

Mice lacking the β 2 adrenergic receptor have a unique genetic profile before and after focal brain ischaemia

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ABSTRACT

The role of the β 2AR (β 2 adrenergic receptor) after stroke is unclear as pharmacological manipulations of the β 2AR have produced contradictory results. We previously showed that mice deficient in the β 2AR (β 2KO) had smaller infarcts compared with WT (wild-type) mice (FVB) after MCAO (middle cerebral artery occlusion), a model of stroke. To elucidate mechanisms of this neuroprotection, we evaluated changes in gene expression using microarrays comparing differences before and after MCAO, and differences between genotypes. Genes associated with inflammation and cell deaths were enriched after MCAO in both genotypes, and we identified several genes not previously shown to increase following ischaemia (Ccl9, Gem and Prg4). In addition to networks that were similar between genotypes, one network with a central core of GPCR (G-protein-coupled receptor) and including biological functions such as carbohydrate metabolism, small molecule biochemistry and inflammation was identified in FVB mice but not in β 2KO mice. Analysis of differences between genotypes revealed 11 genes differentially expressed by genotype both before and after ischaemia. We demonstrate greater Glo1 protein levels and lower Pmaip/Noxa mRNA levels in β 2KO mice in both sham and MCAO conditions. As both genes are implicated in NF- κ B (nuclear factor κ B) signalling, we measured p65 activity and TNF α (tumour necrosis factor α) levels 24 h after MCAO. MCAO-induced p65 activation and post-ischaemic TNF α production were both greater in FVB compared with β 2KO mice. These results suggest that loss of β 2AR signalling

results in a neuroprotective phenotype in part due to decreased NF- κ B signalling, decreased inflammation and decreased apoptotic signalling in the brain.

Key words: β 2 adrenergic receptor, Glo1, microarray, Noxa, nuclear factor κ B, stroke, tumour necrosis factor α .

INTRODUCTION

Stroke is the fourth leading cause of death and the primary cause of chronic neurological disability in the United States (<http://www.strokeassociation.org>). The β 2AR (β 2 adrenergic receptor) is a member of the adrenergic receptor family, a group of GPCR (G-protein-coupled receptors) that respond to endogenous and exogenous catecholamines, primarily noradrenaline (Wallukat, 2002). Previous research examining the role of β 2AR activation after stroke using pharmacological agents has produced contradictory results. Although intraperitoneal pretreatment with clenbuterol, a β 2AR agonist, significantly decreased infarct size after permanent MCAO (middle cerebral artery occlusion) (Semkova et al., 1996), intravenous pretreatment with four individual non-specific β AR blockers resulted in reduced infarct size after transient MCAO (Goyagi et al., 2006). In an effort to better understand how activation of the β 2AR affects stroke outcome, our laboratory used transgenic mice lacking a functional β 2AR in all cell types throughout the body (Chruscinski et al., 1999), and showed that these mice had smaller ischaemic infarcts and improved locomotor recovery following transient MCAO, a model of stroke (Han et al., 2009).

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Abbreviations: AGE, advanced glycation end-product; β 2AR, β 2 adrenergic receptor; CNS, central nervous system; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G-protein-coupled receptor; IL, interleukin; IPA, ingenuity pathway analysis; I κ B α , inhibitory κ B α ; LPS, lipopolysaccharide; MCAO, middle cerebral artery occlusion; MMP9, matrix metalloproteinase 9; NF- κ B, nuclear factor κ B; RT-qPCR, reverse transcription quantitative real-time-PCR; SAGAT, singular value decomposition augmented gene expression analysis tool; SAM, significance analysis of microarrays; TNF α , tumour necrosis factor α ; WT, wild-type.

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The β 2AR is widely expressed throughout the brain and the rest of the body. In the brain, the β 2AR is found on neurons (Vasquez and Lewis, 2003; Ampatzis and Dermon, 2010), astrocytes (Hutchinson et al., 2007; Dong et al., 2012) and microglia (Fujita et al., 1998; Tanaka et al., 2002). β 2AR dysfunction has been implicated in multiple sclerosis (De Keyser et al., 1999) and Alzheimer's disease (Wang et al., 2011), suggesting that it may play a direct role in regulation of neuronal death. In the periphery, the β 2AR is expressed by B- and T-lymphocytes (Kin and Sanders, 2006; Sanders, 2012), in addition to cells in most other organs (Anderson, 2006; Snyder et al., 2008). Treatment with β 2AR agonists for asthma and COPD (chronic obstructive pulmonary disease) induces bronchodilation, although efficacy is reduced over time (Anderson, 2006). An interaction with glucocorticoids has been noted with possible effects on inflammation (Eickelberg et al., 1999).

As the CNS (central nervous system) and peripheral immune system communicate via the release of the adrenergic agonist noradrenaline by the sympathetic nervous system (Sanders, 2012), deletion of the β 2AR would be expected to cause changes in immune response both systemically and in the brain. Predicting likely effects is difficult since, depending on cell type, stimulus and time course, the β 2AR can either have pro-inflammatory effects (Christensen et al., 1999; Frost et al., 2004; Yin et al., 2006; Rohrbach et al., 2007; Tan et al., 2007) or anti-inflammatory effects (Severn et al., 1992; Mori et al., 2002). To explore how the absence of β 2AR receptors is neuroprotective we conducted microarray analysis of brain gene expression in WT (wild-type) (FVB) and β 2AR-deficient (β 2KO) mice 24 h after stroke. By examining gene expression differences between genotypes, we show that β 2KO mice have increased expression of *Glo1* and decreased expression of *Pmaip1/Noxa*, contributing to decreased NF- κ B (nuclear factor κ B) signalling, TNF α (tumour necrosis factor α) production and apoptosis following stroke. Together, these data show that absence of β 2ARs appears to have anti-inflammatory and anti-apoptotic effects in the brain that could contribute to protection following stroke.

MATERIALS AND METHODS

Mice

Male β 2AR-deficient FVB/N (β 2KO) and congenic WT FVB/N (FVB) mice weighing 25–30 g were used. The β 2KO mice are those produced by targeted gene disruption (Chruscinski et al., 1999). All experiments were performed according to a protocol approved by the Stanford Institutional Animal Care and Use Committee.

Transient focal ischaemia

Transient focal ischaemia was induced with an intraluminal suture to occlude the middle cerebral artery, as previously

described (Han et al., 2009) with some modifications. Briefly, mice were anaesthetized with isoflurane in 70% nitrous oxide balance oxygen with spontaneous respiration. Under the operating microscope, the left common carotid artery, internal carotid artery and external carotid artery were exposed through a midline neck incision. The proximal portions of the left common and external carotid arteries were ligated and a 6-0 silicon-coated nylon suture was introduced into the distal common carotid and advanced approximately 9.0 mm beyond the carotid bifurcation to transiently occlude the middle cerebral artery for 60 min. During all surgical procedures mice were maintained normothermic ($37^{\circ}\text{C} \pm 1.0$) using a servo controlled heating blanket (Harvard Apparatus) with rectal temperature monitoring. Animals with no observable deficits immediately after ischaemia, those that died in the first 24 h of reperfusion, and those with subarachnoid haemorrhage at time of killing were excluded from analysis. Sham-treated animals received all surgical procedures but the filament was not inserted and the artery was not opened.

