of either transgenic or wild type mice (see Table 1). By contrast in both types of mice, expression of *bcrp* was significantly higher

in the cerebral cortex than SVZ ($*p < 0.05$ and $**p < 0.01$ in GSK-3 β transgenic and wild type mice, respectively); expression of *mrp4* was also higher in the cerebral cortex than in SVZ ($*p < 0.05$) or hippocampus ($*p < 0.05$) but only in the wild type mice. Conversely, in both transgenic and wild type mice, *mrp1* expression was significantly lower in the cerebral cortex than in the SVZ ($***p < 0.001$ and $**p < 0.01$ in the transgenic and wild type mice, respectively) or hippocampus ($***p < 0.001$ and $**p < 0.01$ in the transgenic and wild type mice, respectively).

Further investigations were undertaken to look for possible differences in Mrp4 expression at the protein level. As

Table 1 – Comparison of ABC transporter transcript expression in GSK-3 β transgenic and wild type mice in different brain regions.

	Cortex			SVZ			Hippocampus		
	TG	WT	p value	TG	WT	p value	TG	WT	p value
<i>mdr1a</i>	0.029 \pm 0.002	0.030 \pm 0.002	$p = 0.1751$, n.s. $n = 5$	0.028 \pm 0.002	0.032 \pm 0.002	$p = 0.2832$, n.s. $n = 5$	0.030 \pm 0.002	0.030 \pm 0.001	$p = 0.6949$, n.s. $n = 5$
<i>mdr1b</i>	0.013 \pm 0.002	0.016 \pm 0.001	$p = 0.0017$ ** $n = 8$	0.013 \pm 0.002	0.015 \pm 0.002	$p = 0.5753$, n.s. $n = 5$	0.012 \pm 0.002	0.012 \pm 0.001	$p = 0.4282$, n.s. $n = 5$
<i>bcrp</i>	0.020 \pm 0.002	0.021 \pm 0.001	$p = 0.3942$, n.s. $n = 5$	0.014 \pm 0.001	0.015 \pm 0.001	$p = 0.2117$, n.s. $n = 5$	0.019 \pm 0.001	0.019 \pm 0.002	$p = 0.6327$, n.s. $n = 5$
<i>mrp1</i>	0.011 \pm 0.0003	0.013 \pm 0.0005	$p = 0.0115$ * $n = 5$	0.025 \pm 0.001	0.026 \pm 0.002	$p = 0.8310$, n.s. $n = 5$	0.026 \pm 0.002	0.023 \pm 0.002	$p = 0.0705$, n.s. $n = 5$
<i>mrp4</i>	0.023 \pm 0.001	0.027 \pm 0.001	$p = 0.0118$ * $n = 5$	0.023 \pm 0.001	0.024 \pm 0.001	$p = 0.2478$, n.s. $n = 5$	0.023 \pm 0.002	0.023 \pm 0.001	$p = 0.9020$, n.s. $n = 5$

Total RNA was extracted and analyzed for expression levels of the genes shown using real time qRT-PCR. Expression of each gene was estimated relative to expression of housekeeper gene, β -actin, and these relative values are shown here as the mean \pm s.e.m. from n different analyses. p values indicate where significant differences exist between transgenic and wild type mice and n.s. states where no statistically significant differences were found.

* $p < 0.02$.

** $p < 0.005$.

revealed from immunofluorescent staining of cerebral cortex of GSK-3 β transgenic and wild type mice (Figs. 1C and D respectively), Mrp4 is located in both blood vessels and on other cell types in the brain parenchyma. When assessed in brain tissue homogenates by Western blot analysis, Mrp4 protein levels were found to be significantly lower per protein in the transgenic mice (band intensity of 0.57 ± 0.06 compared to that in the wild type mice, $n=6$, $p < 0.002$ after normalization to β -actin bands). An example of a typical Western blot is shown in Fig. 1B.

However, lower levels of Mrp4 were not evident when blood vessels only were analyzed. Mrp4 immunofluorescent staining intensity in the vessels was quantified using ImageJ software (as described in the [Experimental procedures, section 4.4.3](#)). By this method, no differences in Mrp4 fluorescent staining intensity of blood vessels between transgenic and wild type mice could be detected in either cerebral cortex (36.4 ± 0.5 , $n=3$ and 31.4 ± 7.3 , $n=3$), SVZ (33.4 ± 1.4 , $n=3$ and 34.6 ± 6.7 , $n=3$) or hippocampus (34.4 ± 3.8 , $n=3$ and 29.4 ± 5.6 , $n=3$).

