Blood–brain barrier dysfunction is implicated in various neurological conditions. Modulating the blood–brain barrier may have therapeutic value. Progress is hindered by our limited understanding of the pathophysiology of the blood–brain barrier in humans, partly due to restricted availability of human tissue, and because human tissue can only provide limited data about temporal patterns of change. We addressed these important challenges by examining surgically resected brain tissue with various lengths of interval between intracranial depth electrode-related injury and resection, and post-mortem whole brain from patients with drug-sensitive or drug-resistant chronic epilepsy and controls. In this valuable set of resources, we found that: (i) there is a highly localized overexpression of P-glycoprotein in the epileptogenic hippocampus of patients with drug-resistant epilepsy; (ii) this overexpression appears specific to P-glycoprotein and does not affect other transporters; (iii) P-glycoprotein is expressed on the vascular endothelium and end-feet of vascular glia (forming a ‘double cuff’) in drug-resistant epileptic cases but not in post-mortem controls or surgical epilepsy tissue with electrode-related injuries; (iv) an acute insult from intracranial electrode recording causes localized inflammation, increased blood–brain barrier permeability and structural changes to vasculature detectable for up to at least 330 days and (v) chronic epilepsy is associated with inflammation, enhanced blood–brain barrier permeability and increased P-glycoprotein expression. The occurrence of seizures appears central to P-glycoprotein overexpression. Our findings have potential clinical impact because they directly improve our understanding of blood–brain barrier disruption and transporter expression in humans. In particular, our findings show that the expression of P-glycoprotein in humans is compatible with the inherent assumptions of one current hypothesis of multidrug resistance, and that the specific upregulation of P-glycoprotein expression is likely to be associated with ongoing chronic seizures. There may be a therapeutic window after initial acute injury for the prevention of P-glycoprotein overexpression, and thus this one potential component of drug resistance. Our findings add to the need for careful consideration of the benefit and risks of invasive electroencephalographic recording in surgical evaluation of drug-resistant epilepsy.
Introduction

Compromise of the human blood–brain barrier is associated with several neurological disorders, including mesial temporal lobe epilepsy (Weis et al., 1996; Dallasta et al., 1999; van Vliet et al., 2007; Ryu et al., 2009). Dysfunction of the blood–brain barrier in such conditions may be causal (Dallasta et al., 1999; Seiffert et al., 2004) or consequential (van Vliet et al., 2007; Ryu et al., 2009; Eugenin et al., 2011) or both, but in any case, dysfunction of the blood–brain barrier may potentially contribute further to disease biology (Abbot et al., 2006). Dysfunction of the blood–brain barrier may be due to several reasons including inflammation, endothelial apoptosis, abnormal endothelial–glial interaction, increased expression of permeability factors, loss of tight junction proteins and/or altered expression of multidrug transporters (Wolburg et al., 2003; Abbott et al., 2006; Eugenin et al., 2011). In particular, here we investigate the dysfunction of the blood–brain barrier in patients with epilepsy because previous studies have shown that multidrug transporters normally located at the vascular endothelium, may contribute to drug resistance (Abbott et al., 2006; Lösch et al., 2011; Zhang et al., 2011).

Multidrug transporters are important in regulating the transcellular movement of various molecules across the tightly sealed blood–brain barrier. According to the transporter hypothesis of drug resistance, altered activity of multidrug transporters may mediate resistance by increased efflux and reduction of anti-epileptic drug concentration below their effective threshold at their targets (Sisodiya, 2003; Lösch and Potschka, 2005). Implicit in this hypothesis are the assumptions that there is an altered (over)expression of multidrug transporters in the brains of patients with drug-resistant epilepsy, which is not observed in drug-sensitive patients or control subjects, and that this (over)expression is localized to the seizure onset region(s). While previous studies have shown that the well-studied transporter P-glycoprotein is ectopically expressed in surgically resected specimens from patients with drug-resistant epilepsy with a variety of structural abnormalities, including hippocampal sclerosis (Tischler et al., 1995; Sisodiya et al., 1999; Dombrowski et al., 2001; Sisodiya et al., 2002; Aronica et al., 2003, 2004), there are few opportunities in humans to examine the global patterns of expression across the brain and critically, no neuropathological studies have examined multidrug transporter expression in the brains of patients with drug-sensitive epilepsy as such patients do not in general undergo neurosurgical removal of brain tissue as a treatment.

Here, we make use of opportunities provided by surgically resected tissue from patients with acute insult caused by the placement of intracranial depth electrodes and post-mortem brain tissue from patients with (drug-resistant or drug-sensitive) mesial temporal lobe epilepsy and unilateral hippocampal sclerosis (henceforth referred to as ‘epilepsy’) to examine: (i) inflammation and changes in blood–brain barrier in acute, subacute and chronic insults in epilepsy; (ii) the expression of multidrug transporters in epileptogenic and non-epileptogenic brain regions of patients with drug-resistant or drug-sensitive epilepsy to formally test the inherent assumptions of the transporter hypothesis and (iii) the neuropathological consequences of the clinical use of invasive intracranial electrode recording, which have not been studied in detail (Dallasta et al., 1999; Seiffert et al., 2004) or consequent (van Vliet et al., 2007; Ryu et al., 2009; Eugenin et al., 2011) or both, but in any case, dysfunction of the blood–brain barrier may potentially contribute further to disease biology (Abbot et al., 2006). Dysfunction of the blood–brain barrier may be due to several reasons including inflammation, endothelial apoptosis, abnormal endothelial–glial interaction, increased expression of permeability factors, loss of tight junction proteins and/or altered expression of multidrug transporters (Wolburg et al., 2003; Abbott et al., 2006; Eugenin et al., 2011). In particular, here we investigate the dysfunction of the blood–brain barrier in patients with epilepsy because previous studies have shown that multidrug transporters normally located at the vascular endothelium, may contribute to drug resistance (Abbott et al., 2006; Lösch et al., 2011; Zhang et al., 2011). Multidrug transporters are important in regulating the transcellular movement of various molecules across the tightly sealed blood–brain barrier. According to the transporter hypothesis of drug resistance, altered activity of multidrug transporters may mediate resistance by increased efflux and reduction of anti-epileptic drug concentration below their effective threshold at their targets (Sisodiya, 2003; Lösch and Potschka, 2005). Implicit in this hypothesis are the assumptions that there is an altered (over)expression of multidrug transporters in the brains of patients with drug-resistant epilepsy, which is not observed in drug-sensitive patients or control subjects, and that this (over)expression is localized to the seizure onset region(s). While previous studies have shown that the well-studied transporter P-glycoprotein is ectopically expressed in surgically resected specimens from patients with drug-resistant epilepsy with a variety of structural abnormalities, including hippocampal sclerosis (Tischler et al., 1995; Sisodiya et al., 1999; Dombrowski et al., 2001; Sisodiya et al., 2002; Aronica et al., 2003, 2004), there are few opportunities in humans to examine the global patterns of expression across the brain and critically, no neuropathological studies have examined multidrug transporter expression in the brains of patients with drug-sensitive epilepsy as such patients do not in general undergo neurosurgical removal of brain tissue as a treatment.

Here, we make use of opportunities provided by surgically resected tissue from patients with acute insult caused by the placement of intracranial depth electrodes and post-mortem brain tissue from patients with (drug-resistant or drug-sensitive) mesial temporal lobe epilepsy and unilateral hippocampal sclerosis (henceforth referred to as ‘epilepsy’) to examine: (i) inflammation and changes in blood–brain barrier in acute, subacute and chronic insults in epilepsy; (ii) the expression of multidrug transporters in epileptogenic and non-epileptogenic brain regions of patients with drug-resistant or drug-sensitive epilepsy to formally test the inherent assumptions of the transporter hypothesis and (iii) the neuropathological consequences of the clinical use of invasive intracranial electrode recording, which have not been studied in detail. We hypothesized that there would be localized blood–brain barrier vascular and inflammatory changes in epilepsy with acute and chronic insults and that there would be a higher expression of multidrug transporters in the epileptogenic, sclerotic hippocampus than in other non-epileptogenic brain regions of the drug-resistant epilepsy brains, but not in the drug-sensitive epilepsy and control brain, in support of a role for multidrug transporters in drug resistance in epilepsy.