Preparation of samples for microarray analysis

At 24 h after MCAO, sham surgery, or no manipulation (naïve) mice were killed under deep isoflurane anaesthesia, transcardially perfused with cold saline, and cerebral cortices were immediately extracted. RNA was extracted from the ischaemic or ipsilateral sham hemisphere using the RNeasy Kit (Qiagen) according to the manufacturer's instructions. Total RNA samples were processed at the Stanford Protein and Nucleic Acid Biotechnology Facility by one-cycle target preparation, labelling and hybridization to Affymetrix 430_2[®] (Affymetrix) whole genome mouse arrays, according to the manufacturer's protocol. Naïve, sham and MCAO groups of each genotype were analysed with 7–8, 3 and 7 biological replicates/group respectively. Each array analysed RNA from an individual mouse.

SAM (significance analysis of microarrays) analysis

The SAM method was used (Tusher et al., 2001) to analyse the array data. Raw image files were processed using Affymetrix GCOS software. Initial chip processing and signal calling were done with R and Bioconductor (Gentleman et al., 2004) using the Affymetrix package (Gautier et al., 2004) with RMA background correction, quantile normalization, and the median polish probe set summary method. A fold-change of 2.0 and false positive rate (q -value) of 0.05 were chosen as the cut-offs for selecting significantly differentially expressed genes. A current gene assignment for the Affymetrix probe sets was done using the Gene ID conversion tool on the DAVID bioinformatic resource (<http://david.abcc.ncifcrf.gov/>) (Dennis et al., 2003). Datasets are posted on the Gene Expression Omnibus (GSE30655).

SAGAT (singular value decomposition augmented gene expression analysis tool) analysis

To analyse the microarray data, we also used the recently developed SAGAT (Daigle et al., 2010). SAGAT extracts transcriptional modules from publicly available microarray data and integrates this information with the dataset of interest. In so doing, it boosts experimental power by increasing the effective sample size of the dataset. SAGAT has been shown to capture additional genes and pathways that may be missed when using methods that ignore data from pre-existing experiments (Daigle et al., 2010).

All available expression data for the Affymetrix Mouse Genome 430 2.0 Array (GPL12610) from the Gene Expression Omnibus were downloaded in March 2009. Probes were mapped to a non-redundant list of Entrez Gene IDs (provided by the Bioconductor R package, *mouse4302*) and expression values for multiple probes of the same gene were averaged. This resulted in a matrix of 21308 genes by 9199 arrays. We excluded 1168 arrays on the basis of missing data. We then ran the R *svd* function to generate the weight matrix for SAGAT.

Using the R *affy* and *bias* packages, we performed background correction, RMA normalization and bias correction on CEL files from the experiments in this study. We used SAGAT to identify genes that were differentially expressed when comparing MCAO with sham. Genes with a false discovery rate less than or equal to 0.05 were considered statistically significant. A list of the top 500 genes, based on SAGAT scores, a measure of statistical significance, was produced.

RT-qPCR (reverse transcription-quantitative real-time-PCR) for mRNA quantification

Total RNA was isolated with TRIzol[®] (Invitrogen) from the ischaemic hemisphere of WT or β 2KO mice 24 h following MCAO. RT was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Equal amounts of RNA (600 ng) were reverse-transcribed with 1.3 mM dNTPs, 50 units of reverse transcriptase, 10 units of RNase inhibitor and random primers at 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. PCRs were then conducted using the TaqMan[®] MicroRNA Assay Kit (Applied Biosystems) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each reaction contained 0.75 μ l of the reaction product and 5 μ l of 2 \times Taqman Universal Master Mix (Applied Biosystems) in a total volume of 10 μ l using the 7900 HT (Applied Biosystems). Predesigned primer/probes (Applied Biosystems) for mRNAs and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were also from Applied Biosystems. The expression of mRNAs was normalized using GAPDH as the internal control. Measurements were normalized to GAPDH ($\Delta\Delta C_t$) and the comparison calculated as the inverse log of $\Delta\Delta C_t$ to give a relative fold change value ($n=3$ per sham group and 4–5 per MCAO group).

Biological function and network analysis

Annotation for biological functions, similar to gene ontology categories, was completed using the IPA (ingenuity pathway

analysis) program (Ingenuity Systems, <http://www.ingenuity.com>). Genes that were differentially expressed, defined as at least a 2-fold change between sham and ischaemic conditions with $P<0.05$, were used for the analysis. IPA was performed to identify the top biological functions and networks enriched in each gene set. To do this, a dataset containing gene identifiers and corresponding expression values was uploaded into the application. The Biological Function Analysis identified the functions in Ingenuity's Knowledge Base that were most significant in the dataset. Right-tailed Fisher's exact test was used to calculate a P -value determining the probability that each biological function assigned to that dataset is due to chance alone. For the Network Analysis, each differentially expressed gene (identifier) was mapped to its corresponding object in Ingenuity's Knowledge Base. These genes, called Network Eligible molecules, were overlaid on to a global molecular network developed from information contained in Ingenuity's Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity. In addition, a comparison analysis between networks enriched after ischaemia in FVB and β 2KO mice was conducted to assess the number of shared genes between networks.

Protein preparation for ELISA and immunoblotting

At 24 h following MCAO or sham treatment mice were transcardially perfused with cold saline, and the cerebral cortices were dissected and placed on dry ice. Protein lysis buffer (10 mM HEPES, 42 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 0.1 mM EGTA and 0.1% Triton X-100) with ProteaseARREST (G Biosciences) was added to tissue at a 1:3 ratio and the mixture was sonicated for 5 s. Following sonication and centrifugation at 15000 g for 50 min the supernatant was collected as the cytoplasmic fraction. Protein concentrations were determined by the BCA (bicinchoninic acid method; Protein Assay Kit).

Immunoblotting

Samples containing equal amounts of protein (100 μ g) were resolved by SDS/4–12% PAGE (NuPage Bis/Tris Gel, Invitrogen). The proteins were transferred on to a PVDF membrane (IPVH00010, Millipore), probed with primary antibodies [rat anti-Glo1 (1:2000, Abcam) and mouse anti- β -actin (1:5000, Sigma) or rabbit anti- β -actin (1:000, Licor Systems)] probed with Licor Fluorescent secondary antibodies (goat anti-rat 680LT and goat anti-mouse 800 or goat anti-rabbit 800, all at 1:15000), and scanned using the Licor Odyssey system. Densitometric analysis of the bands was performed using NIH ImageJ software, with band intensities normalized to β actin.

ELISA

Samples containing equal amounts of protein (100 μ g) were analysed with the mouse TNF α ELISA kit (Invitrogen). As naïve brains contain very low levels of pro-inflammatory

cytokines (Offner et al., 2006), only post-MCAO tissue was analysed. All samples were run in duplicate, and data were fitted to a standard curve from standards provided by the manufacturer.

p65 activity assay

At 24 h following MCAO or sham treatment mice were transcardially perfused with cold saline, and the cerebral cortices were dissected and placed in liquid nitrogen. Complete lysis buffer AM2 from the TransAM NF- κ B p65 Activity Assay (Active Motif) was added to tissue at a 1:5 ratio and the mixture was sonicated for 5 s. In total 20 μ l of the mixture was added to each well and measured according to manufacturer's instructions. All samples were run in duplicate. Data are presented as the fold change in absorbance value from sham to MCAO in each genotype.