It is interesting to note (Figs. 1E and F) that immunofluorescent staining for the blood vessel marker, von Willebrand factor in the same sections as depicted in Figs. 1C and D showed up only the larger blood vessels in both transgenic and wild type mice brains and did not detect the smaller vessels that could be visualized by the anti-Mrp4-antibody.

2.3. Comparison between transgenic and wild type mice: brain vasculature

To compare differences in the brain vasculature, sections from the brains of GSK-3 β transgenic and wild type mice were stained with antibodies to two different endothelial cell markers i.e. Pecam-1 and von Willebrand factor. Figs. 2A and B show immunofluorescent detection of the endothelial cell marker Pecam-1 in the cerebral cortex of GSK-3 β transgenic and wild type mice.

The number of Pecam-1 positive blood vessels in defined areas was counted and (as shown in Fig. 2C) found to be

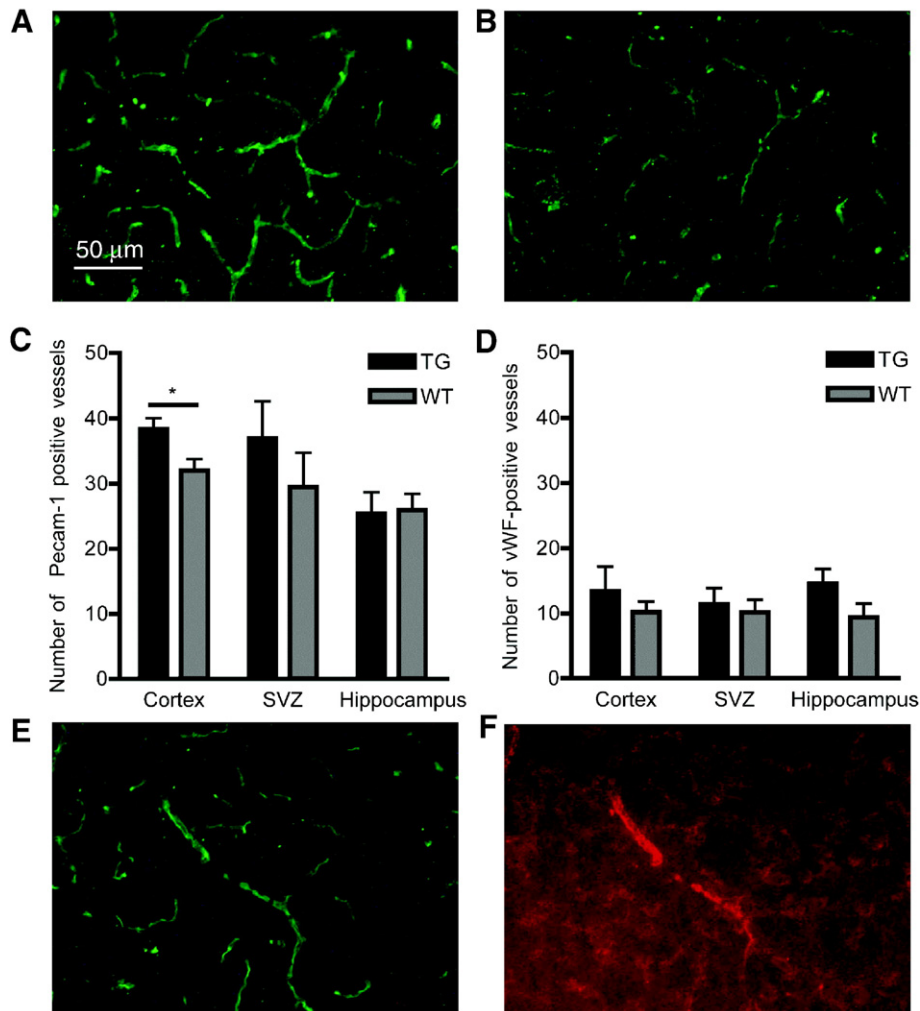


Fig. 2 – Differences in brain vasculature of GSK-3 β transgenic mice compared to wild type mice. (A and B) Immunofluorescent staining for endothelial cell marker, Pecam-1, in the cerebral cortex of (A) GSK-3 β transgenic and (B) wild type mice. (C and D) Graphs depicting the number of (C) Pecam-1 positive blood vessels and (D) von Willebrand positive blood vessels per low power microscopic field in the cerebral cortex, subventricular zone (SVZ), and hippocampus of the transgenic (TG) and wild type (WT) mice. (E and F) Dual immunofluorescent staining for (E) Pecam-1 and (F) von Willebrand factor, in a mouse brain section.