Materials and methods

This study was approved by the Joint Research Ethics Committee of the National Hospital for Neurology and Neurosurgery/UCL Institute of Neurology; use of tissue was in accordance with UK Human Tissue Authority guidelines. Written informed consent was obtained from patients (surgically resected tissue), or relatives of donors (post-mortem tissue) as appropriate, adhering to era-appropriate standards. We studied surgically resected brain tissue from 16 patients with epilepsy who had craniotomy and intracranial depth electrode recording prior to resective surgery (Table 1). The average intracranial electrode recording period was 11 days (range 5–26; median 11 days) and the mean interval between electrode insertion and resective surgery was 130 days (range 8–330; median 72 days). We studied post-mortem brain tissue from eight control subjects with no history of any neurological disorders (Table 2), and nine patients with mesial temporal lobe epilepsy and hippocampal sclerosis, of whom six had drug-resistant epilepsy with no periods of remission (defined here as at least two seizure-free years), two had drug-resistant epilepsy with periods of remission, and one had epilepsy that had been drug-resistant, but with terminal remission (seizure-free for 20 years prior to death; Table 3). All patients with drug-resistant epilepsy had active epilepsy to death and had tried at least three anti-epileptic drugs at maximum tolerated doses. All patients with epilepsy, except Patient EP266, were previous residents at the Chalfont Centre of the Epilepsy Society. Detailed clinical and medical records were available.

Tissue sampling

Surgical brain tissue was fixed in 10% neutral buffered formalin immediately after surgery. Fixed tissue was dissected anteroposteriorly into 5 mm blocks. Blocks with intracranial depth electrode placement injury were selected for further studies. Eleven brain regions from the left and right hemispheres of each post-mortem case were examined. For Cases EP295, N5–N8 and T1, only one hemisphere was available. All brains had been immersed in 10% neutral buffered formalin before...
Table 1  Clinical details of surgical epilepsy cases with intracranial electrode injuries

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Resected brain regions with electrode insults</th>
<th>Interval between electrode insertion and extraction (days)</th>
<th>Interval between electrode insertion and surgery (days)</th>
<th>Complications after electrode recording</th>
<th>Complications after surgery</th>
<th>1-Year postoperative assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>F</td>
<td>9</td>
<td>Frontal lobe</td>
<td>8</td>
<td>300</td>
<td>None</td>
<td>None</td>
<td>Still experienced seizures</td>
</tr>
<tr>
<td>E2</td>
<td>F</td>
<td>12</td>
<td>Frontal lobe</td>
<td>10</td>
<td>180</td>
<td>None</td>
<td>None</td>
<td>Still experienced seizures</td>
</tr>
<tr>
<td>E3</td>
<td>M</td>
<td>18</td>
<td>Occipital and temporal lobes</td>
<td>9</td>
<td>270</td>
<td>None</td>
<td>None</td>
<td>Still experienced seizures</td>
</tr>
<tr>
<td>E4</td>
<td>F</td>
<td>19</td>
<td>Occipital lobe</td>
<td>5</td>
<td>330</td>
<td>None</td>
<td>None</td>
<td>Seizure-free</td>
</tr>
<tr>
<td>E5</td>
<td>F</td>
<td>22</td>
<td>Frontal and temporal lobes</td>
<td>10</td>
<td>240</td>
<td>None</td>
<td>None</td>
<td>Seizure-free</td>
</tr>
<tr>
<td>E6</td>
<td>F</td>
<td>23</td>
<td>Temporal lobe</td>
<td>10</td>
<td>10</td>
<td>None</td>
<td>None</td>
<td>Seizure-free</td>
</tr>
<tr>
<td>E7</td>
<td>M</td>
<td>25</td>
<td>Occipital lobe</td>
<td>26</td>
<td>52</td>
<td>None</td>
<td>None</td>
<td>Seizure-free</td>
</tr>
<tr>
<td>E8</td>
<td>M</td>
<td>26</td>
<td>Temporal lobe</td>
<td>11</td>
<td>11</td>
<td>None</td>
<td>None</td>
<td>Not available</td>
</tr>
<tr>
<td>E9</td>
<td>F</td>
<td>30</td>
<td>Temporal lobe</td>
<td>8</td>
<td>8</td>
<td>None</td>
<td>None</td>
<td>Seizure-free</td>
</tr>
<tr>
<td>E10</td>
<td>F</td>
<td>30</td>
<td>Temporal lobe</td>
<td>8</td>
<td>14</td>
<td>None</td>
<td>None</td>
<td>Not available</td>
</tr>
<tr>
<td>E11</td>
<td>F</td>
<td>31</td>
<td>Temporal lobe</td>
<td>10</td>
<td>10</td>
<td>None</td>
<td>None</td>
<td>Still experienced seizures</td>
</tr>
<tr>
<td>E12</td>
<td>F</td>
<td>31</td>
<td>Hippocampus</td>
<td>14</td>
<td>14</td>
<td>None</td>
<td>None</td>
<td>Still experienced seizures</td>
</tr>
<tr>
<td>E13</td>
<td>F</td>
<td>38</td>
<td>Temporal lobe</td>
<td>8</td>
<td>91</td>
<td>None</td>
<td>None</td>
<td>Still experienced seizures</td>
</tr>
<tr>
<td>E14</td>
<td>F</td>
<td>45</td>
<td>Hippocampus</td>
<td>10</td>
<td>10</td>
<td>None</td>
<td>None</td>
<td>Still experienced seizures</td>
</tr>
<tr>
<td>E15</td>
<td>F</td>
<td>47</td>
<td>Temporal lobe</td>
<td>7</td>
<td>217</td>
<td>None</td>
<td>None</td>
<td>Seizure-free</td>
</tr>
<tr>
<td>E16</td>
<td>M</td>
<td>49</td>
<td>Temporal lobe</td>
<td>14</td>
<td>325</td>
<td>None</td>
<td>None</td>
<td>Psychosis, memory loss, pain and tremor attacks</td>
</tr>
</tbody>
</table>

BBB and pharmaco-resistance in humans Brain 2012: 135; 3115–3133 | 3117

Downloaded from https://academic.oup.com/brain/article-abstract/135/10/3115/295213 on 17 February 2018
**Table 2 Clinical details of post-mortem control cases**

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Gender</th>
<th>Age at death (years)</th>
<th>Cause of death as stated in coroner’s report</th>
<th>Clinical symptomsa</th>
<th>Medications taken throughout life</th>
<th>Post-mortem brain death (years) through life</th>
<th>In coroner’s report</th>
<th>Post-mortem brain examination</th>
<th>Clinical symptoms in coroner’s report</th>
<th>Post-mortem brain examination</th>
<th>Clinical symptoms found throughout life</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>M</td>
<td>29</td>
<td>Sudden death</td>
<td>None</td>
<td>No notes available</td>
<td>Normal. No metastases</td>
<td>No notes available</td>
<td>Normal</td>
<td>Normal</td>
<td>No notes available</td>
<td>Normal. No metastases</td>
</tr>
<tr>
<td>N2</td>
<td>F</td>
<td>35</td>
<td>Cardiac arrest</td>
<td>None</td>
<td>No notes available</td>
<td>Normal. No metastases</td>
<td>No notes available</td>
<td>Normal</td>
<td>Normal</td>
<td>No notes available</td>
<td>Normal. No metastases</td>
</tr>
<tr>
<td>N3</td>
<td>F</td>
<td>37</td>
<td>Gastric carcinoma</td>
<td>Diagnosed with advanced gastric squamous cell carcinoma</td>
<td>No notes available</td>
<td>Normal. No metastases</td>
<td>No notes available</td>
<td>None</td>
<td>None</td>
<td>No notes available</td>
<td>Normal. No metastases</td>
</tr>
<tr>
<td>N4</td>
<td>M</td>
<td>57</td>
<td>Intracerebral haemorrhage</td>
<td>Diagnosed with advanced gastric squamous cell carcinoma</td>
<td>No notes available</td>
<td>Normal. No metastases</td>
<td>No notes available</td>
<td>None</td>
<td>None</td>
<td>No notes available</td>
<td>Normal. No metastases</td>
</tr>
<tr>
<td>N5</td>
<td>M</td>
<td>78</td>
<td>Gastric carcinoma</td>
<td>Diagnosed with advanced gastric squamous cell carcinoma</td>
<td>No notes available</td>
<td>Normal. No metastases</td>
<td>No notes available</td>
<td>None</td>
<td>None</td>
<td>No notes available</td>
<td>Normal. No metastases</td>
</tr>
<tr>
<td>N6</td>
<td>F</td>
<td>53</td>
<td>Adenocarcinoma of recto-sigmoid</td>
<td>Diagnosed with advanced gastric squamous cell carcinoma</td>
<td>No notes available</td>
<td>Normal. No metastases</td>
<td>No notes available</td>
<td>None</td>
<td>None</td>
<td>No notes available</td>
<td>Normal. No metastases</td>
</tr>
<tr>
<td>N7</td>
<td>M</td>
<td>57</td>
<td>Mesotheloma</td>
<td>Mesotheloma</td>
<td>No notes available</td>
<td>Normal. No metastases</td>
<td>No notes available</td>
<td>None</td>
<td>None</td>
<td>No notes available</td>
<td>Normal. No metastases</td>
</tr>
<tr>
<td>N8</td>
<td>M</td>
<td>71</td>
<td>Mesotheloma</td>
<td>Mesotheloma</td>
<td>No notes available</td>
<td>Normal. No metastases</td>
<td>No notes available</td>
<td>None</td>
<td>None</td>
<td>No notes available</td>
<td>Normal. No metastases</td>
</tr>
</tbody>
</table>