Statistics

For biochemical experiments data are presented as means \pm S.E.M. ELISA, immunoblotting, RT-qPCR and p65 activity data were analysed using Student's *t* tests. Statistical significance was set at $P < 0.05$.

RESULTS

Focal ischaemia alters gene expression in both FVB and β 2KO mice, sham versus MCAO

The Affymetrix Mouse 430A 2.0 array assesses 45037 probes for approximately 14000 genes (1–4 probes per gene comprising a probeset). To compare gene expression levels, the median of each probeset was determined to produce a single value for each gene. Genes were considered differentially regulated if the fold change between sham and ischaemia was ≥ 2 with a P -value of < 0.05 . Using these criteria, SAM analysis yielded a total of 414 genes differentially regulated after MCAO in the FVB group and 347 genes differentially regulated after MCAO in the β 2KO group (Figures 1A and 1B, Supplementary Tables S1 and S2 at <http://www.asnneuro.org/an/004/an004e096add.htm>). Of these, 260 were shared between genotypes (243 up, 17 down), which was 80% of the FVB genes and 75% of the β 2KO genes. In the FVB group, 305 genes were up-regulated after ischaemia (74%) and 109 were down-regulated (26%). In the β 2KO group, 324 were up-regulated (93%) and 23 were down-regulated (7%).

The SAGAT analysis generates a list of genes ranked by SAGAT score, a value derived both from information from the current arrays and publicly available mouse microarray data (Daigle et al., 2010). To match the number of differentially regulated genes identified by the SAM analysis, the top 414 genes ranked by SAGAT score in the FVB mice and the top 347

genes in the β 2KO mice were analysed. This revealed that 234 up-regulated genes and 32 down-regulated genes were shared between genotypes, 67% of FVB and 88% of β 2KO up-regulated genes and 48% of FVB and 40% of β 2KO down-regulated genes (Figure 1C and Supplementary Tables S3 and S4 at <http://www.asnneuro.org/an/004/an004e096add.htm>). In the FVB group, 347 genes were up-regulated after ischaemia (84%) and 67 were down-regulated (26%). In the β 2KO group, 266 were up-regulated (77%) and 81 were down-regulated (23%). We compared the genes identified by the SAM and SAGAT analyses, and found for FVB mice, 251 up-regulated and 51 down-regulated genes were shared between analyses (Figure 1D). For β 2KO mice, 208 up-regulated and 13 down-regulated genes were shared between analyses (Figure 1E). The top 10 genes up-regulated and down-regulated in each genotype from the SAGAT and SAM analyses are shown in Table 1.

Prg4, Ccl9 and Gem are novel genes up-regulated following MCAO in both genotypes

A list of the top 20 genes up-regulated by MCAO (compared with sham) by SAM analysis is given in Table 2. Genes previously implicated in ischaemia are noted. We identified several genes not previously described as changing after MCAO, two in the top 20 genes (Prg4 and Ccl9), one differentially expressed at a lower fold-change (Gem), and two previously identified by microarray but not previously validated by RT-PCR (Ch25h and Ifi202b). We confirmed the changes in expression of these nine genes by RT-qPCR from sham to MCAO for both genotypes (Table 3).

Genes related to inflammatory response and cell death are enriched in both genotypes following MCAO

Using IPA, we assessed the top biological functions enriched after ischaemia using gene lists generated from the SAM analysis, for genes increased ≥ 2 -fold (Figures 2A and 2B). Overall these were similar for the two genotypes, differing by only 1 of the 6 top biological functions.

A network composed largely of GPCR is differentially regulated after MCAO in FVB but not β 2KO mice

IPA Network Analysis groups genes based on relationships previously identified in the literature. For example, genes differentially regulated after ischaemia and that were previously shown to associate with each other will be identified as members of a network. If the network members are over represented in our differentially expressed gene list compared with the fraction of the genome represented by all members of the network, then the network is ranked as over-represented. To determine how these networks were different between genotypes, we compared the top 10 networks enriched after ischaemia in each genotype. The analysis examines the number

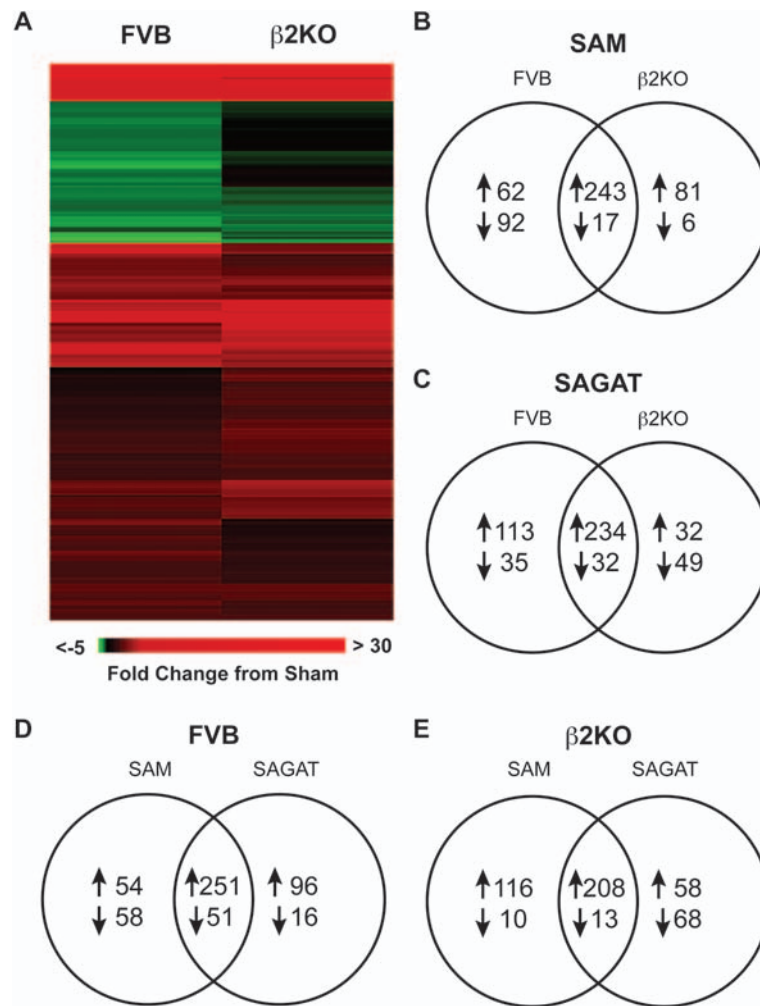


Figure 1 Focal ischaemia alters gene expression in both FVB and β 2KO mice, sham versus MCAO

(A) Heatmap showing genes significantly regulated from sham to MCAO with a fold-change of ≥ 2 . Red represents up-regulated genes and green represents down-regulated genes. (B, C) Venn diagrams showing genes differentially regulated from sham to ischaemia with a fold-change of at least 2-fold by SAM analysis (B) and the top 414 (FVB) and 347 (β 2KO) genes identified by SAGAT (C). (D, E) Venn diagrams showing genes identified as differentially regulated by SAM or in the top 414 or 347 genes by SAGAT in FVB (D) and β 2KO (E) mice.

of shared genes between each network and forms a graphical representation linking networks containing shared genes (Figure 3A). The biological functions associated with each ranked network are listed in Table 4. Notably, network 1 of FVB does not share any members with the other identified networks. This network is shown in Figure 3(B). This network included both up- and down-regulated genes with the GPCR family as the core of the network (Figure 3B).