significantly higher in the cerebral cortex of the transgenic mice (38.40 ± 1.66 , $n=5$, $*p < 0.05$) compared to that in the wild type mice (32.05 ± 1.72 , $n=5$). However, no statistical differences were found in the SVZ (36.95 ± 5.67 , $n=5$ and 29.50 ± 5.23 , $n=5$ in the transgenic and wild type mice, respectively) and hippocampus (25.40 ± 3.29 , $n=5$ and 25.95 ± 2.46 in the transgenic and wild type mice, respectively).

A similar analysis for the von Willebrand factor (Fig. 2D) showed there was to be no difference between transgenic and wild type mice in the number of von Willebrand factor-positive blood vessels per low power microscopic field in either cerebral cortex (13.38 ± 3.81 , $n=4$ and 10.19 ± 1.63 , $n=4$ respectively), SVZ (11.38 ± 2.51 , $n=4$ and 10.13 ± 1.95 , $n=4$ respectively) or hippocampus (14.56 ± 2.22 , $n=4$ and 9.38 ± 2.07 , $n=4$ respectively). It is interesting to note that, as noted during dual staining for Mrp4 (see previous section 2.2), antibodies against von Willebrand factor only stained the larger blood vessels (Fig. 2E) and not the smaller vessels detectable with the anti-Pecam-1 antibody (Fig. 2F).

Total RNA extracted from the cerebral cortex, SVZ and hippocampus of GSK-3 β transgenic and wild type mice was analysed for *pecam-1* expression using real time qRT-PCR. There was no significant difference in the relative expression of *pecam-1* in the cerebral cortex of the GSK-3 β transgenic (0.026 ± 0.001 , $n=5$) and wild type mice (0.027 ± 0.003 , $n=5$). Similarly no differences were noted in the two other brain regions of the transgenic (0.026 ± 0.002 , $n=5$ and 0.026 ± 0.003 , $n=5$ in the SVZ and hippocampus, respectively) and wild type mice (0.029 ± 0.005 , $n=5$ and 0.025 ± 0.001 , $n=5$).

Hence, differences in vessel number were not reflected in the increases in *pecam-1* transcript level expression per total RNA. This implies that the increase in blood vessel density seen by the fluorescent staining in the cerebral cortex of the transgenic mice might relate to a general increase in cell density. As described in the following, comparisons of this aspect were made between transgenic and wild type mice.

2.4. Comparison between transgenic and wild type mice: brain cell density and relative expression of neuronal to glial cell markers

To determine whether the observed differences in vessel density might relate to differences in general cellular density as mentioned above, comparisons were made between transgenic and wild type mice. The cerebral cortex of the GSK-3 β transgenic mice (Fig. 3A) showed a higher cellular density compared to that in the wild type mice (Fig. 3B). Cellular density was also quantified by counting the number of DAPI-stained nuclei within defined areas of these images. As shown in the graph (Fig. 3C), increased cellular density was noted in the cerebral cortex of GSK-3 β transgenic (310 ± 14 , $n=3$, $*p < 0.05$) compared to that in wild type mice (269 ± 11 , $n=3$), i.e. having a cellular density of 1.2 \times greater than that of the wild type. However, the cellular densities in the SVZ (324 ± 14 , $n=3$ and 301 ± 15 , $n=3$) and hippocampus (192 ± 11 , $n=3$ and 159 ± 6 , $n=3$) were not statistically different between the GSK-3 β transgenic and wild type mice.