* a Seizures were not documented in any control cases.

---

**Immunohistochemistry**

Multidrug transporter and blood–brain barrier-related antibodies that labelled positive controls strongly and specifically were selected for immunohistochemical studies (Supplementary Table 2). Anti-CD34, anti-caveolin 1, anti-claudin 5, anti-CD3 and anti-CD20 antibodies are sensitive to fixation duration (Liu et al., 2010) and were employed only on surgical or post-mortem tissue fixed in formalin for less than a year. Selected antibodies were optimized for automated and manual immunohistochemistry (Supplementary Table 2).

For immunofluorescence, surgical and post-mortem sections from the sclerotic and contralateral hippocampus and ipsilateral superior temporal gyrus of epilepsy and control cases were deparaffinized in xylene and alcohol, and incubated in hydrogen peroxide, unmasking and blocking solutions as for immunohistochemistry. Sections were incubated overnight at 4°C in a primary antibody solution consisting of anti-CD34 (1:25; Dako), anti-P-glycoprotein (1600; Alexis Biochemicals), anti-glial fibrillary acidic protein (GFAP; 1:1500; Dako) or anti-human leucocyte antigen (HLA-DR; 1:100; Dako) antibody in Dako antibody diluent. On the following day, Dako EnVision™ horseradish peroxide solution was applied for 30 min before fluorescein-labelled antibody in tyramide signal amplification buffer (1:500, Perkin Elmer) was applied for 8 min. The tyramide signal amplification system is a sensitive detection system that has been used in previous studies on post-mortem human tissue (Loup et al., 1998; Thom et al., 2009). Sections were thoroughly washed before anti-fibrinogen (1:600; Dako), anti-caveolin 1 (1:200; Santa Cruz Biotech) and/or anti-GFAP (1:100, Dako) antibodies in Dako diluent were applied on sections for 1 h at room temperature. Alexa Fluor® 564 conjugated anti-rabbit (1:100; Molecular Probes, Invitrogen) and/or Alexa Fluor® 633 conjugated anti-mouse secondary antibodies (1:50; Molecular Probes, Invitrogen) in Dako diluent were incubated on sections for 3 h at room temperature. Sections were cover-slipped in DAPI-mounting media (Vector).

**Qualitative and quantitative assessments**

The first part of the study assessed the immunoreactivity of various markers in surgical epilepsy cases with depth electrode-related injuries. The second part of the study examined immunoreactivity in the post-mortem epilepsy and control cases. In both studies, all...
<table>
<thead>
<tr>
<th>Identifier</th>
<th>Gender</th>
<th>Age at death (years)</th>
<th>Responsiveness to AED a</th>
<th>Age of onset of habitual seizures (years)</th>
<th>History of febrile seizures</th>
<th>Types of seizures experienced</th>
<th>Seizure frequency 1 year before death (total seizures per month)</th>
<th>AED and other medications taken throughout lifetime b (dosage where available)</th>
<th>Cause of death as stated in coroner’s report</th>
<th>Hippocampal pathology (hemisphere)</th>
<th>Comorbidity</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP061</td>
<td>M</td>
<td>69</td>
<td>Sensitive with terminal remission (seizure-free for 20 years before death)</td>
<td>4.0</td>
<td>45</td>
<td>Not documented</td>
<td>SPS (epigastric aura), CPS (dyscognitive), SGTCS</td>
<td>No CPS and SGTCS since 1978. (0)</td>
<td>PHT (1500), PHT (500), levetiracetam, oxcarbazepine, aspirin, folic acid, benzodiazepines.</td>
<td>HS, left ventricular failure; ischaemic heart disease</td>
<td>Type 2 diabetes; left ventricular failure; ischaemic heart disease</td>
</tr>
<tr>
<td>EP199</td>
<td>F</td>
<td>69</td>
<td>Resistant with no history of remission</td>
<td>1.5</td>
<td>68</td>
<td>Yes</td>
<td>SPS (sensory R arm), CPS (with R arm dystonia), SGTCS (starting with L head version)</td>
<td>1–2 CPS/month, 6 SGTCS/year (2.5)</td>
<td>CBZ (1200), ACZ (1000), VA, PB, PHT, lamotrigine, aspirin, folic acid, vitamin D, folic acid, ranitidine, calcium, etidronate</td>
<td>HS, right Heart failure, chronic airways disease</td>
<td></td>
</tr>
<tr>
<td>EP200 b</td>
<td>M</td>
<td>79</td>
<td>Resistant with a period of remission lasting 6 years while on PB + PHT</td>
<td>0.3</td>
<td>73</td>
<td>No notes available</td>
<td>CPS</td>
<td>Infrequent seizures</td>
<td>PHT, PB</td>
<td>HS, left Congestive heart failure; hypertension</td>
<td></td>
</tr>
<tr>
<td>EP231</td>
<td>M</td>
<td>58</td>
<td>Resistant with no history of remission</td>
<td>1.3</td>
<td>57</td>
<td>No notes available</td>
<td>CPS (automotor, vocalization and vacant stare), SGTCS (no aura described)</td>
<td>CPS (automotor, vocalization and vacant stare), SGTCS</td>
<td>CBZ (1200), VA (7500), SUDEP</td>
<td>HS, left Intracerebral thalamic haemorrhage in 1999</td>
<td></td>
</tr>
<tr>
<td>EP234</td>
<td>F</td>
<td>67</td>
<td>Resistant with a period of remission lasting 9 years while on a high level of PB</td>
<td>2.5</td>
<td>56</td>
<td>No notes available</td>
<td>CPS (epigastric aura), CPS (automotor), SGTCS (no aura described)</td>
<td>2–6 CPS/month, 1–2 SGTCS/month (8)</td>
<td>CBZ (325), CBZ (VA, OX (900), CLB)</td>
<td>HS, left Learning disability</td>
<td></td>
</tr>
<tr>
<td>EP266 b</td>
<td>M</td>
<td>38</td>
<td>Resistant with no history of remission</td>
<td>7.0</td>
<td>31</td>
<td>No notes available</td>
<td>No notes available</td>
<td>No notes available</td>
<td>CBZ, TPM, CLB</td>
<td>HS, left Learning disability</td>
<td></td>
</tr>
<tr>
<td>EP286</td>
<td>M</td>
<td>32</td>
<td>Resistant with no history of remission</td>
<td>1.1</td>
<td>31</td>
<td>Yes</td>
<td>CPS (automotor, vocalization and vacant stare), SGTCS</td>
<td>1–2 CPS/month, 5–6 SGTCS/month (8)</td>
<td>OXG (1350), VA (7500), CLB (10), CBZ, ACZ, VA, PB, PHT, lamotrigine, oxcarbazepine, aspirin, folic acid, vitamin D, ranitidine, calcium, alendronate</td>
<td>HS, left Learning disability</td>
<td></td>
</tr>
<tr>
<td>EP295</td>
<td>F</td>
<td>50</td>
<td>Resistant with no history of remission</td>
<td>5.0</td>
<td>45</td>
<td>No notes available</td>
<td>SPS (epigastric aura; autonomic aura), CPS (automotor), SGTCS</td>
<td>1–2 CPS/month, 1 SGTCS/month (3)</td>
<td>CBZ (1000), PB (10), LEV (5000), NO, VA, PB, PHT, lamotrigine, oxcarbazepine, aspirin, folic acid, vitamin D, ranitidine, calcium, alendronate</td>
<td>HS, left Psychosis</td>
<td></td>
</tr>
</tbody>
</table>

(continued)
immunolabelled sections were qualitatively examined under a light microscope (Nikon Eclipse 80i) using ×10, ×20 and ×60 objective lenses.

