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Brain inflammation is induced by co-morbidities and risk factors for stroke

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Chronic systemic inflammatory conditions, such as atherosclerosis, diabetes and obesity are associated with increased risk of stroke, which suggests that systemic inflammation may contribute to the development of stroke in humans. The hypothesis that systemic inflammation may induce brain pathology can be tested in animals, and this was the key objective of the present study. First, we assessed inflammatory changes in the brain in rodent models of chronic, systemic inflammation. PET imaging revealed increased microglia activation in the brain of JCR-LA (corpulent) rats, which develop atherosclerosis and obesity, compared to the control lean strain. Immunostaining against Iba1 confirmed reactive microgliosis in these animals. An atherogenic diet in apolipoprotein E knock-out (ApoE −/−/−) mice induced microglial activation in the brain parenchyma within 8 weeks and increased expression of vascular adhesion molecules. Focal lipid deposition and neuroinflammation in periventricular and cortical areas and profound recruitment of activated myeloid phagocytes, T cells and granulocytes into the choroid plexus were also observed. In a small, preliminary study, patients at risk of stroke (multiple risk factors for stroke, with chronically elevated C-reactive protein, but negative MRI for brain pathology) exhibited increased inflammation in the brain, as indicated by PET imaging. These findings show that brain inflammation occurs in animals, and tentatively in humans, harbouring risk factors for stroke associated with elevated systemic inflammation. Thus a “primed” inflammatory environment in the brain may exist in individuals at risk of stroke and this can be adequately recapitulated in appropriate co-morbid animal models.

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1. Introduction

Clinical and experimental evidence implicates inflammation in multiple phases of stroke aetiology and pathology (Allan et al., 2005; Amor et al., 2010; Denes et al., 2010a,b; McColl et al., 2009; Muir et al., 2007). Several of the risk factors for stroke, such as atherosclerosis, hypertension and diabetes/obesity are triggered and/or propagated by dysregulated systemic inflammatory processes (Dandona et al., 2004; Ross, 1999; Savoia and Schiffirn, 2006). Markers of elevated systemic inflammation are associated with increased stroke risk and brain lesions detected by magnetic resonance imaging (MRI) (Fornage et al., 2008; van Dijk et al., 2005). Like other statins, rosuvastatin, has multiple anti-inflammatory properties. It reduces cerebrovascular events in patients without hyperlipidemia but with raised C-reactive protein (CRP) levels
2.1. Pre-clinical studies

2.1.1. Positron emission tomography

Rats were anaesthetised by isoflurane inhalation (induction, 5%; maintenance, 2–2.5%) in oxygen. $[^{18}\text{F}]$DPA-714, a specific tracer for the TSP0 (Boutin et al., 2008; Chauveau et al., 2009) was synthesised (James et al., 2008), and injected intravenously in the tail vein as a bolus (10.8–19.8 MBq, 0.03–2.79 nmol). Respiratory and temperature was monitored throughout using a pressure sensitive pad and rectal probe, Model 1025L interface and PC-SAM software (SA Instruments, NJ, USA). Body temperature was maintained at 37 ± 0.5 °C by use of a heating pad and the heating and fan module connected to the rectal probe via the interface and controlled by the PC-SAM software. Whole-body images were acquired in list-mode with a non-rotating 16-module quad-HIDAC PET camera (Oxford Positron Systems, UK) for 1 h (Hastings et al., 2007). The list-mode data were reconstructed directly into 5 min time-frame images (without resorting to histogramming) via the one-pass-list-mode-expectation maximisation (OPL-EM) algorithm (Reader et al., 2002) with one iteration of 16 sub-sets into images of dimensions $120^3$ (transaxially) $\times 240$ (axially) with isotropic $1\text{mm}^3$ voxels. Absolute calibration of the images was achieved by reference to a $^{24}\text{Na}$ source imaged in the field of view in each scan. This had been validated with a uniformly filled mouse-sized $[^{18}\text{F}]$ phantom imaged over 2 h. Dynamic images were calibrated in kBq/cm$^3$.