The idea was also considered that the observed differences in transporter expression might be due to differences in cellular composition between the brains of the GSK-3 β

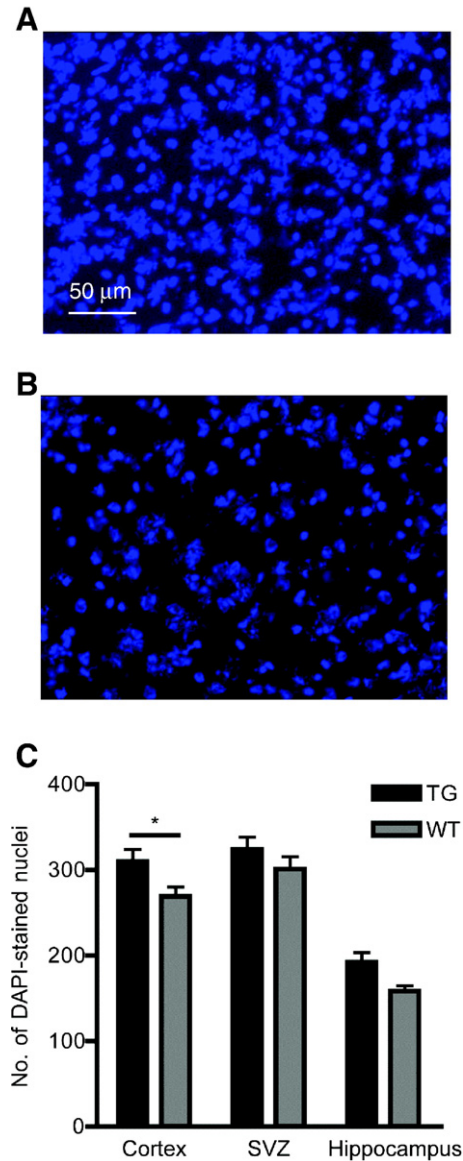


Fig. 3 – Cellular density in brains of GSK-3 β transgenic mice compared to wild type. (A and B) DAPI-stained nuclei in sections of cerebral cortex from (A) GSK-3 β transgenic mice and (B) wild type mice. (C) Graph showing the number of DAPI-stained nuclei per low power microscopic field in the cerebral cortex, subventricular zone (SVZ), and hippocampus of the transgenic (TG) and wild type (WT) mice.

transgenic and wild type mice. To investigate this, comparisons were made of expression levels of the glial marker, Gfap, and neuronal marker, Map2, at both protein and transcript levels. Staining for Gfap and for Map2 was undertaken on immediately adjacent sections of cerebral cortex and the staining intensities quantified using ImageJ analyses and ratios of Map2/Gfap then calculated. However, no differences could be detected in these ratios between the transgenic and wild type mice (1.48 ± 0.21 , $n=4$ and 1.64 ± 0.13 , $n=4$, respectively). Similar analyses was undertaken to investigate expression of these glial and neuronal cell markers at the RNA level. Total RNA was isolated from the cerebral cortex of

the transgenic and wild type mice and subjected to RT-PCR analysis to determine the transcript levels of *map2* and *gfap*. Again, no differences could be detected in these ratios of *map2* to *gfap* between the transgenic and wild type mice (3.81 ± 0.40 , $n=4$ and 4.23 ± 0.70 , $n=4$, respectively).

3. Discussion

Multidrug efflux transporters of the ABC family of proteins, in particular P-glycoprotein, breast cancer resistance protein, and certain multidrug resistance associated proteins have a major influence on drug absorption, distribution, and excretion in many tissues including the brain (Dallas et al., 2006; Doyle and Ross, 2003; Lee et al., 2001). Several of the drugs used in the treatment of neurological disorders such as epilepsy, schizophrenia, depression, and infection of the brain with HIV are substrates for these ABC transporters. As such, treatments that inadvertently alter their expression either at the blood-brain barrier or within the brain are likely to impact on drug access to and distribution inside the brain. Thus an understanding of the mechanisms underlying their regulation is important in the event of pharmacotherapeutic failure in the treatment of these disorders (Loscher and Potschka, 2005).

There are many pathways that may influence the expression of ABC transporters particularly at the transcriptional level. One recently recognized pathway is that involving Wnt/ β -catenin signaling, a pathway already known to be important in development and differentiation (Scotto, 2003). *In vitro* work has already shown the possible role of this signaling pathway in the regulation of some ABC transporters including P-glycoprotein, BCRP and some of the MRPs (Lim et al., 2008). The present model of a transgenic mouse overexpressing a constitutively active GSK-3 β developed by Spittaels et al. (2002) permits the *in vivo* study of the effects of changes to this pathway on ABC transporter expression. Several transgenic mouse models in which GSK-3 β is overexpressed have been developed recently (Engel et al., 2006). In the Spittaels et al. model, expression of this mutant gene is steered by the mouse *thy-1* promoter (Vidal et al., 1990). Expression of the *Thy-1* gene varies considerably in its tissue specificity between species and is under tight developmental control. Thus as shown by Spittaels et al. (2000, 2002) expression of the transgene is restricted to the post-natal period and predominantly to neurons. However, some glial cells are also *Thy-1*-positive, especially at later stages of their ontogeny (Kemshead et al., 1982). As already shown by others (Lucas et al., 2001) and demonstrated also in this present study, such GSK-3 β transgenic mice exhibit reduction in total β -catenin levels compared to wild type littermates. Hence, these mice models are good tools for investigating *in vivo* target genes transactivated by β -catenin.