Multiple genes differ between β 2KO and FVB mice in naïve, sham and MCAO conditions

To directly analyse genotype effects, SAM analysis was performed to compare differences in gene expression between genotypes by treatment group (naïve, sham and MCAO). Venn diagrams illustrate the number of genes differentially regulated

between genotypes in the treatment conditions and how those genes are similar or different (Figures 4A and 4B). With a 2-fold change and $P < 0.05$ cut-off, there were five common genes up-regulated in all conditions in β 2KO mice compared with FVB (Figures 4A–4C) and six common genes down-regulated in all conditions in β 2KO compared with FVB (Figures 4B and 4C).

The genes up-regulated in β 2KO mice were: *Adrb2*, *Fxyd2*, *Glo1*, *Nedd4L* and *Vps52*. Genes down-regulated in the β 2KO mice were: *Al506816*, *Magi2*, *Pmaip1*, *Ppcdc*, *Tcf4* and *Zfp398*. *Glo1* and *Pmaip1/Noxa* were chosen for verification because both are known to affect cell survival. *Glo1* inhibits NF- κ B activation (El-Osta et al., 2008) and is protective after renal ischaemia (Kumagai et al., 2009), while *Noxa* is pro-apoptotic, and has been previously implicated as up-regulated in brain ischaemia and downstream of NF- κ B activation (Inta et al., 2006).

Table 1 The top 10 genes down- and up-regulated after ischaemia in each genotype using SAM and SAGAT analysis
Genes in normal text are in both the SAGAT- and SAM-generated lists. Those in bold do not appear on the other list using the other analysis.

Genotype	SAM	SAGAT
FVB up	1. Spp1	1. Spp1
	2. Lcn2	2. Lcn2
	3. Il6	3. Il6
	4. Lilrb4	4. Lilrb4
	5. Cd14	5. Cd14
	6. Tgm1	6. Hspa1a
	7. Hspa1a	7. Tgm1
	8. Soc3	8. Ccl3
	9. Timp1	9. Cxcl2
	10. S100a9	10. Mmp3
FVB down	1. Gpr6	1. Gpr6
	2. P2ry12	2. P2ry12
	3. Grp34	3. Grp34
	4. Gm1337	4. GM1337
	5. Slc35d3	5. OTTMUSG00000006514
	6. Serpina9	6. LOC100039795
	7. 2310076G05Rik	7. C330006P03Rik
	8. Drd2	8. Rxrg
	9. Pde10a	9. 2310076G05Rik
	10. Rxrg	10. Drd2
β 2KO up	1. Spp1	1. Spp1
	2. Lcn2	2. Lcn2
	3. Soc3	3. Ifi202b
	4. Hspa1a	4. Hspa1b
	5. Cd14	5. Tgm1
	6. Il6	6. Cd14
	7. Lilrb4	7. Soc3
	8. Prg4	8. Serpine1
	9. Tgm1	9. Hmox1
	10. Hspa1b	10. Lilrb4
β 2KO down	1. Gpr6	1. P2ry12
	2. Pde10a	2. Itpka
	3. P2ry12	3. Pcdhb9
	4. Fibcd1	4. Rnf112
	5. Gm1337	5. LOC100039864
	6. Indo	6. Nov
	7. Otof	7. Aldh1a1
	8. Itpka	8. Dbp
	9. Sh3rf2	9. Shox2
	10. Al853363	10. Hes5

Glo1 protein expression is increased in β 2KO mice

Using immunoblotting, the sham or MCAO cerebral hemispheres of FVB and β 2KO mice 24 h after surgery were probed with a specific antibody against Glo1 protein. Glo1 protein levels in both sham-operated and MCAO-treated β 2KO mice were significantly higher (approximately twice) than in FVB brains (Figure 5).

Noxa mRNA is decreased in β 2KO mice

Noxa is decreased 75% in sham and 60% in MCAO conditions in β 2KO mice compared with FVB (Figure 6) by RT-qPCR. Previous work found that Noxa was transcriptionally up-regulated by NF- κ B activation in the setting of ischaemia (Inta et al., 2006).

NF- κ B activity and TNF α production are attenuated in β 2KO mice compared with FVB after MCAO

NF- κ B plays a complex role in cerebral ischaemia as a regulator of both apoptotic cell death and inflammation

(Ridder and Schwaninger, 2009). In our microarray data, there were no significant differences between genotypes in p65, p50 or I κ b α (inhibitory κ B α) and levels of p65 and p50 were not changed by MCAO. Conversely, I κ B α was significantly increased from sham to MCAO in both genotypes (3.1-fold in FVB and 2.9-fold in β 2KO mice). As NF- κ B transcription factor activity is regulated post-translationally, we measured p65 activity in tissue harvested 24 h after sham or MCAO surgery. β 2KO mice had a non-significant trend to increased p65 activity in sham compared with FVB ($P=0.17$, data not shown), while p65 activity increased significantly (20%) more in FVB than in β 2KO mice from sham to MCAO (Figure 7A).

TNF α is a pro-inflammatory cytokine produced early after ischaemia which induces NF- κ B activation, and is one of the inflammatory cytokines downstream of NF- κ B activation. In the microarray data, TNF α was 1.4-fold higher in sham β 2KO compared with sham FVB mice ($P=0.03$), with no differences between genotypes after MCAO. Other genes downstream of NF- κ B such as IL-1 β (interleukin-1 β) (1.4-fold, $P=0.05$), IL-6 (1.3-fold, $P=0.07$) and MMP9 (matrix metalloproteinase 9) (1.4-fold, $P=0.05$) were all increased in sham β 2KO mice compared with sham FVB mice, with no differences between genotypes after MCAO. Increased baseline TNF α , IL-1 β , IL-6 and MMP9 levels in β 2KO mice might act as a preconditioning stimulus, limiting NF- κ B activation following ischaemia. To assess this we measured TNF α protein levels in the ischaemic brain tissue of the FVB and β 2KO mice and found that TNF α protein levels were significantly lower (50%) in β 2KO mice compared with FVB mice (Figure 7B).

DISCUSSION

Using microarray analysis, we assessed gene expression changes 24 h after MCAO in FVB and β 2KO mice. We used both SAM, a well-established analysis tool, and SAGAT, an alternate strategy that uses pre-existing mouse microarray data to reduce measurement uncertainty. Both methods identified that the top-most up-regulated genes after ischaemia were similar in both groups. This is not surprising, as both genotypes suffered significant injury following MCAO. The SAGAT method is most useful in increasing effective group size for $n < 4$ (Daigle et al., 2010). As we had $n=3$ in the sham groups but $n=7$ in our MCAO groups, identification of similar groups of genes by the two methods is expected. The majority of the top up-regulated genes identified in our microarrays have been previously implicated in stroke (Table 2). However, Prg4, Ccl9 and Gem, all of which we confirmed using RT-qPCR, had not been previously shown to increase after MCAO.