Images were segmented using the Local Means Analysis method and the organ mean Time Activity Curves were corrected for Partial Volume Effect using the Geometric Transfer Matrix (GTM) method with a selection of 20% of the organ voxels (GTM20) (Mangat et al., 2007; Vannucci et al., 2001). Here we undertook a translational study to determine if risk factors for stroke, which involve chronic systemic inflammation, also induce brain inflammation in rodents and humans. We show that brain inflammation is present in rats and mice harbouring systemic vascular and/or metabolic disease and that analogous changes may be present in patients with clinical risk factors and evidence of systemic inflammation, as indicated by a raised concentration of circulating CRP.

2.1.2. Tissue processing

Under terminal anesthesia, mice and rats were perfused transcardially with saline followed by 4% paraformaldehyde.
(PFA; Sigma, UK). Brains were removed and postfixed in 4% PFA at 4 °C for 24 h. Brains were subjected to cryoprotection in phosphate-buffered saline containing 20% sucrose for 24 h. Five alternate sets of 20 μm (mice) or 30 μm (rats) thick coronal brain sections were cut on a sledge microtome (Bright series 8000; Bright Instruments, Huntingdon, UK). All sections were collected into an antifreeze solution containing 30% ethylene glycol (Sigma, UK) and 20% glycerol (Fisher, UK) in phosphate-buffered saline and stored at −20 °C until processing.

2.1.3. Immunohistochemistry

Immunohistochemistry was performed on free-floating brain sections. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide (Sigma) in dH₂O and sections were treated with 2% normal serum (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Sections were incubated overnight in antibody diluent (0.1 M PBS + 0.3 % Triton X-100, Sigma) using the following primary antibodies: goat anti-mouse VCAM-1 1:250 (R&D Systems, UK), goat anti-mouse ICAM-1 1:250 (R&D Systems, UK), goat anti-mouse Iba1 1:500 (Abcam, UK), rabbit anti-Iba1 (Wako Chemicals, Germany) and rat anti-mouse CD45 1:250 (Serotec, UK). Sections were then incubated in appropriate biotinylated secondary antibody for 1 h (rabbit anti-goat 1:1000 and rabbit anti-rat 1:750, Vector Laboratories, UK). Sections were then incubated in Vectastain ABC solution (Vector laboratories, UK) and colour was developed by nickel enhanced diaminobenzidine (50 mg/ml) incubation (Vector Laboratories, UK) and 54% glycerol in water). Haematoxylin & Eosin (H&E) staining was performed on mounted brain sections. Following staining sections were dehydrated and cover-slipped with Depex mounting medium.

Activated microglia were identified as showing: (1) increased Iba1 immunopositivity, (2) enlarged and/or amoeboid cell body, (3) complete or partial loss of thin, elongated processes. Round shaped, small Iba1-positive cells with leucocyte morphology were not counted. Regions analysed for microglial activation were also stained with mouse anti-rat CD68 (corpulent rats) and rat anti-mouse CD45 (mice) to assess the number of parenchymal macrophages and other leucocytes. Activated microglia were counted throughout the striatum and expressed as activated microglia per mm².

Fluorescently labelled CD45 positive cells were counted in two randomly selected fields of view of the caudal choroid plexus (−1.82 mm from Bregma) and the lateral ventricle (−1.58 mm from Bregma). The choroid plexus and ventricular ependyma were visualised by using VCAM immunofluorescence.

2.1.6. Histology

After CD45 immunohistochemistry (see above) sections were rinsed in dH₂O and incubated in 60% v/v isopropanol/dH₂O (Fischer, UK) for 2 min. Sections were transferred to Oil red O (ORO; Sigma, UK) (0.05% w/v ORO/99% isopropanol) for 15 min, rinsed in 60% v/v isopropanol, rinsed in dH₂O and coverslipped with an aqueous glycerol jelly mount (7.7% w/v Gelatine (BDH, UK) and 54% glycerol in water). Haematoxylin & Eosin (H&E) staining was performed on mounted brain sections. Following staining sections were dehydrated and cover-slipped with Depex mounting medium.

2.1.7. Statistical analysis

Quantitative analysis of data was performed in a blinded manner. PET image quantifications were analysed using Mann–Whitney for comparison between lean and corpulent animals and for comparing 9 vs 15 and 9 vs 12 month age groups. Because the same group of animals was scanned at 12 and 15 months of age, a non-parametric paired Wilcoxon test was used to compare these two groups.