No differences between the GSK-3 β transgenic and wild type mice were observed in either *mdr1a* or *bcrp* expression in brain homogenates. Both the *mdr1a* form of P-glycoprotein and Bcrp located in brain tissue are found primarily in the endothelial cells lining the blood vessels (Regina et al., 1998; Schinkel, 1997; Tanaka et al., 2004; Cisternino et al., 2004). These are cells in which *Thy-1* is not thought to be present, hence not likely to overexpress the mutant constitutively

active GSK-3 β . By contrast, a significant decrease was observed in *mdr1b* expression in the cortex of the transgenic mice. The *mdr1b* form of P-glycoprotein is known to be expressed in nonvascular cells in the brain, i.e. cells in which *Thy-1* is present, hence likely to overexpress the mutant GSK-3 β . It has been demonstrated by Regina et al. that *mdr1b* is expressed in the parenchyma, i.e. astrocytes, neurons and microglia, of rat brain homogenates (Regina et al., 1998). Thus the observations with each of the transporters suggest it is primarily those transporters located in brain cells where GSK-3 β is likely to be overexpressed that show downregulation.

In the case of the MRP4 transporter, the situation is more complex. There appeared to be a significantly lower *mrp4* expression in the cortex of transgenic compared to the wild type mice. However, it is evident from staining for MRP4 that it is present in both glial and microvascular endothelial cells where, like Pecam-1, it occurs both in larger vessels and in the smaller vessels that do not stain for von Willebrand factor. Measurements of the intensities of MRP4 staining in the vessels did not reveal any significant differences in amounts between transgenic and wild type mice, a result to be expected since endothelial cells lining the blood vessels are not thought to contain *Thy-1*, hence not likely to overexpress the mutant constitutively active GSK-3 β . However as mentioned above, glial cells may also contain *Thy-1* and hence overexpress activated GSK-3 β , so this may account for the observed decreases overall in *mrp4* expression detected in RNA extracted from cortical brain homogenates and in Western blots.

It is interesting to note the existence of regional differences in expression with some i.e. Bcrp *mrp1* and *mrp4* but not all i.e. *mdr1a* transporters investigated. For example, *bcrp* appeared to be expressed to lower levels in the subventricular regions compared to the other two regions in both transgenic and wild type animals. This cannot relate to blood vessel density, as it is not seen with either Pecam-1 staining or *mdr1a* expression, and may suggest regional differences in expression of this transporter. Regional differences were also observed with *mrp1* where a significantly lower expression was detected in the cortex of both transgenic and wild type mice compared to the other brain regions. *mrp1* has not been associated with any particular cell type so it is not as yet possible to interpret this observation. It has however been detected in astrocytes (Dallas et al., 2006; Mercier et al., 2004; Regina et al., 1998). In addition, it has been detected in neurons in epileptic brains, so it may be normally present in neurons though in low amounts (Ak et al., 2007; Sisodiya et al., 2002).

With *mrp4*, a significantly higher expression of this transporter was found in the cortex of wild type mice compared to the other brain regions.

Regarding the possible effects of GSK-3 β overexpression on blood vessel density, it appeared that the number of Pecam-1-positive vessels was significantly higher per section area in the cortical region of the brains of the transgenic mice. There was however no difference in *pecam-1* expression relative to β -actin expression at the transcript level. This suggests that the density of cells overall may have been higher in the transgenic mouse brain rather than there being more vessels in relation to other cell types. This idea was supported by observations

showing significantly higher numbers of DAPI-stained nuclei per brain section area in the cortex. Although there was greater blood vessel density in the cortex of the brains of the transgenic mice, this paralleled the increase in cell density in this region, hence no change in cell to vessel proportions but a general compaction of the brain parenchyma. This is in agreement with a previous report showing that these transgenic mice exhibit volume loss in the brain, a phenomenon related to reduction in size of the somatodendritic compartment (Spittaels et al., 2002). Though GSK-3 β is known to be involved in the control of vascular cell migration and differentiation (Skurk et al., 2005), the constitutively active mutant GSK-3 β transgene is not expressed in the vascular endothelial cells, thus no change in blood vessel proliferation was to be expected.