For quantitative studies, the region of interest was outlined using image analysis software (ImagePro Plus, Media Cybernetics) under ×2.5 objective. For surgical tissue, we were interested in multidrug transporters and the blood–brain barrier-related changes in the vicinity of electrode insertion, so the regions of interest were the intracranial electrode placement track or the area of injury, where tissue was missing or a coagulum had formed, the immediate area of immunopositivity surrounding the area of injury, and a similar-sized area of normal-appearing, undamaged cortex in an adjacent gyrus. For post-mortem tissue, we were interested in multidrug transporters and blood–brain barrier-related changes in the epileptogenic compared to non-epileptogenic regions, so the regions of interest outlined were the grey matter of the superior temporal gyrus and the hippocampal head, inclusive of the dentate gyrus and all cornu ammonis subfields. Images were acquired in a random systematic sequence within the region of interest under ×20 objective using a CCD camera (JVC). Random systematic sampling of 10% of the region of interest gave similar results to sampling 80% (Supplementary Fig. 1). The percentage of immunopositive labelling did not differ between sections taken at different depths (z-axis) within the same tissue block. All images of a given case were acquired and processed in the same run. Positive immunolabelling was segmented (thresholded) using the ColorCube module of the ImagePro Plus software (Media Cybernetics, Inc.): positive labelling was manually selected by centring a 5 × 5 pixel selection grid on the darkest pixel in the image with the highest sensitivity option selected. The final results for each region of each case were expressed as the percentage area of the region of interest positively immunolabelled per case per marker. The mean number of immunolabelled structures per area (µm^2), and the mean area (µm^2), width (µm) and length (µm) per immunolabelled structure within each region of interest was also measured and recorded, as appropriate, for surgical tissue. Sample numbers were small. The non-parametric paired-sample Wilcoxon test (SPSS 17.0 statistical software) was used to identify significant differences between groups. Correction for multiple comparisons was applied (corrected significance P < 0.004).

Correlation analysis was performed using the Spearman rank correlation test (SPSS 17.0) with significance level at P < 0.05. Bonferroni correction for multiple comparisons was applied to surgical (P < 0.01) and post-mortem data (P < 0.008). Correlation was tested between percentage area of immunopositive labelling and age, gender, interval between electrode insertion and extraction, interval between electrode insertion and surgery, and the location of electrode tissue injuries. For post-mortem cases, correlation was assessed between the percentage difference of immunolabelling in the sclerotic hippocampus and/or the contralateral hippocampus and superior temporal gyrus and age, age at onset of habitual seizures, duration of seizure history, seizure frequency per year and the number of P-glycoprotein inducers or substrates taken throughout life. Medications were categorized as P-glycoprotein inducers, substrates or inhibitors based on findings from previous studies (Supplementary Table 3).

Immunofluorescent labelled sections were viewed under a confocal laser scanning microscope (Zeiss LSM610 Meta) equipped with blue diode (405 nm), argon (458, 477, 488, 514 nm) and helium/neon (546 and 633 nm) lasers. For immunofluorescent quantitative studies, the hippocampal head or the grey matter of the superior temporal gyrus was outlined. Ten sets of stacked images were taken inside the outlined region using a ×40 oil-immersion objective lens. Each set of stacked images consisted of four optical slices through the z-axis in
Results

Surgical tissue with depth electrode-related injuries

Haematoxylin and eosin, and Luxol fast blue stains show 4/16 surgical cases had electrode-related injuries to the surface of the cortex (Fig. 1A), 10 had injuries to the deep cortical layers or white matter (Fig. 1B) and two had injuries to the hippocampus. We found that injuries to two of the four cases with superficial cortical injuries might have been due to subdural grids rather than depth electrodes, so these two cases were removed from quantitative analysis. Electrode-related injuries had missing tissue, or were either haemorrhagic with leakage of red blood cells and infiltration of white blood cells, or in the form of an acellular coagulum surrounded by white blood cells. Neuronal loss was noted at all sites of electrode-related injury.

Immunohistochemistry using various markers was then conducted and the immunopositive labelling at the electrode-related injury sites, and the adjacent normal cortex, of all surgical cases was examined. We intended to investigate inflammation, blood–brain barrier and multidrug transporter changes in response to electrode track injury only, partly as a way of establishing the time course of alterations in these parameters after a defined insult. In the epileptogenic target of electrodes, such as the hippocampus, epileptogenicity itself could have altered these parameters. We therefore excluded such regions from our analysis.

Increased vasculature in the injury site

The blood vessel markers, anti-CD34 (P = 0.001) and anti-caveolin 1 (P < 0.001), showed a significantly higher percentage area labelling at the injured site than in the adjacent normal-appearing cortex of each surgical case (Fig. 1C). CD34 immunoreactivity was limited to the endothelium of blood vessels in all regions. CD34-immunopositive blood vessels at the injured sites of all surgical cases often formed clusters and appeared smaller and shorter than immunopositive vessels in the adjacent normal cortex (Fig. 1D), but a significant difference in the size of individual CD34-immunopositive structures in the injured sites compared with the adjacent normal cortex was not noted. Caveolin 1-immunopositive vessels at the injured sites of all surgical cases were long, and had immunopositive glial-like processes projecting from the endothelium (Fig. 1E), which were not observed in the adjacent normal cortex (Fig. 1F). At the injury sites of all surgical cases, caveolin 1-immunopositive multipolar cells were also observed, but were not seen in adjacent normal cortex.

The percentage area of claudin 5 (an endothelial tight junction protein) immunopositivity was marginally not significantly higher in the injured sites compared to the adjacent cortex (Fig. 1C; P = 0.005). Claudin 5-immunopositive vessels at injured sites had an over-expression of claudin 5 (Fig. 1F(i) and(ii)). In the adjacent normal cortex of all surgical cases, claudin 5 expression was limited to vascular endothelium only.

No change was observed in the expression of multidrug transporters in the injury site compared with adjacent cortex

The percentage area of immunoreactivities of the multidrug transporter markers, anti-P-glycoprotein (P = 0.414) and anti-breast cancer resistance protein (BCRP; P = 0.363) was not significantly different between the injured site and the adjacent normal cortex (Fig. 1C). P-glycoprotein (Fig. 1G) and BCRP immunoreactivity (Fig. 1H) were observed strictly in vessel endothelium in both injured sites and adjacent normal cortex, with no marked difference between the two regions. The ratio of percentage area of claudin 5, P-glycoprotein and BCRP immunoreactivities to the percentage area of CD34 immunoreactivity was near 1:1 in the normal adjacent cortex (Fig. 1I). The ratio of percentage area of caveolin 1 to CD34 immunoreactivity was near 2:1 in the normal adjacent cortex, which suggests that caveolin 1 is normally expressed in a larger area than CD34 in blood vessels. In the injured region, the ratio of percentage area of claudin 5, P-glycoprotein and BCRP immunoreactivities to CD34 was slightly lower than ratios observed in the normal adjacent cortex. This suggests that the expression of claudin 5, P-glycoprotein and BCRP may not be as widely distributed as CD34 in new vessels at the injured site. Certainly, there was no spatial overexpression of P-glycoprotein in or around vessels at or near the electrode tracks.

