Is neural activation within the rescued penumbra impeded by selective neuronal loss?

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After stroke, penumbral salvage determines clinical recovery. However, the rescued penumbra may be affected by selective neuronal loss, as documented both histopathologically in animals and using the validated in vivo positron emission tomography marker ¹¹C-flumazenil in humans. However, whether the non-infarcted penumbra is capable of neuronal activation, and how selective neuronal loss may interfere, is unknown. Here we prospectively mapped the topographical relationships between functional magnetic resonance imaging responses and non-infarcted penumbra, and tested the hypothesis that the former do take place in the latter, but only in its subsets spared selective neuronal loss. Seven patients (mean age 74 years; three thrombolysed) with first-ever acute anterior circulation stroke, presence of penumbra on computed tomography perfusion performed within 6 h of onset, and substantial deficit on admission but good outcome at 1–3 months (National Institute of Health Stroke Score range 6–13 and 0–1, respectively, P = 0.001), were studied. At follow-up, patients underwent structural magnetic resonance imaging to map the infarct, functional magnetic resonance imaging (three tasks selected to probe the right or left hemisphere), and ¹¹C-flumazenil positron emission tomography generating binding potential maps. Patients with significant carotid or middle-cerebral artery disease or impaired vasoreactivity were excluded. Following image coregistration, the non-infarcted penumbra comprised all acutely ischaemic voxels (identified on acute computed tomography perfusion using previously validated thresholds) not part of the final infarct. To test our hypotheses, the overlap between functional magnetic resonance imaging activation clusters and non-infarcted penumbra was mapped, and binding potential values then computed both within and outside this overlap. In addition, the overlap between functional magnetic resonance imaging activation clusters and areas of significantly reduced binding potential (determined using Statistical Parametric Mapping against 16 age-matched control subjects) was assessed in each patient. An overlap between non-infarcted penumbra and functional magnetic resonance imaging clusters was present in seven of seven patients, substantial in four. Binding potential was significantly reduced in the whole non-infarcted penumbra (P < 0.01) but not within the functional magnetic resonance imaging overlap. Clusters with significantly reduced binding potential showed virtually no overlap with functional magnetic resonance imaging activation compared with 12 age-matched controls (P = 0.04). The results from this proof of principle study suggest that 1–3 months after stroke the non-infarcted penumbra is capable of neuronal activation, consistent with its established role in recovery of neurological functions. However, although the non-infarcted penumbra as a whole was affected by selective neuronal loss,
activations tended to occur within portions spared selective neuronal loss, suggesting the latter impedes neuronal activation. Although its clinical correlates are still elusive, selective neuronal loss may represent a novel therapeutic target in the aftermath of ischaemic stroke.

**Keywords:** PET; $^{11}$C-flumazenil; fMRI; brain activation; cerebral ischaemia; stroke

**Abbreviations:** BPND = non-displaceable binding potential; FMZ = flumazenil; NIHSS = National Institutes of Health Stroke Scale; NIP = non-infarcted penumbra

**Introduction**

After ischaemic stroke, clinical recovery is determined by the volume of penumbral tissue that escapes infarction, termed ‘non-infarcted penumbra’ (NIP) (Astrup et al., 1981; Baron, 1999, 2001; Heiss, 2000). Accordingly, reperfusion therapy, notably using thrombolytic agents, is the mainstay of acute management. Given it largely sustains behavioural recovery, the NIP is widely assumed capable of neuronal activation, a view indirectly supported by the presence of immediate peri-infarct activations in the post-stroke subacute stage in both rats (Dijkhuizen et al., 2003) and humans (Cao et al., 1999; Warburton et al., 1999; Cramer et al., 2000; Rosen et al., 2000; Jaillard et al., 2005). However, probably because of the comprehensive multimodal imaging data set required, namely acute perfusion imaging to identify the penumbral, infarct mapping using late structural imaging to identify the NIP, and finally functional MRI to ascertain the presence of brain activation within the latter, only one clinical study to date has directly tackled this assumption (Kleiser et al., 2005), yet without definitive conclusions as only two patients were reported, in one of whom spontaneous hyperperfusion was found on perfusion imaging, precluding assessment of the penumbral. This question is all the more important to resolve as neuronal activation is believed to be the main driver of use-dependent plastic processes in peri-infarct cortical areas (Cramer, 2008). In the present prospective investigation involving patients assessed in the hyperacute stage, multi-modality imaging and voxel-based analyses were implemented to directly assess whether activation at follow-up functional MRI mapped to the NIP.