Quantitative data from immunohistochemical and immunofluorescence studies were analysed by one- or two-way analysis of variance (ANOVA) followed by post-hoc Bonferroni’s correction. All data are expressed as mean ± SD. Statistical significance is reported at the 0.05 level.

2.2. Clinical study

2.2.1. Patients

This small, preliminary study was undertaken to assess cerebral inflammation in humans with multiple risk factors for stroke, but no evidence of cerebral damage, in order to investigate the relevance of our experimental findings in a translational context. One hundred and twenty-one subjects were screened, and rigorous criteria were applied to identify patients at risk of stroke, while excluding patients with existing brain pathology. Subjects were deemed eligible if having multiple (three or more) risk factors for stroke, and/or established arterial disease (hypertension, dyslipidaemia, atrial fibrillation, left ventricular hypertrophy, ischaemic heart disease, diabetes mellitus, peripheral vascular disease, carotid disease and smoking), and CRP >3 mg/L on two separate occasions. All subjects underwent MRA scans to exclude any intracranial pathology, and subjects with a history of a previous cerebrovascular event were not involved in the study. MRA scans were reviewed by neuroradiologists. Only four patients fulfilled all inclusion criteria and were subjected to PET imaging to assess microglial activation in the brain (see below). Age matched control participants were chosen on the basis of having two or fewer major vascular risk factors and plasma CRP ≤1 mg/L (see Table 1). All participants were also screened to exclude cognitive impairment and a
telephone consultation was used to exclude symptoms of acute infection prior to PET scanning. All participants gave written informed consent.

2.2.2. Positron emission tomography

Participants underwent MRI scans on a 3 T Philips Achieva system using a T1 weighted inversion recovery SENSE sequence for co-registration of PET images and to exclude visible evidence of stroke. PET studies were performed on a high resolution research tomograph (CTI/Siemens). \[^{11}C\](R)-PK11195 (TSPO ligand) was used to assess microglial activation in the brain. Following a 6 min transmission scan, \[^{11}C\](R)-PK11195 was injected as a slow bolus over 20 s and data were acquired during a 60 min emission scan. The injected radioactivity dose was 465 ± 121 MBq and radiochemical purity was always greater than 98.9%. The injected mass of cold (R)-PK11195 was 2.4 ± 1.0 μg. Binding potential (BPND) images were generated using the simplified reference tissue model and a supervised clustering algorithm was used to extract a reference tissue input function (Turkheimer et al., 2007). The study was approved by the local research and ethics committee.

3. Results

3.1. PET imaging reveals neuroinflammation in cp/cp JCR-LA cp rats

There was no significant difference in microglial activation as determined by PET imaging between lean and corpulent rats at 9 months of age (Fig. 1A). By 12 months of age, microglial activation was increased significantly in the brains of the corpulent rats in the ROI with the lowest tracer uptake (+35%, Fig. 1B). \[^{18}F\]DPA-714 uptake increased further in 15 month old animals (+32% and +53% in low and high uptake ROI respectively; Fig. 1C). We also observed a trend for an increase in neuroinflammation with age in both lean and corpulent animals, although this was significant only in the corpulent in the low uptake ROI when comparing 15 with 9 month old animals (+28%, P < 0.05, Fig. 1C). Although the \[^{18}F\]DPA-714 uptake was increased by a similar magnitude (+29 to 33%, Fig. 1B and C) between 12 and 15 months in the corpulent rats, the differences were not significant. In reference organs, known to express high level of TSPO (heart, lungs and kidneys), there were no significant differences between lean and corpulent rats (Supplementary Fig. 1).

3.2. Immunohistochemical evidence of microglial activation in rodents with peripheral disease

Immunohistochemistry revealed activated microglial cells in the brains of 15 month old corpulent rats (Fig. 2A). We found no activated microglial cells in the brains of corpulent rats aged 9 months or in heterozygous (lean) rats at any age (9–15 months) examined.

In ApoE\(^{-/-}\) mice fed Paigen diet lba1 immunohistochemistry revealed activated microglial cells (Fig. 2B). Microglia displaying thickened processes and increased levels of lba1 were observed in multiple brain regions such as the cerebral cortex, striatum, hypothalamus, periventricular areas and meninges. Chow or Paigen-diet fed C57BL/6 control mice and chow-fed ApoE\(^{-/-}\) mice lacked activated brain microglia.