The expression of another well-known multidrug transporter, multidrug resistance related protein 1 (MRP1, now known as ABCG2), was predominantly observed in glial-like cells in cortical layer I and white matter of surgical epilepsy cases. MRP1-immunopositive glial cells and blood vessels were also observed throughout the cortex, and they were not seen to be upregulated in number in the area near the electrode tracks.

Upregulation of inflammatory mediators at the injury site

The injury sites of all surgical cases were infiltrated by darkly labelled, large, CD68-immunopositive reactive microglial cells with amoeboid morphology and retracted processes, which were not, or only occasionally, observed in adjacent normal cortex (Fig. 2A). CD68 is expressed in human monocytes and macrophages. The percentage area of CD68 immunolabelling (P < 0.001), and the mean μm² per CD68-immunopositive structure (P = 0.002) were significantly higher in injury sites than in adjacent normal cortex for all surgical cases (Fig. 1C). The spread of CD68 immunoreactivity was, on average, 13 times greater than the area of injury (Fig. 1C). In contrast, immunolabelling with lymphocyte markers (anti-CD3 and -CD20 antibodies) revealed few immunopositive cells at the injury sites and no...
**Figure 1** Vascular structures in the electrode-injured site of surgical cases. (A and B) Haematoxylin and eosin (H + E) stain showing electrode-related injuries at the superficial cortex of Case E4 (A) and at the hippocampus of Case E7 (B). Arrows point to the injury site. (C) Quantitative findings from the immunohistochemical study. Only surgical cases with depth electrode-related injuries were included in the analysis. Parameters refer to the average percentage (%) area or area (μm²) of immunoreactivity across all surgical cases with depth electrode-related injuries. Z is a value from the Wilcoxon test; #area (μm²) of injury or spread of immunoreactivity of respective markers. (D) Anti-CD34 immunopositive blood vessels often clustered at the electrode-injured site, but not in the adjacent normal cortex.
immunopositive cells in the adjacent, normal cortex of surgical cases with electrode-related injuries. The injured sites of all surgical cases were also surrounded by a dense matrix of GFAP- (Fig. 2B) and connexin 43 (Cx43)-immunopositive astrocytes and processes (Fig. 2C), which were comparably fewer in the adjacent normal cortex. GFAP is an established marker of astrocytes. Cx43 is a gap junction protein, expressed mostly on astrocytes and is considered to be associated with the spread of inflammation and/or seizures after acute brain injury (Fonseca et al., 2002; Collignon et al., 2006; Cronin et al., 2008; Chew et al., 2010).

Immunoreactivity for the serum proteins, albumin and fibrinogen, was seen in parenchyma at or around the injured sites of all cases. The area of blood–brain barrier leakage as marked by such immunoreactivity significantly exceeded the area of injury in all surgical cases (P < 0.000, albumin and fibrinogen). The spread of albumin and fibrinogen immunoreactivities was 30 and 31 times larger than the area of the injury, respectively (Fig. 1C). Within the

Figure 2

Immunoreactivities of anti-CD68, anti-GFAP, anti-Cx43 and anti-fibrinogen. (A) The injury site of a surgical case, Case E9, showed a high number of intensely labelled anti-CD68 positive cells. Anti-CD68 immunopositive cells were only occasionally observed in the adjacent normal cortex. (B) A dense matrix of anti-GFAP immunopositive cells and processes cells were shown in areas immediately surrounding the injury site. In contrast, small, individual anti-GFAP immunopositive cells were seen throughout the adjacent normal cortex. (C) Anti-Cx43 immunolabelling was strongly observed in the injury site but not in the adjacent normal cortex. (D) A surgical case showed intense, anti-fibrinogen (FR) immunoreactivity at the injured site and in the immediate parenchymal area around the injured site. Within the darkly labelled parenchymal area at the injured site, anti-fibrinogen immunoreactivity was found in neurons, glia and blood vessels. The extravasation of fibrinogen was confirmed by triple label immunofluorescence, which showed anti-fibrinogen immunopositive labelling outside anti-P-glycoprotein (E), or anti-CD34 (F) immunopositive blood vessels and inside anti-HLA-DR microglial cells (G, arrows). Scale = 100 μm (A–C), 25 μm (D); 20 μm (E–G).

Figure 1 Continued

(E) At the injured site, anti-caveolin 1 immunoreactivity was observed in the vascular endothelium and also in glial-like ‘off-shoots’ near to the endothelium. In contrast, anti-caveolin 1 immunopositive labelling was restricted only to the endothelium of blood vessels in the adjacent normal cortex. (F) Case E4 showed over- (i) or distorted expression (ii, *missing region) of anti-claudin 5 immunopositive labelling in the injury site. In contrast, anti-claudin 5 immunolabelling was found only in the endothelium of blood vessels in the adjacent normal cortex. (G) Anti-P-glycoprotein (Pgp) immunoreactivity was only shown in vascular structures in the injured site and the adjacent normal cortex. (H) Similar to anti-P-glycoprotein immunolabelling, anti-BCRP immunoreactivity was only found in vascular structures in the injured site and adjacent normal cortex. (I) Ratios between the percentage area of immunoreactivity of respective antibodies and anti-CD34. Sterr = standard error of means. Scale = 100 μm (A and B), 50 μm (D–H).
spread, there were albumin and fibrinogen-immunopositive vessels, neurons and small gial cells (Fig. 2D). Triple-labelled immunofluorescent studies confirmed fibrinogen immunoreactivity outside P-glycoprotein- (Fig. 2E) or CD34-immunopositive blood vessels (Fig. 2F), and inside some GFAP- or HLA-DR-immunopositive gial cells at the injury sites (Fig. 2G), confirming breached blood–brain barrier integrity. Apart from five cases that showed albumin and fibrinogen immunoreactivities in the parenchyma, neurons or glia of the adjacent normal cortex, all other cases had albumin and fibrinogen immunoreactivities only within blood vessels in the normal cortex.

The interval between intracranial electrode insertion and extraction was associated with the extent of fibrinogen immunoreactivity at the injured sites ($R = 0.69$, $P = 0.004$). The relationships between other clinical factors and immunoreactivities of various markers did not reach significance.

**Post-mortem tissue**

**No changes in vasculature in the hippocampus of drug-resistant or drug-sensitive patients or in controls**

Anti-CD34, anti-caveolin 1 and anti-claudin 5 antibodies labelled vascular structures in all short-fixed, post-mortem epilepsy and control tissue. Unlike surgical tissue with electrode-related tissue injuries, no distinctive clustering of CD34-immunopositive blood vessels or caveolin 1-immunopositive glial cells was seen in sclerotic or contralateral hippocampus of any post-mortem epilepsy or control cases.

**P-glycoprotein is expressed throughout the human brain**

P-glycoprotein immunolabelling was seen predominantly in blood vessels in all regions of all post-mortem cases (Fig. 3A). The anti-P-glycoprotein antibody also labelled many small, gial-like cells in the cortical subpial layer, white matter and hippocampus of all epilepsy cases (Fig. 3B). A small number of P-glycoprotein-immunopositive glial-like cells were located in these areas in controls. The sclerotic hippocampus of all drug-resistant epilepsy cases had P-glycoprotein-immunopositive vascular structures that were surrounded by a halo of diffuse P-glycoprotein-immunopositive labelling (Fig. 3C and D), which was not as strongly or as often seen in the contralateral, non-sclerotic hippocampus or other brain regions of drug-resistant epilepsy cases or any brain regions of drug-sensitive epilepsy or control cases. In one post-mortem, drug-resistant epilepsy case (Case EP200), darkly labelled, P-glycoprotein-immunopositive deposits were observed along P-glycoprotein-immunopositive vascular endothelium of all regions (Fig. 3E).