Prg4, a proteoglycan found in cartilage, lung, bone and heart (Ikegawa et al., 2000), has never been documented in brain. However, tendon Prg4 mRNA is up-regulated in response to transforming growth factor β (Rees et al., 2002), a factor that increases in the brain following stroke (Dhandapani and

Table 2 The top 20 genes up-regulated after ischaemia and their documentation in previous literature

Each of the top 20 genes up-regulated after ischaemia (by SAGAT analysis) is listed by gene symbol and gene name. FC, fold change from sham to ischaemia. Time up, time at which gene or protein is increased after ischaemia. Injury/species, the ischaemia type and animal species. Method of detection, the way in which gene expression change was assessed. MCAO, middle cerebral artery occlusion; HI, hypoxia/ischaemia; RT-PCR, reverse transcription-PCR; IHC, immunohistochemistry; ISH, *in situ* hybridization. Genes in bold have previously been implicated using protein analysis, genes in normal type have previously been implicated using RNA analysis, and those in italics have not been previously implicated in ischaemia.

Gene	Name	FC FVB	FC β 2	Time up	Injury/species	Detection	Reference
Spp1	Secreted phosphoprotein 1	81.7	10.3	4D	MCAO/mouse	RT-PCR	Schroeter et al., 2006
				5D	MCAO/rat	Northern blot	Wang et al., 1998
				24H	MCAO/rat	IHC	Ellison et al., 1998
Lcn2	Lipocalin 2	42.3	7.7	24H	MCAO/mouse	Western blot	MacManus et al., 2004
IL6	Interleukin 6	22.6	3.8	24H	MCAO/rat	IHC	Block et al., 2000
Lilrb4	Leucocyte immunoglobulin-like receptor, subfamily B, member 4	19.1	5.7	24H	MCAO/mouse	Microarray	Chen et al., 2006
Cd14	CD14 antigen	18.8	5.9	24H–48H	Cortical ischaemia/human	IHC	Beschorner et al., 2002
<i>Tgm1</i>	<i>Transglutaminase 1, K polypeptide</i>	14.7	6.3				
Lgals3	Lectin, galactose binding, soluble 3	13.9	3.6	48H	pMCAO/rat	Western blot	Walther et al., 2000
Timp1	Tissue inhibitor of metalloproteinase 1	13.6	5	6D	Global ischaemia/rat	IHC	Rivera et al., 2002
Ccl3	Chemokine (C-C motif) ligand 3	13.4	4.9	4H	Photothrombosis/rat	cDNA array	Kury et al., 2004
				24H	tMCAO/mouse	ELISA	Pang et al., 2001
Cxcl2	Chemokine (C-X-C motif) ligand 2	13	2.6	6H	tMCAO/mouse	Microarray	Takami et al., 1997
							Bowler et al., 2002
<i>Mmp3</i>	<i>Matrix metalloproteinase 3</i>	12.6	5.3				
Socs3	Suppressor of cytokine signalling 3	12.4	5.9	24H	MCAO/rat	RT-PCR	Tureyen et al., 2007
				12H	tMCAO/rat	RT-PCR	Dinapoli et al., 2010
<i>Prg4</i>	<i>Proteoglycan 4</i>	11.5	4.9				
<i>Ccl9</i>	<i>Chemokine (C-C motif) ligand 9</i>	11.2	5				
Hmxo1	Haem oxygenase (decycling) 1	8.7	5.8	3D	tMCAO/rat	IHC	Sutherland et al., 2009
<i>Irf202b</i>	<i>Interferon induced gene 202b</i>	8.2	7.7				
Atf3	Activating transcription factor 3	8	4.3	4H	MCAO/mouse	ISH	van der Weerd et al., 2009
					pMCAO/mouse	Microarray/RT-PCR	Tang et al., 2006
<i>Ch25h</i>	<i>Cholesterol 25-hydroxylase</i>	7.6	4				
Serpine1	Serine (or cysteine) peptidase inhibitor, clade E, member 1	7.2	5.8	2H	Embolic cerebral ischaemia/mouse	RT-PCR	Ahn et al., 1999
Vim	Vimentin	5.9	4.6	3D	tMCAO/rat	IHC	Shen et al., 2010
Cd44	CD44 antigen	5.7	4	6H	pMCAO/rat	Western blot	Wang et al., 2001
				12H	MCAO/mouse	RT-PCR	Wang et al., 2002
Hspa1b	Heat shock protein 72	4.6	6.5	24H	MCAO/mouse	Western blot	Han et al., 2009

Brann, 2003). Although Ccl9 has not been implicated in stroke, brain levels increase in response to infections including pneumococcal meningitis (Klein et al., 2006) and herpes simplex virus (Aravalli et al., 2005). Gem is a guanidine triphosphate binding protein that is induced in response to mitogens (Maguire et al., 1994), suggesting that it may play a role in post-ischaemic cell proliferation. Additional studies will be necessary to determine the roles and potential therapeutic applications of these novel genes. A limitation of our study is that we only performed array analysis and biochemical analyses at 24 h after MCAO, whereas the process of ischaemic brain injury has a much longer course that still needs to be characterized.

When all genes differentially regulated after stroke were examined by grouping the genes into biological functions, both groups showed increases in inflammatory response and cellular death, similar to previous microarray studies (Bowler et al., 2002; Chen et al., 2011). Only β 2KO mice had cell proliferation as one of the top five biological functions. This is consistent with previous literature describing increased astrocyte proliferation in the β 2KO mice (Chesik et al., 2007). Network analysis revealed that networks of genes enriched after MCAO were largely similar in both genotypes, with the exception of a GPCR-centred network enriched in the FVB mice but not the β 2KO mice.

Table 3 Genes increased 24 h after ischaemia by RT-qPCR

Values reflect fold change from sham to ischaemia \pm S.E.M. ($n=6$ /group in sham and 10–12/group in MCAO). Genes in bold have not previously been implicated in brain ischaemia.

Gene	WT	β 2KO
Ccl9	21.24 \pm 7.11	28.19 \pm 6.68
Cebpa	1.91 \pm 0.19	2.58 \pm 0.29
Ch25h	24.85 \pm 6.73	23.17 \pm 4.55
Cxcl1	76.05 \pm 29.13	118.3 \pm 47.82
Gem	2.30 \pm 0.44	3.26 \pm 0.69
Ifi202b	32.33 \pm 8.27	57.98 \pm 12.87
Mmp3	1244 \pm 306.1	2880 \pm 1094
Prg4	6.71 \pm 2.20	17.69 \pm 6.38
Tgm1	517.7 \pm 143.0	633.6 \pm 141.9

To assess differences in gene expression by genotype, we analysed genes up- or down-regulated in β 2KO mice compared with FVB in naïve, sham and MCAO conditions. Gene expression differences between genotypes revealed multiple genes altered between β 2KO and FVB mice in naïve, sham and MCAO conditions, with 11 genes differentially expressed in all conditions, indicative of changes in response to the chronic absence of the β 2 receptor. Perhaps surprisingly, *Adrb2*, the gene for the β 2AR, was significantly increased on the arrays in the β 2KO mice compared with FVB. The probeset for *Adrb2* targets nucleotides 1608–1835, outside the fourth transmembrane segment sequence targeted and replaced in the KO (Chruscinski et al., 1999). It suggests that the β 2KO mice may produce elevated levels of this aberrant RNA perhaps as an attempted compensatory mechanism.