3.3. Atherogenic mice develop vascular inflammation and leucocyte infiltration in the brain

C57BL/6 or ApoE\(^{-/-}\) mice fed a chow diet did not show elevated vascular ICAM or VCAM expression in the brain. A trend for increased vascular ICAM and VCAM expression was observed in C57BL/6 mice fed the Paigen diet (not significant). In contrast, ICAM and VCAM expression was significantly augmented in ApoE\(^{-/-}\) mice on the Paigen diet (Fig. 3A and B). Increased VCAM staining was present mainly on medium sized or large blood vessels in the cerebral cortex, striatum, thalamus and hippocampus. Quantitative analysis of VCAM immunohistochemistry revealed significantly stronger staining in Paigen fed groups compared to chow diet \((P < 0.01,\) data not shown). Post-hoc comparison revealed significant differences between ApoE\(^{-/-}\) chow and Paigen fed animals (Fig. 3C), but not in C57BL/6 mice, indicating that diet-induced pro-inflammatory changes are augmented in ApoE\(^{-/-}\) mice.

We also investigated the possibility that diet-induced atherosclerosis was associated with leucocyte infiltration into the brain parenchyma and ventricles, using immunofluorescent staining of the common leucocyte antigen CD45. Microglial CD45 expression was relatively dim throughout the brain and was well discriminated from that of bright and round shaped or elongated leukocytes. Profound enrichment of ventricular leucocytes was found in ApoE\(^{-/-}\) mice fed with Paigen diet, and this was associated with increased VCAM expression in the choroid plexus (Fig. 4A). Invasion of the choroid plexus by CD45-positive cells was significantly elevated in ApoE\(^{-/-}\) animals on the Paigen diet compared to ApoE\(^{-/-}\) animals on normal diet, but this was not observed in C57BL/6 mice fed with Paigen diet (Fig. 4B). In Paigen-fed ApoE\(^{-/-}\) mice, CD45-positive cells were numerous in the choroid plexus of the lateral ventricles from the \emph{fimbria hippocampi} to the caudal areas of the ventricle. Caudally, infiltration of ventricular-associated cells into the surrounding parenchyma was also observed in ApoE\(^{-/-}\) mice (Fig. 4C). The size of the lateral ventricle was not significantly different among experimental groups and no correlation between CD45-positive cells and ventricle size was found in individual mice. The choroid plexus was found to contain a number of different cell types including granulocytes (identified by an anti-neutrophil serum, SJC) and CD3-positive T cells (Fig. 4D). Granulocytes represented a large proportion of the cells and were uniformly distributed along the VCAM-positive areas of the choroid plexus, in partial overlap with T cells. Activated microglia/macrophages lined the walls of the caudal lateral ventricle, showed increased CD45 expression (Fig. 4E).

3.4. Atherogenic diet results in focal lipid deposition and inflammation in ApoE\(^{-/-}\) mice

In peripheral tissues, particularly in large blood vessels, ApoE\(^{-/-}\) mice develop atherosclerotic plaques, as identified by lipid deposition, leucocyte infiltration and vascular stenosis (Stoll and Bendszus, 2006; Zadelaar et al., 2007). However, it is not known whether such focal vascular pathologies appear in the brain in these animals or not. In 40% of the Paigen fed ApoE\(^{-/-}\) mice, focal pathologies were observed in the brain parenchyma (typically in the hypothalamus, near the third ventricle). Oil red staining identified blood vessel-associated lipid deposition (Fig. 5A and B).
accompanied by microglial activation and leucocyte recruitment (identified by H&E), as well as CD45 and Iba1 staining (Fig. 5C). VCAM expression was increased focally around lipid rich areas and also in the ipsilateral wall of the third ventricle, indicating ongoing inflammatory responses in the brain (Fig. 5D).