The possibility was considered that the observed changes in expression profiles of *mdr1b*, *mnp1*, and *mnp4* between the transgenic and wild type mice might be the result of alterations in cell composition in the brains of the transgenic mice. Thus measurements were made at both protein and transcript levels of the glial and neuronal markers, Gfap and Map2, in order to determine whether relative proportions of these particular cell types had changed in the transgenic mice but no differences were detected. However, use of these criteria may be complicated by the observations of Spittaels et al. who demonstrated a marked decrease in Map2 levels, as determined by Western blot analysis, but an absence of neuronal loss in the transgenic mice (Spittaels et al., 2002). Thus Map2 levels cannot be used to indicate number of neurons. However, given the evidence of Spittaels et al. that there is no neuronal cell loss in the transgenic mice, then the observed decreases in *mdr1b*, *mnp1* and *mnp4* are more likely to reflect decreases in expression per cell than decreases in numbers of the cell types in which they are expressed.

In conclusion, results of this study suggest through the use of a transgenic mouse model overexpressing GSK-3 β that events downstream in the Wnt/ β -catenin signaling pathway may contribute to the regulation of expression of ABC transporters in the brain *in vivo*. Decreased expression is observed of those ABC efflux transporters that are associated with the brain cell types likely to overexpress GSK-3 β but those transporters that are present in the brain blood vessels appear to remain unaffected. The implications of these observations are that whether or not drug access across the blood–brain barrier is affected in these transgenic animals that drug entry into certain cell types within the brain, i.e. neurons and glial cells, might be enhanced thereby altering drug distribution within the brain.

4. Experimental procedures

Reagents were of analytical, tissue culture, or molecular biology grade, as appropriate and were obtained from Sigma Aldrich Co. Ltd. (Poole, Dorset, UK), except where stated.

4.1. Cryostat sectioning of frozen tissue

Snap-frozen mouse brains of wild type and transgenic mice from the same litter were obtained from mice raised at Johnson

and Johnson, Pharmaceutical Research and Development, Beerse, Belgium. Upon receipt of the samples, these were immediately stored at -80°C until use for experiments.

For immunohistochemistry, 20 μM sections were cut from regions of each of the brains corresponding to the cortex, subventricular zone (SVZ) and the hippocampus. For total RNA isolation, 50 μM sections were cut from the cortex, SVZ and hippocampus. Slices taken from the SVZ and hippocampal regions of the brain were dissected at -16°C (i.e. within the cryostat) in order to remove cortical areas. The slices were mixed with 1.0 ml Tripure Isolation Reagent (Roche Applied Sciences, Indianapolis, USA), broken up by vortexing for 1 min and placed in dry ice, before storage at -80°C . For Western blot analysis, thirty 30 μM sections were cut from the cortex and lysed in ice-cold 1 mM Tris-buffer with 1 mM AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride, a protease inhibitor, Roche). After leaving on ice for 15 min, the mixture was subjected to mechanical disruption by shearing through 21 gauge and 24 gauge needles, and stored at -80°C until further analysis.

4.2. Quantitative real time RT-PCR

1 μg of RNA was used for cDNA synthesis using random hexamers (Promega) and BioScript MMLV reverse transcriptase according to the manufacturer's instructions (Biolone, London, UK). PCR amplification mixtures (20 μl) contained 8 μl template cDNA, 2 \times SensiMix (Quantace Ltd., Watford, UK) containing SYBR Green (12 μl) and 500 nM forward and reverse primers. Sequences of these primers (shown in Table 2) were either obtained from RTPrimerDB (<http://medgen.ugent.be/rtpriimerdb>), a public data base for primer and probe sequences used in real-time PCR assays (Pattyn et al., 2006) or designed using Primer3 software. Amplification was allowed to proceed in a Rotor-Gene 3000[™] (Corbett Research, Sydney, Australia) using the following cycle conditions: an initial denaturation step for 10 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with fluorescent detection at 72°C , and a final step of 72°C for 2 min. Melt curve analysis was performed from 72°C to 99°C in 1°C steps, 15 s for the first step and 5 s for each step thereafter. Results were quantified with the Rotor-Gene 6000 software (version 1.7) (Corbett Research, Sydney, Australia) using the "comparative quantitation" feature to compare expression of genes of interest with that of β -actin.