Of the genes differentially expressed between genotypes, two were related to NF- κ B activity: *Glo1* and *Pmaip1/Noxa*. This suggested that activation of NF- κ B by MCAO might differ between the genotypes, which we confirmed by a p65 activity assay. Furthermore, *Glo1* protein levels are increased

in β 2KO compared with FVB mice. *Glo1* detoxifies methylglyoxal, a toxic by-product of glycolysis known as an AGE (advanced glycation end-product) (Thornalley, 1996). AGEs are produced not only by hyperglycaemia but are also produced in the preinfarct area following cerebral ischaemia (Qiu et al., 2008; Harada et al., 2009). Hyperglycaemia-induced NF- κ B activation is attenuated by *Glo1* overexpression (El-Osta et al., 2008), and *Glo1* overexpression is protective in renal ischaemic injury (Kumagai et al., 2009). Thus *Glo1* overexpression in the β 2KO mice may be protective by attenuating NF- κ B signalling and/or decreasing toxic AGEs.

Noxa mRNA levels are decreased in β 2KO compared with FVB mice. Noxa, a member of the BH3 (Bcl2 homology domain 3) family of pro-apoptotic proteins, targets the anti-apoptotic Bcl2 molecule Mcl1 for proteasomal degradation, resulting in increased cytochrome c release and caspase activation (Ploner et al., 2008). Noxa antisense significantly decreased infarct size after rat MCAO (Kim et al., 2004), and Noxa was implicated in the neurotoxicity of cerebral ischaemia, where it was shown to be transcriptionally up-regulated by NF- κ B activation (Inta et al., 2006; Ridder and Schwaninger, 2009). Together, these results suggest that Noxa directly affects ischaemia-induced cell death, and levels of Noxa are at least partially dependent upon NF- κ B activation. This is consistent with our current observation that NF- κ B activity and levels of TNF α are both lower in β 2KO mice compared with FVB after MCAO, and supports the idea that reduced NF- κ B activation leading to lower levels of downstream genes Noxa and TNF α may contribute to protection in the absence of β 2AR.

Although NF- κ B signalling can protect against oxidative stress-induced neuronal death (Kratsovnik et al., 2005), the majority of the literature suggests that neuronal NF- κ B

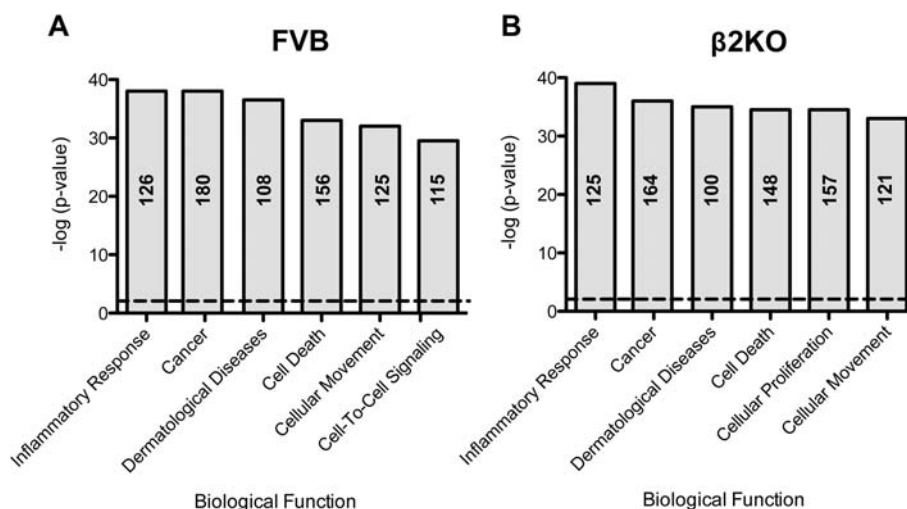


Figure 2 Biological functions enriched in all genes regulated after ischaemia by genotype (A) FVB mice or (B) β 2KO mice. Dotted line indicates threshold for significance. The number of genes from the microarray in each category are shown in each bar.