Quantitated P-glycoprotein expression was not directly compared between cases as variations in immunolabelling were known to exist between post-mortem cases; instead, we compared normalized levels of P-glycoprotein labelling (i.e. percentage area of P-glycoprotein immunopositivity) between different regions for each case and sought consistent trends in regional differences. Drug-resistant epilepsy cases showed a higher and a broader range of percentage area of P-glycoprotein-immunopositive labelling across all 11 brain regions compared with drug-sensitive epilepsy cases and controls (Fig. 3F). The percentage area of P-glycoprotein immunopositivity was highest in frontal, parietal and/or occipital neocortices and lowest in mesial temporal structures within each case. The regional difference in P-glycoprotein expression might be influenced by the varying vascularity in each brain region: a previous study reported that cortical grey matter had a higher vessel density compared with cortical white matter, cerebellar grey and white matter or the globus pallidus (Dallasta et al., 1999). Normalization of P-glycoprotein data for local vascular density was not possible in our study because all the blood vessel markers examined (anti-CD34, anti-caveolin 1, anti-claudin 5 and anti-von Willebrand factor antibodies) are sensitive to duration of fixation (Liu et al., 2010). We performed anti-CD34 immunohistochemistry on two epilepsy cases and four controls with short post-mortem delay and fixation duration and found that the sclerotic hippocampus and ipsilateral temporal cortex (or left hippocampus and temporal cortex) had similar percentage area of CD34-immunopositive labelling (Fig. 3G). Thus, changes in temporal lobe P-glycoprotein immunoreactivity in this study were unlikely to be due to a regional difference in vascularity.

**Focal increased expression of P-glycoprotein in the drug-resistant, epileptogenic hippocampus**

We compared quantified P-glycoprotein immunoreactivity between the epileptogenic, sclerotic hippocampus and non-epileptogenic, undamaged brain regions that had a similar level of vascular density, such as the contralateral hippocampus and superior temporal gyrus of both hemispheres of all epilepsy cases, and between the left and right hippocampus and superior temporal gyrus of all control cases. All drug-resistant epilepsy cases with no history of remission showed a higher percentage area of P-glycoprotein-immunopositive labelling in the sclerotic hippocampus than in the contralateral hippocampus, and ipsilateral and contralateral superior temporal gyrus, while an average of 38% of control cases showed a higher percentage area of P-glycoprotein-immunopositive labelling in the left hippocampus than the right hippocampus (2/4 control cases), and left (3/8 control cases) and right superior temporal gyrus (1/4 control cases; Fig. 4A–C). We did not find the pattern of P-glycoprotein-immunopositive labelling to be different between different hippocampal subfields of either epilepsy cases or controls. The amount of asymmetry (percentage difference of P-glycoprotein-immunopositive labelling) between sclerotic hippocampus and contralateral hippocampus, ipsilateral or contralateral superior temporal gyrus was greater in drug-resistant epilepsy cases with no history of remission compared with control cases: range of percentage difference between both hippocampi, epilepsy 74–600%, control −18 to 17%; between sclerotic hippocampus and ipsilateral superior temporal gyrus in epilepsy 104–733%, left hippocampus and superior temporal gyrus in controls −40 to 74%; between sclerotic hippocampus and contralateral superior temporal gyrus in epilepsy 96–269%; left hippocampus and right superior temporal gyrus in controls −24 to 20%.

The two drug-resistant epilepsy cases with periods of remission and the drug-sensitive epilepsy case with terminal remission had a smaller increase of percentage area of P-glycoprotein immunopositive labelling in the sclerotic hippocampus than in the
Figure 3 Anti-P-glycoprotein immunolabelling in the post-mortem human brain. (A and B) Anti-P-glycoprotein antibody labelled blood vessels and small, glial-like cells in the subpial, white matter and hippocampus of epilepsy tissue. (C and D) In the sclerotic hippocampus of a drug-resistant epilepsy case, anti-P-glycoprotein immunopositive vascular structures (BV) appeared to be surrounded by two ‘barriers’ of anti-P-glycoprotein immunopositive labelling; the inner barrier (arrowheads) and the more diffuse, outer ‘cuff’ (arrows). Box in C is shown magnified in D. (E) In one drug-resistant post-mortem case with epilepsy, Case EP200, darkly labelled, anti-P-glycoprotein immunopositive

(continued)
contralateral hippocampus, ipsilateral or contralateral superior temporal gyrus. The amount of asymmetry in these cases was clearly less than that observed in drug-resistant epilepsy cases with no history of remission, and more consistent with the range of percentage difference shown by control cases: range of percentage difference between sclerotic hippocampus and contralateral hippo- 
campus, ipsilateral superior temporal gyrus and contralateral superior temporal gyrus.

**P-glycoprotein and clinical factors**

We investigated whether the amount of asymmetry or the percentage difference of P-glycoprotein-immunopositive labelling between the sclerotic hippocampus and contralateral hippocampus or superior temporal gyrus correlated with the clinical factors listed in Tables 2 and 3 (refer to the ‘Materials and methods’ section). Correction for multiple comparisons was applied (corrected threshold of significance \( P < 0.008 \)).

The percentage difference of P-glycoprotein-immunopositive labelling between the sclerotic hippocampus and contralateral

**Figure 4** Quantification of anti-P-glycoprotein immunolabelling in the hippocampus and temporal cortex of drug-resistant and drug-sensitive epilepsy and control cases. (A–C) A higher percentage area of anti-P-glycoprotein immunopositive labelling was observed in the sclerotic hippocampus (HPs) than the contralateral hippocampus (HPc, A), the superior temporal cortex ipsilateral (STGi, B) or contralateral (STGc, C) of all cases with drug-resistant epilepsy with no history of remission. The percentage increase in percentage area of anti-P-glycoprotein immunopositive labelling in the five cases with drug-resistant epilepsy with no remission ranged from 74% to 600% between sclerotic hippocampus and contralateral hippocampus; from 104% to 733% between sclerotic hippocampus and ipsilateral superior temporal cortex; and from 96% to 269% between sclerotic hippocampus and contralateral superior temporal cortex. Pgp = P-glycoprotein.

**Figure 3** Continued

deposits (arrows) were evident along the immunopositive labelled vascular endothelium of all regions. Scale = 50 μm (A and B), 100 μm (C), 10 μm (D and E). (F) Each box and whisker plot shows the range of percentage area of anti-P-glycoprotein immunopositive labelling in 11 brain regions of each case. Drug-resistant epilepsy cases had the highest and broadest range of percentage area of anti-P-glycoprotein immunopositive labelling compared with drug-sensitive epilepsy and control cases. GCL = granule cell layer; mol = molecular layer.
hippocampus ($R = 0.68, P = 0.007$) or ipsilateral superior temporal gyrus ($R = 0.71, P < 0.001$) or contralateral superior temporal gyrus ($R = 0.77, P = 0.001$) of all cases increased with the number of medications taken throughout life that are P-glycoprotein-inducers. There is ongoing debate as to which anti-epileptic drugs are P-glycoprotein-inducers. We considered that phenytoin, carbamazepine, phenobarbital and valproate are P-glycoprotein-inducers based on previous studies (Supplementary Table 1). The percentage difference of P-glycoprotein-immunopositive labelling between the sclerotic hippocampus and ipsilateral superior temporal gyrus ($R = 0.71, P < 0.001$) of all cases also increased with monthly seizure frequency (includes all types of seizures experienced). No other significant relationships were observed. Patients with higher seizure frequency may have been exposed to more anti-epileptic drugs.

**P-glycoprotein expression on peri-vascular glia**

P-glycoprotein-immunopositive vascular structures in the sclerotic hippocampus of drug-resistant epilepsy cases were sometimes surrounded by a diffuse halo of P-glycoprotein-immunopositive labelling, forming a Type 2 or ‘double cuff’ pattern of immunoreactivity. Blood vessels are often surrounded by end-feet of astrocytic glial cells (Abbott et al., 2006). To characterize P-glycoprotein distribution further, we used immunofluorescent techniques and antibodies against P-glycoprotein, caveolin 1 (blood vessel marker), glial fibrillary acidic antigen (GFAP, astrocyte marker) and the nuclear stain, DAPI, on the sclerotic hippocampus, contralateral hippocampus and superior temporal gyrus in two, randomly chosen, drug-resistant epilepsy cases with no history of remission (Cases EP266, EP286), one drug-resistant epilepsy case with periods of remission (Case EP234), the drug-sensitive epilepsy case and two controls (Controls N2, N3).