3.5. PET imaging: pilot study reveals neuroinflammation in human subjects with risk factors for stroke

Peripheral inflammatory markers increased in both groups of subjects between screening and time of PET but remained higher in the at risk group (Table 1). Visual inspection of the participants’ BPND maps revealed increased $^{[11]}$C(R)-PK11195 binding in three of the subjects with increased risk factors (Fig. 6). The distribution of the $^{[11]}$C(R)-PK11195 signal showed individual differences and was seen across neocortical areas and other brain regions, including the thalamus and brain stem. There was no evidence of raised $^{[11]}$C(R)-PK11195 binding in periventricular or deep white matter regions. There was no pattern of activity in a particular vascular territory, as one might see with established stroke. These preliminary results indicate that neocortical inflammation is present in the brain of subjects with chronic

![Fig. 1. Sum images (20–60 min post-injection; left panel) and respective quantification (graphs on the right panel) of $^{[18]}$FDP-714 uptake in the brain of lean (+/?) and corpulent (cp/cp) rats at 9 (A), 12 (B) and 15 months (C) of age. * and † indicate a significant difference between lean and corpulent animals of the same age in respectively low and high uptake regions of interest ($P < 0.05$, Mann–Whitney test). # indicates a significant difference between 9 (A) and 15 (B) months old animals ($P < 0.05$, Mann–Whitney test). Data are expressed as mean ± SD (filled symbols correspond to the respective image on the left panel).]
Fig. 2. Rodent models of atherosclerosis involve microglial activation in the brain. (A) Activated microglia as identified by increased Iba1 immunopositivity, thickened processes and irregular cell bodies were seen in the striatum of 15 month old corpulent rats, but not in 9 month old animals. Aged corpulent rats had a significantly increased number of activated microglia compared to young corpulent, or 15 month old heterozygous rats. (B) Activated, Iba1-positive microglia was numerous in ApoE−/− mice fed a Paigen diet. Insets show representative images of microglial cells from the different groups of mice. Quantitative analysis revealed significantly more activated microglial cells in the striatum of ApoE−/− mice fed a Paigen diet compared with ApoE−/− mice fed chow diet. *P < 0.05. Scale bars: 200 and 10 μm (insets).

Fig. 3. Cerebrovascular activation occurs in the brain in association with peripheral atherosclerosis. Vascular activation was assessed in the cerebral cortex using immunostaining to the adhesion molecules (A) ICAM and (B) VCAM. Unlike mice fed a chow diet, mice fed a Paigen diet showed an increased number of ICAM and VCAM-positive blood vessels in the brain. (C) Quantitative analysis of VCAM-positive blood vessels in the cerebral cortex. Scale bars: 200 and 50 μm (inset).
systemic inflammation, which is consistent with our findings in rodents with risk factors for stroke.

4. Discussion

Here we show that major risk factors for stroke such as atherosclerosis, hyperlipidemia and obesity, which involve chronic systemic inflammation, are associated with brain inflammation in relevant animal models and in a small cohort of humans, in the absence of any cerebrovascular events. These data suggest that systemic inflammation can drive brain inflammation prior to stroke presentation, leading to a "primed" inflammatory environment in the brain.

We used PET imaging to identify microglial activation, because these cells are early responders to pathological changes in the CNS and microglial activation is a hallmark of multiple brain diseases in patients and rodent models (Hanisch and Kettenmann, 2007; Teeling and Perry, 2009). The advantage of assessing neuroinflammation by in vivo PET imaging is that these measurements are comparable with clinical imaging data, and is therefore highly translatable to clinical settings. Both [11C]PK11195 and [18F]DPA-714 bind to TSPO, but despite that [18F]DPA-714 has the advantages of better signal to noise ratio (Chauveau et al., 2009) and the longer half-life of [18F], which allow PET imaging of 2–3 animal per batch of tracer, [18F]DPA-714 is not yet available for clinical use in our facilities. Corpulent rats exhibited focal areas of microglial activation, as assessed by increased [18F]DPA-714 binding in vivo. Increased TSPO-ligand binding was observed in various brain areas, including periventricular regions and some subcortical and cortical regions in the corpulent rats (Fig. 1B and C). Imaging data correlated well with the immunohistochemistry findings, which revealed an increase in the number of activated microglial cells. In line with the experimental data, the presence of [11C](R)-PK11195 binding indicated neuroinflammation in subjects with multiple risk factors.