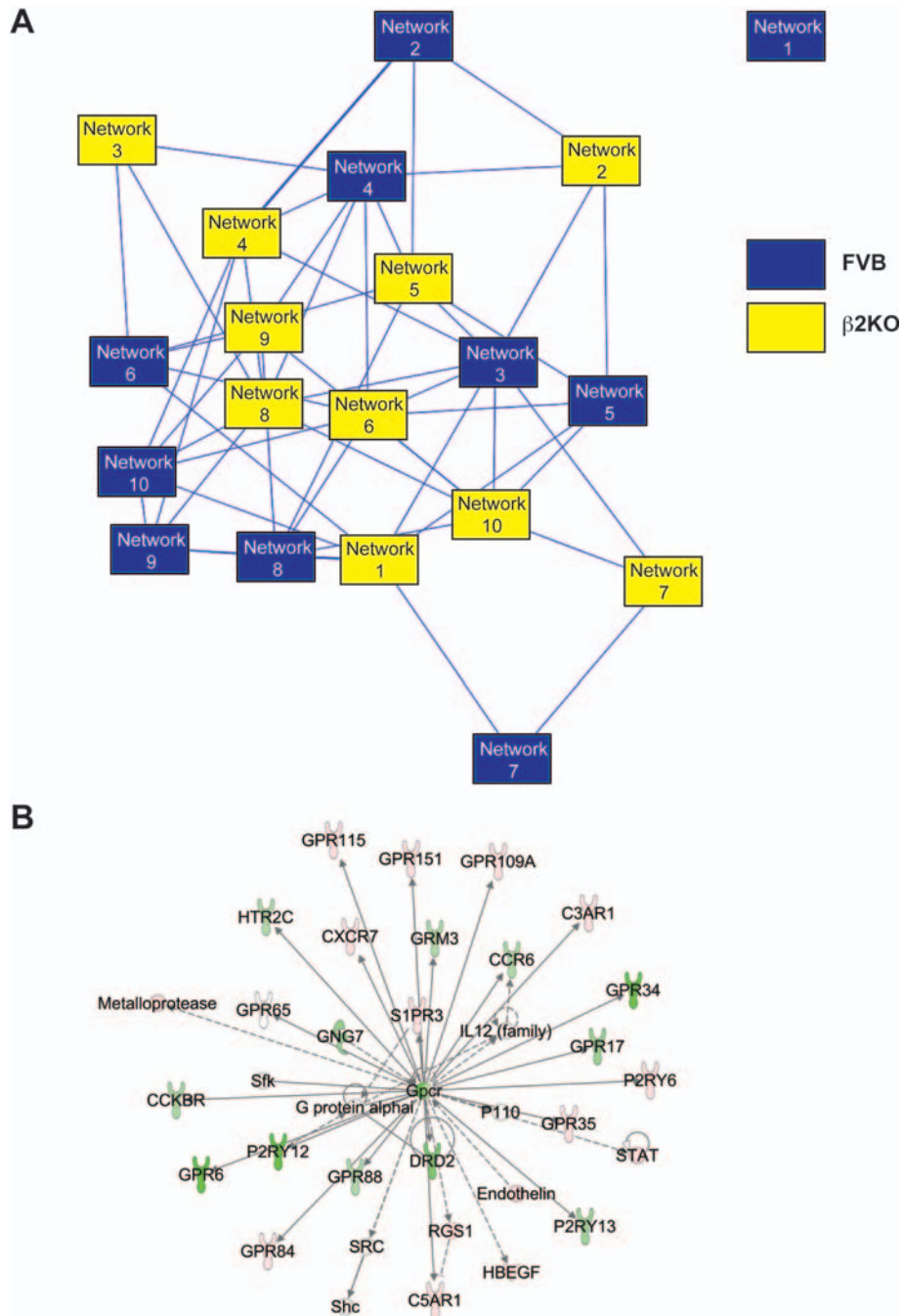


Figure 3 A GPCR network is differentially regulated after MCAO in FVB but not β 2KO mice
 (A) Network analysis shows that most networks enriched after MCAO are similar between genotypes with the exception of Network 1 in the FVB mice. Lines between networks indicate shared genes between those networks. (B) Schematic diagram showing the unique network enriched in the FVB mice, consisting mostly of GPCR. Genes in red are up-regulated, and genes in green are down-regulated following ischaemia. Genes in white are part of the network but were not differentially regulated in the dataset. Direction of arrow indicates previously documented relationships between genes; genes at the end of each arrow are affected by the source of the arrow.

activation (Zheng et al., 2008; Ridder and Schwaninger, 2009) and $\text{TNF}\alpha$ are detrimental following stroke. Inhibition of $\text{NF-}\kappa\text{B}$ activation is protective after rat MCAO (Xu et al., 2002), and transgenic mice specifically overexpressing a dominant

non-inactivable $\text{I}\kappa\text{B}\alpha$ in neurons have significantly smaller infarcts following permanent MCAO (Zhang et al., 2005). Rats overexpressing $\text{TNF}\alpha$ have greater injury after stroke (Pettigrew et al., 2008), while $\text{TNF}\alpha$ -neutralizing antibodies significantly

Table 4 Biological functions associated with networks enriched after ischaemia in FVB and β 2KO mice
For each network, the biological functions associated with genes in the network and the number of genes from the microarray that appear in the network are shown.

Network	Biological functions	No. of genes
FVB		
1	Carbohydrate metabolism, small molecule biochemistry and inflammatory response	25
2	Drug metabolism, lipid metabolism and molecular transport	24
3	Organ development, organ morphology, respiratory system development and function	20
4	Cellular movement, haematological system development and function, and immune cell trafficking	19
5	Cardiovascular disease, haematological disease and cell death	18
6	Tissue morphology, cancer, skeletal and muscular disorders	18
7	Cancer, reproductive system disease, cell-to-cell signalling and interaction	18
8	Cell-to-cell signalling and interaction, nervous system development and function, cellular assembly and organization	17
9	Neurological disease, skeletal and muscular disorders, genetic disorder	16
10	Cellular movement, cellular development and cell cycle	15
β 2KO		
1	Cardiovascular disease, haematological disease and embryonic development	23
2	Cell death, drug metabolism and lipid metabolism	23
3	Cellular movement, haematological system development and function, immune cell trafficking	19
4	Cell cycle, cancer and reproductive system disease	19
5	Inflammatory response, cell movement, haematological system development and function	19
6	Cardiovascular disease, inflammatory disease and renal nephritis	16
7	Post-translational modification, cardiac damage and cardiovascular disease	15
8	Drug metabolism, small molecule biochemistry, endocrine system development and function	15
9	Cell morphology, cellular development and embryonic development	16
10	Connective tissue development and function, skeletal and muscular system development and function. Tissue morphology	16

reduce damage (Yang et al., 1998). Furthermore, activation of NF- κ B signalling can lead to further production of harmful pro-inflammatory cytokines (Tak and Firestein, 2001) and chemokines (Thompson and Van Eldik, 2009), contributing to

further neuronal death following the initial injury. NF- κ B activation has a complicated and variable dynamic timeline, which we have modelled previously (Sheppard et al., 2011). The kinetics of activation is known to influence which downstream

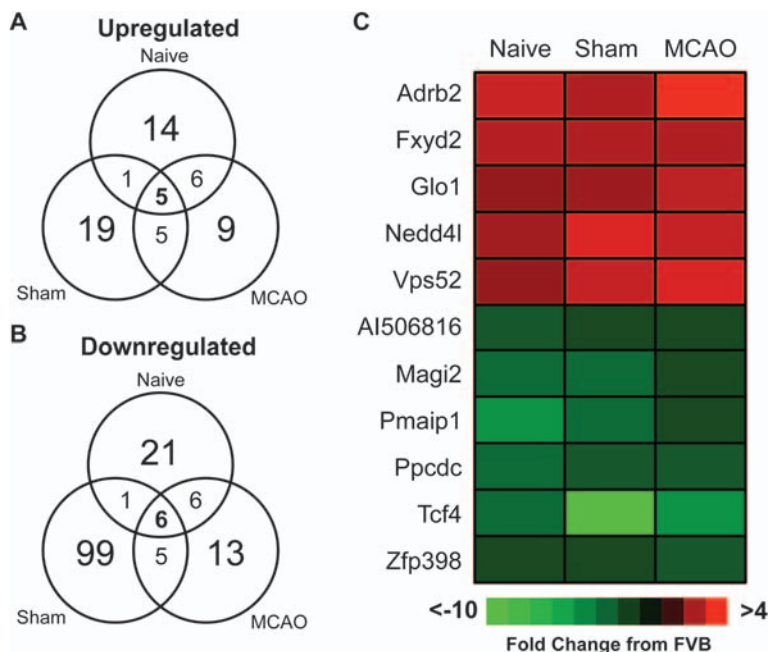


Figure 4 Multiple genes differ between β 2KO and FVB mice in naïve, sham and MCAO conditions
(A, B) Venn diagrams showing genes up-regulated (A) and down-regulated (B) in β 2KO mice. (C) Heatmap showing genes up-regulated or down-regulated in β 2KO compared with FVB mice. Red indicates up-regulated genes and green indicates down-regulated genes. The genes are: Adrb2 (β 2 adrenergic receptor), Fxyd2 (FXD domain containing ion transport regulator 2), Glo1 (glyoxalase 1), Nedd4l (neural precursor cell expressed, developmentally down-regulated 4-like), Vps52 (vacuolar protein sorting 52 homologue), Al506816 (hypothetical protein), Magi2 (membrane associated guanylate kinase, WW and PDZ domain containing 2), Pmaip1/Noxa (phorbol-12-myristate-acetate-induced protein 1), Ppcdc (phosphopantothienoylcysteine decarboxylase), Tcf4 (transcription factor 4) and Zfp398 (zinc finger protein 398).