4.3. Immunohistochemistry

Tissue sections from the brains of transgenic and wild type mice were processed for immunohistochemistry as described in Cooray et al. (Cooray et al., 2002) and stained with the desired antibodies listed in Table 3.

4.4. Analyses of images

4.4.1. Analysis of cell density

Images of DAPI-stained nuclei were used to count the total number of cells within a given image area under low power magnification. Since the size of the microscopic field and the magnification were kept constant, these values arbitrarily

Table 2 – Primers used in the polymerase chain reaction.

Gene	Strand	Sequence	RTPrimerDB ID or Reference
<i>mdr1a</i>	Forward	GCAGGTTGGCTAGACAGGTTGT	511
	Reverse	GAGCGCCACTCCATGGATAA	
<i>mdr1b</i>	Forward	GCTGGACAAGCTGTGCATGA	512
	Reverse	TGGCAGAATACTGGCTTCTGCT	
<i>bcrp</i>	Forward	AATCAGGGCATCGAACTGTCA	1265
	Reverse	CAGGTAGGCAATTGTGAGGAAGA	
<i>mrp1</i>	Forward	CCTGGAGCTTGCTCACCTAAAG	1191
	Reverse	TTCAAACCTGCGTCCGGATG	
<i>mrp4</i>	Forward	CATACCATTTGGTTCCGCTCT	
	Reverse	TGCATCAAACAGCTCCTGAC	
<i>pecam-1</i>	Forward	GAGCCCAATCACGTTTCAGTTT	3302
	Reverse	TCCTTCTGCTTCTTGTAGCT	
<i>map2</i>	Forward	AAAGACCACCACCCTCTG	
	Reverse	TGTTGCTGTGGTTGGGAATA	
<i>gfap</i>	Forward	AGAAAACCGCATCACCATTC	
	Reverse	TCACATCACACGTCCTTGT	
<i>β-actin</i>	Forward	CCACTGCCGCATCCTCTTCC	3581
	Reverse	CTCGTTGCCAATAGTGATGACCTG	

denoting cellular density could be compared across all images analysed. For each section, two representative DAPI-stained images were analysed from each of the three regions, i.e. cortex, SVZ, and hippocampus, and then averaged for each region. This process was repeated for a total of three pairs of GSK-3 β transgenic and wild type mouse brains. Results were pooled from two independent blind evaluations.

4.4.2. Analysis of blood vessel density

Microvessel quantification was performed according to the criteria of Bossi et al. in which clusters of endothelial cells, with or without a lumen, were considered as individual vessels (Bossi et al., 1995). Microvessel counts were expressed as the number of vessels per microscopic field under low power magnification.

Images of sections stained with anti-vWF antibody and of sections stained with anti-PECAM-1 antibody were used to assess the density of blood vessels. The number of stained vessels within the microscopic field displayed by each image was counted, and taken as an arbitrary value of blood vessel density that could be compared across sections, given that the

magnification and size of the microscopic field in each image was constant. Briefly, slides were examined at low power magnification ($\times 40$) to identify the areas with the highest density of microvessels. In each case, the two most vascularized areas from each of the three regions investigated, i.e. cortex, SVZ, and hippocampus, were selected, and the microvessels in a $\times 200$ field of these two areas were counted. The average counts of the two $\times 200$ fields were recorded for analysis. A total of four pairs of GSK-3 β transgenic and wild type mouse brains were analysed. Results were pooled from two independent blind evaluations.

4.4.3. Analysis of MRP4 staining intensity in blood vessels

Blood vessels on images of sections stained with anti-MRP4 antibody were identified and well-defined areas of these blood vessels were selected for analysis. The intensity of fluorescence was measured using the Image J software (Abramoff et al., 1994). Briefly, 4–6 representative blood vessels were selected per low power microscopic field. At least 2 microscopic fields were analyzed for each of the 3 brain regions, i.e. cerebral cortex, SVZ and hippocampus. A region of interest was drawn surrounding these blood vessels

Table 3 – Antibodies used for immunohistochemistry.