Microglial activation was also detected in several brain regions in atherosclerotic ApoE<sup>−/−</sup> mice, indicating that neuroinflammation is likely to be a common link among animal models of chronic systemic inflammatory diseases. Although the exact mechanism of microglial activation needs to be further investigated, such "priming" of microglia in response to peripheral inflammatory changes has important implications to multiple cerebrovascular diseases. It is now established that microglia primed by central neurodegeneration or amyloidosis respond more vigorously to subsequent systemic or central inflammatory insults. For example, in a murine...
model of prion disease, intracerebral or systemic LPS challenge induced augmented microglial activation and cytokine expression compared to control mice (Cunningham et al., 2005). Our data indicate that systemic influences are also capable of priming the inflammatory response of the brain. The presence of activated microglia and cerebrovascular inflammation may not only lead to irreversible neuroinflammatory alterations in the brain, but probably contribute to outcome if an ischaemic event occurs. Given that the vast majority of experimental stroke studies are undertaken in “normal” animals with no underlying inflammation this might explain the lack of translation of potential treatments to the clinic.

We performed further characterisation of the vascular and cellular response in the brain of C57BL/6 and ApoE−/− mice, to explore the possible effects of atherogenic diet on neuroinflammation. Although the atherogenic “Paigen” diet alone reportedly induces inflammation in peripheral organs (Desai et al., 2008), we found significant vascular activation or enrichment of CD45-positive cells in the choroid plexus only in ApoE−/− mice fed the Paigen diet, not in C57BL/6 mice. No sign of intraluminal plaques was observed in cerebral blood vessels in our study, which is in line with a report showing increased oxidative stress and endothelial dysfunction in cerebral arterioles in high-fat fed ApoE−/− mice, but in the absence of atherosclerotic lesions (Kitayama et al., 2007).

Our results indicate that brain inflammation is associated with chronic systemic inflammation, and an atherogenic diet further augments this process. In Paigen-fed ApoE−/− mice an increase in T lymphocytes in the choroid plexus at the areas of granulocyte recruitment was seen. A recent report in experimental autoimmune

Fig. 5. Focal pathological changes are present in the brain in response to peripheral atherosclerosis. Haematoxylin & Eosin (H&E) staining (A) reveals vascular inflammation as indicated by dilated blood vessels and inflammatory infiltrates in the hypothalamus adjacent to the third ventricle in Paigen fed ApoE−/− mice. Focal lipid deposition as identified by Oil red O staining is observed in the vicinity of perivascular CD45-positive leucocytes (B). This is associated with an increase in the number of activated, Iba1-positive microglia (C) recruitment of CD45+ cells (D, red) and focally upregulated VCAM immunostaining (D, green). VCAM expression is also seen in the ipsilateral wall of the third ventricle but not in the contralateral part. Parallel brain sections from a representative brain are shown. *P < 0.05. Scale bars: 100 and 12.5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)
encephalomyelitis highlights a key role of interleukin-17-producing T helper cells in recruiting immune cells into the choroid plexus (Reboldi et al., 2009). It is intriguing to speculate, therefore, that our data also highlight the possibility that such a process may occur as a result of chronic systemic inflammation alone. Alternatively, the brain inflammation may be driven by metabolic disturbances alone without the need for systemic inflammation. Some ApoE/- mice fed a Paigen diet also displayed brain peri-vascular areas with focal lipid deposition and with microglial and vascular inflammation, similar to that seen in large peripheral blood vessels in these mice (Stoll and Bendszus, 2006; Zadelaar et al., 2007). Our data cannot confirm whether lipid deposition in the brain is a trigger of focal inflammatory changes or only a consequence of an ongoing inflammatory response. Nevertheless, we show that atherogenic diet is associated with focal inflammatory changes in the brain of animals that develop systemic vascular disease.

In summary, we demonstrate that chronic systemic inflammatory diseases, which are primary risk factors for stroke, are associated with inflammatory changes in the brain of rodents and humans. Our data support the existence of a causal relationship between systemic inflammation and brain inflammation that may contribute to stroke and other neurological disorders. An augmented inflammatory environment in the brain of stroke-prone individuals could aggravate post-ischaemic damage if stroke occurs and further studies will address this issue. Our translational approach has shown that appropriate co-morbid animal models exist that replicate important aspects of the stroke-prone state in humans, and that these co-morbid models could help facilitate translation from experimental studies to the clinic by providing a more realistic pre-clinical setting for testing novel therapies.

Conflict of interest

Prof. N. Rothwell is a non-executive director of AstraZeneca, but there was no involvement of the company in any of these studies.

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Appendix A. Supplementary data


References