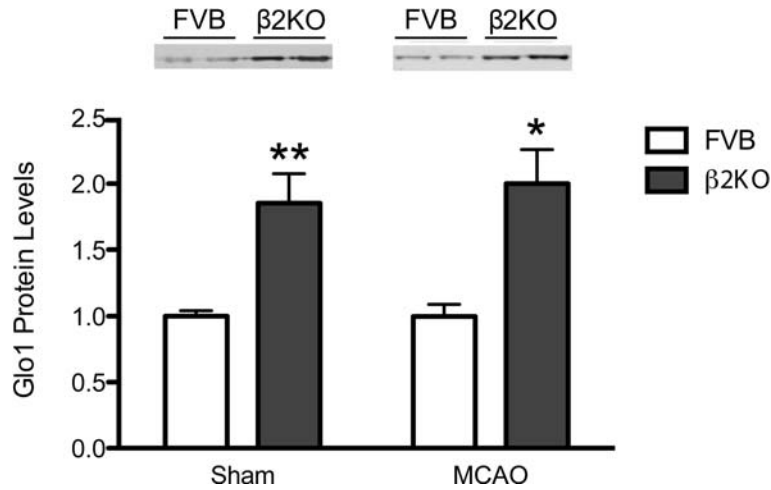


Figure 5 Glo1 is up-regulated in the β 2KO mice compared with FVB
 Immunoblots of sham and MCAO tissue probed for an antibody against Glo1. β -Actin protein levels were consistent across samples (data not shown). Graph indicates quantification of blots in sham and MCAO conditions, normalized to β -actin levels ($n=3-4$ /sham group, 4/MCAO group). * $P<0.05$, ** $P<0.01$.

targets will be induced (Fine et al., 1999). As this study only assessed NF- κ B 24 h following MCAO, additional studies are needed to determine the full kinetics of these processes after stroke.

The reasons for altered NF- κ B signalling, cell death and inflammatory response in the β 2KO mice could be due to direct, indirect or combined effects of the chronic absence of β 2AR signalling. The role of the β 2AR in modulating inflammation and NF- κ B activation is controversial. Multiple studies have shown that treating cells with selective or non-selective β AR agonists has an anti-inflammatory effect, decreasing TNF α (Severn et al., 1992; Mori et al., 2002), IL-8 (Farmer and Pugin, 2000) and IL-6 (Mohamed-Ali et al., 2000) in response to LPS (lipopolysaccharide). But treatment with agonists alone was also shown to increase IL-6 via NF- κ B signalling (Christensen et al., 1999; Frost et al., 2004; Yin

et al., 2006; Rohrbach et al., 2007; Tan et al., 2007), and the β 2AR-specific agonist terbutaline increased microglial production of harmful ROS (reactive oxygen species) (Qian et al., 2009). Furthermore, treatment with the agonist isoprenaline increased p65 and p50 activity in rat salivary gland 1 h after administration (Yeh et al., 2012).

A previous study (Rough et al., 2009) has begun to shed light on this controversy, demonstrating that pre-treatment with adrenaline for 18 h prior to LPS stimulation significantly increases TNF α production, an effect that is completely abolished by treatment with a β 2AR-specific inhibitor. Thus acute and chronic blockade of the β 2AR may have different and even opposite effects on NF- κ B signalling and inflammation. This is consistent with the known unique switch in coupling of β 2AR from G_s to G_i with chronic exposure to agonist (Daaka et al., 1997; Xiao et al., 2006). In our study,

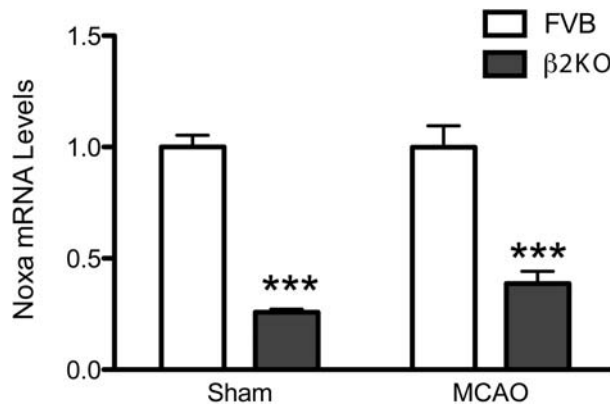


Figure 6 Noxa mRNA is decreased in β 2KO mice compared with FVB in sham and MCAO conditions
 Quantification of RT-qPCR showing a significant decrease in Noxa mRNA in sham and MCAO conditions in β 2KO mice ($n=6$ /sham group and 10-12/MCAO group). *** $P<0.001$.

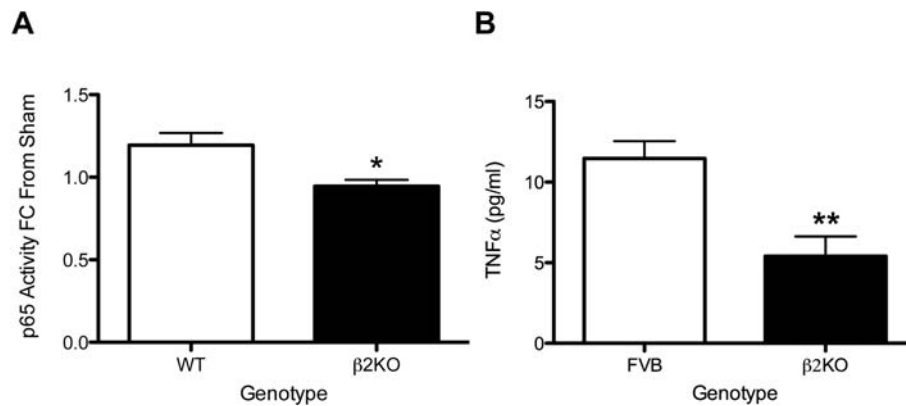


Figure 7 Ischaemia-induced p65 activity and TNF α production is attenuated in β 2KO mice
 (A) Fold-change of OD value representing p65 activity shows a significant decrease in β 2KO mice compared with FVB ($n=3$ /sham group, 6–7/MCAO group). FC=fold change. (B) TNF α levels as measured by ELISA were significantly lower following MCAO in the β 2KO mice compared with FVB controls ($n=4$ /group). ** $P<0.01$.

chronically absent β 2AR signalling contributes to damping production of TNF α and thereby likely attenuating injury. This would be consistent with the idea that chronic absence of β 2AR produces a kind of preconditioning. Earlier observations demonstrated that preconditioning is associated with NF- κ B activation which then limits NF- κ B activation by the more severe ischaemia (Ridder and Schwaninger, 2009).

Preconditioning with low levels of systemic LPS significantly decreases brain inflammation and infarct size following MCAO (Vartanian et al., 2011), while higher doses given before and/or after MCAO significantly exacerbate damage (McColl et al., 2007; Langdon et al., 2010). Thus, chronically mildly elevated levels of systemic inflammation may lead to protection by a form of preconditioning, while high levels of inflammation prior to stroke are likely to be detrimental. Interestingly, our arrays identified a modest increase in TNF α , IL-1 β , IL-6 and MMP9 in the sham β 2KO compared with FVB mice, suggesting that higher basal brain inflammation might be present in the β 2KO mice.

The effects of abolishing β 2AR signalling in the periphery as well as in the CNS must be considered, as inflammation in the periphery can affect the CNS and vice versa (Denes et al., 2010). Earlier studies in the β 2KO mice found that the contact sensitivity response and T-cell-dependent antibody response in β 2KO compared with FVB mice were similar, despite defects demonstrable in isolated immune cells, but the mechanism of compensation was not identified (Sanders et al., 2003). Thus it is unclear whether changes in peripheral inflammation might play a role in the difference between the genotypes.

CONCLUSIONS

Microarray analysis revealed that β 2KO mice have a unique genetic profile both before and after ischaemia. The current

study demonstrates reduced cell death signalling, reduced inflammation and reduced NF- κ B activation in β 2KO mice compared with FVB mice. Altered levels of Glo1 and Noxa may contribute to the neuroprotection observed.

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