Antibody	Raised in	Working dilution	Source
Anti-von Willebrand Factor (ab6994)	Rabbit	1:100	Abcam, Cambridge, UK
Anti-MRP4 (ab15602)	Rat	1:80	Abcam, Cambridge, UK
Anti-PECAM-1 (CD31)	Rat	1:100	Chemicon International, CA, USA
Anti-Map2			
AntiGfap			
Anti- β actin	Mouse	1:1000	Abcam, Cambridge, UK
Anti- β catenin	Rabbit	1:2000	Abcam, Cambridge, UK
Alexa Fluor 594 Anti-rabbit IgG	Goat	1:100	Invitrogen-Molecular Probes, OR, USA
Alexa Fluor 488 Anti-rat IgG	Goat	1:100	Invitrogen-Molecular Probes, OR, USA
Peroxidase linked-anti-mouse IgG	Goat	1:10,000	Abcam, Cambridge, UK
Peroxidase linked-anti-rat IgG	Goat	1:10,000	Abcam, Cambridge, UK
Peroxidase linked-anti-rabbit IgG	Goat	1:2,500	Abcam, Cambridge, UK

and a histogram was generated to represent the distribution of gray values in the selection. To eliminate noise intensity, a Gaussian intensity distribution was fitted for each of the histogram using an Excel macro (developed by Dr. S Hladky, University of Cambridge). Mean fluorescent intensities for Mrp4 staining in blood vessels in the cerebral cortex, SVZ and hippocampus for both GSK-3 β transgenic and wild type mice were determined. Results were pooled from two independent blind evaluations.

4.4.4. Analysis of Gfap and Map2 staining intensity

The intensity of Gfap and Map2 fluorescent staining intensities were measured using the Image J software (Abramoff et al., 1994). Images of sections from the cortex of transgenic and wild type mice stained with either Gfap or Map2 were taken under low power magnification. The corresponding no primary antibody control for each section was also taken to account for background staining. For each image, the mean intensity of the red component was measured by splitting the photo into a red-green-blue (RGB) stack. From this value, the fluorescent intensity of the no primary antibody control was subtracted. The ratios of Gfap to Map2 fluorescent staining intensities of the entire field in each photograph were measured and comparisons made between that of the cortex of GSK-3 β transgenic and that of wild type mouse brains.

4.5. Western blot analysis

To analyze expression at the protein level, Western blot analysis was performed on tissue lysates prepared from cryostat sections of brains of transgenic and wild type mice. Protein concentrations were assayed using the BCA™ Protein Assay kit (Pierce), equivalent amounts then applied to each well, separated through 7.5% polyacrylamide, and transferred to 0.45 μ m nitrocellulose membranes. Following a blocking step with Tris-buffered saline (50 mM Tris, 250 mM NaCl, pH 7.5) supplemented with 0.1% Tween-20 and 5% non-fat milk powder, blots were probed overnight with one of the following antibodies diluted as described in the same buffer: rat anti-MRP4 antibody, M41-10 (1:100, Abcam, Cambridge, UK), rabbit anti- β -catenin antibody (1:2000, Abcam, Cambridge). Following three washes in the buffer, the blots were subsequently probed for 30 min with the appropriate peroxidase-conjugated secondary antibody, i.e. anti-mouse, anti-rat and anti-rabbit IgG (all from Abcam, Cambridge, UK) and the proteins then detected using the ECL system (Amersham, UK). All blots were stained with Ponceau S prior to probing with antibody and also probed with anti- β -actin antibody (1:10000, Abcam, Cambridge UK) to verify equal protein loading in each well. Band intensities were estimated using ImageJ software and normalized to that of the appropriate β -actin band before comparisons between wild type and transgenic mice were done. Blots were performed five times using samples from separate pairs.

4.6. Statistical analyses

All values are expressed as the mean \pm standard error of the mean (SEM). All statistical analyses were performed using GraphPad Prism 4 (GraphPad Software, Inc., CA, USA). Comparisons of mean values of cell and blood densities,

expression of genes of interest, and staining intensities between age- and gender-matched GSK-3 β transgenic and wild type mice were done using a paired t-test. Comparisons between the three brain regions were done using one-way analysis of variance (ANOVA), followed by Bonferroni's test for multiple comparisons.

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