Type 1 vessels had distinct P-glycoprotein-immunopositive labelling at the luminal surface of the vascular endothelial cells, which co-labelled with anti-caveolin-1 in all cases (Fig. 5A). Type 2 vessels (or ‘double cuff’ vessels) had an additional ‘cuff’ of P-glycoprotein-immunolabelling at the periphery of the blood vessel which co-labelled with anti-GFAP but not anti-caveolin-1 (Fig. 5B and C). We counted the first 50 blood vessels per region per case to determine the proportion of Type 1 and Type 2 blood vessels (Fig. 5D). The majority of blood vessels in the sclerotic hippocampus of drug-resistant epilepsy cases with no history of remission were Type 2 ‘double cuffs’ (proportion of Type 2 vessels = 86% in Case EP266, 78% in Case EP286) while most blood vessels in the contralateral hippocampus and the ipsilateral superior temporal gyrus were Type 1 (proportion of Type 1 vessels = 53% contralateral hippocampus, 92% ipsilateral superior temporal gyrus for Case EP266; 92% contralateral hippocampus, 100% ipsilateral superior temporal gyrus for Case EP286). In contrast, almost all blood vessels in the sclerotic hippocampus and contralateral hippocampus and ipsilateral superior temporal gyrus of the drug-resistant epilepsy case with periods of remission, the drug-sensitive epilepsy case and the left and right hippocampus and left superior temporal gyrus in control cases were Type 1.

No consistent trends in breast cancer resistance protein expression in drug-resistant epilepsy

Anti-BCRP antibody labelled vascular structures in all brain regions of all cases (Fig. 6A). The percentage area of BCRP-immunopositive labelling was noticeably different across brain regions of all cases, which as for P-glycoprotein immunolabelling, might be due to different vascular densities in each region. The range of percentage area of BCRP-immunopositive labelling was not markedly different between drug-resistant and drug-sensitive epilepsy cases and controls (Fig. 6B). The number of cases that showed a higher percentage area of BCRP-immunopositive labelling in the sclerotic hippocampus (or left hippocampus in controls) than in the contralateral hippocampus and superior temporal gyrus was similar between epilepsy cases and controls (average percentage of cases, 58% epilepsy; for controls, 50%; Fig. 6C). Of those cases that showed asymmetry of BCRP-immunopositive labelling, the range of percentage difference of BCRP-immunopositive labelling between sclerotic hippocampus and contralateral hippocampus or ipsilateral superior temporal gyrus was not obviously different between epilepsy cases and controls (range of percentage difference, −3 to 500% mesial temporal lobe epilepsy; −2 to 225% controls).

**Upregulation of multidrug resistance related protein 1**

MRP1 immunolabelling was sensitive to long-term formalin fixation. Therefore, MRP1-immunolabelling was only qualitatively assessed. We found many intensely labelled MRP1-immunopositive glial cells and processes in the sclerotic hippocampus, contralateral hippocampus and the subpial layer of ipsilateral and contralateral superior temporal gyrus of drug-resistant and drug-sensitive epilepsy cases (Fig. 6D and E). In contrast, no or only weak MRP1-immunopositive labelling was observed in the left and right hippocampus and temporal cortex of control cases. Triple-labelled immunofluorescence experiments using anti-MRP1, anti-GFAP and DAPI showed that MRP1-immunopositive labelling around blood vessels in the sclerotic hippocampus of epilepsy cases co-localized with anti-GFAP (Fig. 6F).

**Strong presence of inflammatory mediators and blood–brain barrier leakage in cases with epilepsy**

GFAP-immunopositive astrocytes (Fig. 7A), HLA-DR-immunopositive microglial cells (Fig. 7B) and Cx43-immunopositive glial-like cells (Fig. 7C) were more intensely immunolabelled in the sclerotic hippocampus than in the contralateral hippocampus or superior temporal gyrus of all drug-resistant and drug-sensitive epilepsy post-mortem cases. In control cases, there were a few small GFAP- and HLA-DR-immunopositive cells in the CA4 region of the control hippocampus and in the superior temporal gyrus. No Cx43-immunopositive cells were observed in the control hippocampus and only a few Cx43-immunopositive cells were observed in the subpial layer of the control superior temporal gyrus.

Albumin- and fibrinogen-immunopositive blood vessels were observed in all regions of drug-resistant and drug-sensitive epilepsy and control post-mortem cases. Albumin- and fibrinogen-immunopositive neurons and glial cells, and immunoreactivities in the parenchyma, were more frequently observed in drug-resistant and drug-sensitive epilepsy cases than in controls (Fig. 7D).
Figure 5  Characterizing P-glycoprotein expression in the hippocampus and temporal cortex of drug-resistant and drug-sensitive epilepsy and control cases. (A–C) Quadruple-label immunofluorescence using markers of P-glycoprotein (anti-Pgp), blood vessels (anti-caveolin 1), astrocytes (anti-GFAP) and the nuclear stain, DAPI showed P-glycoprotein expression in the anti-caveolin-1 immunopositive vascular endothelium (white arrows, Type 1) and on the anti-GFAP immunopositive endfeet of astrocytes (pink arrows, Type 2). Type 2 blood vessels had a ‘double cuff’ or barrier of P-glycoprotein (pink arrows) around the vessels. Scale = 10 μm (A–C). (D) Of the first 50 vessels examined per region per case, an average of > 78% of blood vessels had Type 2 morphology in the sclerotic hippocampus (HPs) of drug-resistant epilepsy cases. In contrast, a higher proportion of Type 1 blood vessels than Type 2 were evident in the undamaged, contralateral hippocampus (HPc) and ipsilateral superior temporal cortex (STGi) of the drug-resistant epilepsy cases and all regions of the drug-resistant epilepsy case with a history of remission, drug-sensitive epilepsy case and control cases. HPI = left hippocampus; HPr = right hippocampus.
Figure 6 Anti-BCRP and anti-MRP1 immunopositive labelling in epilepsy and control post-mortem cases. (A) Anti-BCRP labelled vascular structures in the epilepsy and control hippocampus. Scale = 50 μm. (B) The range of percentage area of anti-BCRP immunopositive labelling in different brain regions of the epilepsy brains was similar to control cases. (C) A higher percentage increase in anti-BCRP immunopositive labelling between sclerotic hippocampus (HPs) and contralateral hippocampus (HPc) or ipsilateral superior temporal cortex (STGi) was not observed in all drug-resistant epilepsy cases. (D and E) Anti-MRP1 immunopositive glial-like cells were evident in the sclerotic hippocampus and subpial layer of the superior temporal cortex of all cases with epilepsy. In contrast, only weak anti-MRP1 immunoreactivity was evident in the hippocampus and temporal cortex of post-mortem controls. Scale = 100 μm. (F) Immunofluorescent labelling using antibodies against MRP1, GFAP and DAPI revealed many MRP1 and GFAP-immunopositive co-labelled cells and processes in the sclerotic hippocampus of epilepsy cases. Scale = 20 μm. BV = blood vessel; CA = cornu ammonis; GCL = granule cell layer.
We investigated the relationship between human blood–brain barrier disruption and anti-epileptic drug resistance using surgical and post-mortem human brain tissue from patients with epilepsy. We showed that: (i) there is a highly localized overexpression of P-glycoprotein in the epileptogenic hippocampus of patients with drug-resistant epilepsy, which is compatible with the current hypothesis of multidrug resistance; (ii) this overexpression appears specific to P-glycoprotein and does not affect other transporters, such as BCRP and MRP1; (iii) the ‘double cuff’, Type 2, expression of P-glycoprotein is likely to be associated with ongoing, chronic seizures, being absent in sustained remission; (iv) a single, acute insult from intracranial electrode recording causes localized inflammation, increased blood–brain barrier permeability and structural changes to blood vessels (such as the irregular expression of claudins and caveolins), but not P-glycoprotein overexpression; (v) exposure to P-glycoprotein inducers is associated with increased P-glycoprotein expression in human brain, though the number of cases studied is small; and (vi) chronic epilepsy is associated with inflammation, enhanced blood–brain barrier permeability and increased P-glycoprotein expression, particularly in perivascular gliia of the epileptogenic hippocampus. We are aware that our study is a cross-sectional study, necessarily limited to small case numbers, and requiring the statistical comparisons to be interpreted cautiously. Fixed autopsy materials also restrict the type of experiments that may be performed. Without functional studies, we cannot conclude that P-glycoprotein causes drug resistance, but our data support assumptions implicit in the transporter hypothesis and give it further strong plausibility. Our study also provides information on the distribution and location, at high resolution, of P-glycoprotein in controls and brain tissue from drug-resistant and drug-sensitive mesial temporal lobe epilepsy, which will be useful for in vivo imaging studies of P-glycoprotein (Bauer et al., 2012). We have also examined and quantified various aspects of blood–brain barrier disruption and multidrug transporter expression in the same human tissue across the whole brain from patients with drug-resistant and drug-sensitive epilepsy, and we have explored the temporal relationship of blood–brain barrier disruption and multidrug transporter expression in response to a specific insult, neither of which have been investigated in previous human studies due to limited tissue resources. Thus, our findings have potential clinical impact because they directly improve our understanding of blood–brain barrier disruption and transporter expression in humans.

Blood–brain barrier dysfunction may contribute to drug resistance in epilepsy. Evidence comes from animals studies (Marchi et al., 2006; van Vliet et al., 2007) and from human studies using surgically resected specimens from patients with drug-resistant epilepsy, which show plasma leakage and increased expression of multidrug transporters at the blood–brain barrier (Tishler et al., 1995; Sisodiya et al., 2002, 2006; Aronica et al., 2004, 2005; van Vilet et al., 2007), and this has contributed to the ‘transporter hypothesis’ of drug resistance. It has been challenging to progress further partly due to the limited availability of human tissue, especially from patients with drug-sensitive epilepsy, and the lack of appropriate controls (Lösch and Potschka, 2005), and also because, in general, human tissue can only provide limited data about temporal patterns of change. Findings from animal and in vitro studies in blood–brain barrier disruption and drug resistance (Abbott et al., 2006), although valuable, still require confirmation in the human brain. We acknowledge that
electrode-related injury and chronic epilepsy may induce different severity and types of brain response, but the use of surgical tissue with electrode-related injuries provides an opportunity to examine blood-brain barrier integrity, inflammation and P-glycoprotein expression at known intervals after a defined, sterile initial insult. Electrode placements with a defined follow-up (i.e. up to 30 days after injury) may lead to inflammation, blood-brain barrier damage and angiogenesis; new vessels have a reduced expression of P-glycoprotein and BCRP per vessel, and this localized insult is not sufficient to generate blood-brain barrier leakage and inflammation more widely, nor the ‘double cuff’ expression pattern of P-glycoprotein around blood vessels seen in chronic active epilepsy. This suggests a long history of accumulated insults, such as persistent seizures, local inflammation and breaches of blood-brain barrier, may be required to upregulate P-glycoprotein expression in epileptogenic brain regions. Thus, there may be a therapeutic window after an initial acute injury (at least of this type) that may be of use in prevention of increased P-glycoprotein expression and thus, potentially, prevention of at least one putative mechanism of development of drug resistance. A more comprehensive synthesis of our results is also possible. The asymmetrical increase in Type 2 ‘double cuff’ redistribution of P-glycoprotein is seen in brain tissue from patients who had active epilepsy up to the point of death. Indeed, we found that a higher seizure frequency is associated with increased asymmetry of P-glycoprotein expression in the hippocampus. This ‘double cuff’ pattern of P-glycoprotein expression is seen in a specific distribution in epileptogenic rather than other brain regions, is not seen in the brain tissue of people with the same type of epilepsy who have been seizure-free at death, in brain tissue of people who do not have seizures, or in injured but non-epileptogenic brain tissue. The more comprehensive interpretation is that the specific, local upregulation of P-glycoprotein expression seen occurs in the vicinity of chronic seizures. The use of particular anti-epileptic drugs may also play a role. Previous studies have shown that tumour necrosis factor alpha and interferon alpha may increase P-glycoprotein expression and activity in cultured cells (McRae et al., 2003; Fernandez et al., 2004). The administration of the anti-inflammatory cyclo-oxygenase-2 inhibitor, celecoxib, before seizure induction in animals decreases inflammation, reduces the frequency and severity of seizures, prevents the increase in P-glycoprotein expression and restores the efficacy of anti-epileptic drugs in animal models of mesial temporal lobe epilepsy (Schlichtiger et al., 2010; van Vliet et al., 2010). While more research is required into the effect of pro-inflammatory cytokines on regulation of P-glycoprotein, these data suggest inflammation may modulate P-glycoprotein expression and activity. If, subsequently, P-glycoprotein expression patterns do contribute to drug resistance, our findings further emphasize the importance of achieving prompt seizure control after seizure onset (Kwan and Brodie, 2000; Sillanpaa and Schmidt, 2009). Indeed, a previous study has found P-glycoprotein-expressing astrocytes cultured from surgical epileptic human tissue to be more functionally active at extruding the P-glycoprotein substrates, phenytoin and doxorubicin, compared with P-glycoprotein expressed on control astrocytes (Marchi et al., 2004). More studies are still needed to substantiate the function of P-glycoprotein expression on astrocytes in epileptogenic regions of the epileptic human brain (Kovacs et al., 2011).

Our study also highlights the importance of examining injuries caused by invasive depth electrode recording. Electrode-related brain injuries are important to consider because the clinical use of invasive EEG recordings in epilepsy is increasing (Engel et al., 2005). Animal studies have shown that the implantation of biosensors (e.g. for real time in vivo measurements) may activate the ‘wound healing process’, which involves haemostasis, inflammation, repair and remodelling events (Kim et al., 2004; Biran et al., 2005; Pollkov et al., 2005; Stonecek and Reichert, 2008). In humans, only one previous study has investigated (microglial) inflammatory responses in surgical tissue with intracranial electrode placement (Stephan et al., 2001), but the extent of this inflammatory response, and the pathology of the blood-brain barrier and vasculature after such insults, have not been investigated. We show that electrode-related injuries may lead to the generation of aberrant blood vessels (with reduced expression of P-glycoprotein and BCRP), the upregulation of active inflammatory cell types and extravasation of plasma proteins into an area that is ~31 times the area of the physical insult. The augmented release of pro-inflammatory cytokines after insults may alter blood-brain barrier permeability by reducing the amount of endothelial tight junction proteins (Minagar et al., 2003) and increase the expression of adhesion molecules (ICAM1 and LFA1), directing the transmigration of blood leukocytes into the brain (Wong and Dorovini-Zis, 1992; Merrill and Murphy, 1997; Vezzani et al., 2011). It is conceivable that blood-brain barrier disruption may underlie some complications associated with the placement of intracranial electrodes, such as severe haemorrhage, infection, oedema, hypersensitivity subdural haemorrhage and infarction (Stephan et al., 2001; Fountas et al., 2007; Wong et al., 2009). Therefore, it is important to carefully weigh the benefit of using intracranial depth electrodes to localize seizure focus to optimise surgical outcome against the risk of damage and complications caused by such placement, which increase with the number of electrodes used (Wong et al., 2009). Many of these complications have also been reported in patients who have undergone deep brain stimulation, a surgical procedure that requires the placement of electrodes into subcortical structures (Guehl et al., 2003).

Acknowledgements

We thank the Queen Square Brain Bank (London, UK) for providing brain tissue from five post-mortem control cases; Prof N. Joan Abbott for helpful discussion on the blood-brain barrier in epilepsy; Dr Lisa Vaugier for collecting patient data; Miss Tharnitha Vasavan for her technical assistance and Dr Zoe Fox for her advice on the statistical analysis.

Funding

This work was undertaken at University College London Hospitals/University College London, which received a
proportion of funding from the Department of Health’s National Institute for Health Research Biomedical Research Centres funding scheme; European Commission within the 7th Framework Programme; EURIPIDES.

Supplementary material

Supplementary material is available at Brain online.

References