An important feature of the NIP—and one highly relevant to this discussion—is that this tissue may not be completely spared of neuronal damage, which may in turn impede optimal clinical recovery (Baron, 2005). Effectively, recent quantitative PET studies using the in vivo neuronal marker $^{11}$C-flumazenil (FMZ) have documented that the cortical NIP may be affected in a patchy fashion by selective neuronal loss (Guadagno et al., 2008; Morris et al., 2012), consistent with extensive experimental literature (see Baron, 2005 for a review) and two earlier semi-quantitative clinical SPECT studies (Nakagawa et al., 1997; Saur et al., 2006). In relation to this, one key question is whether or not selective neuronal loss hampers neuronal activation in the NIP in the mid-to-long term. This issue is clinically relevant as, if it did, selective neuronal loss would be considered a novel post-acute therapeutic target. However, this clinical issue has never been directly addressed before, which may reflect the significant challenge of adding FMZ-PET to the already extensive set of imaging modalities listed above.

In the present investigation we therefore addressed in sequence the following two questions:

(i) Is the NIP capable of functional activation, as identified by functional MRI, in the early chronic phase after stroke? Based on the above reasoning that the NIP sustains a good part of clinical recovery, we made the hypothesis that functional MRI activation does map to the NIP. Assuming this was indeed true, we then addressed the following, connected question:

(ii) Does selective neuronal loss impede neuronal activation in the NIP? Because neuronal activation is believed to reflect synaptic activity within the local neuronal networks (Logothetis et al., 2001), we hypothesized that by damaging such local networks, selective neuronal loss would impede neuronal activation.

The present prospective multi-modality imaging study was therefore designed to assess functional MRI responses in the NIP affected or not by selective neuronal loss. Fig. 1 illustrates the different tissue compartments studied as well as the two main hypotheses to be tested.

**Materials and methods**

**Subjects**

Patients admitted to Addenbrooke’s Hospital, Cambridge, UK, were prospectively recruited in this investigation according to the following inclusion criteria: (i) first-ever acute anterior circulation stroke; (ii) age $>18$ years; (iii) native English speaker; (iv) right-handedness, based on the Edinburgh Questionnaire; and (v) perfusion CT obtained on admission as part of standard clinical procedures. In addition, to maximize the chance of having substantial areas of NIP and for the patients to be able to perform the functional MRI tasks, recruited patients were to have significant neurological deficit at admission (National Institutes of Health Stroke Scale (NIHSS) $>5$) but good clinical outcome (NIHSS $\leq 1$) at follow-up, i.e. at the time of the functional MRI and PET studies, 1–3 months after onset. Exclusion criteria included: (i) history of other major neurological or non-neurological disorder; (ii) recent use of benzodiazepines; (iii) any contraindication to MRI; (iv) carotid artery stenosis $\geq 50\%$ on acute stage cervical ultrasound or magnetic resonance angiography; (v) stroke subsequent to recruitment; (vi) middle cerebral artery occlusion on follow-up.
intracranial magnetic resonance angiography; and (vii) inadequate vasoreactivity breath-holding test on follow-up transcranial doppler.

Control subjects for functional MRI included 12 right-handed healthy subjects (seven male and five female, age 45.5 years, mean age 62.8 ± 2.4 years) with no history of stroke or other major neurological or psychiatric disease, on no current medication including benzodiazepine.

As controls for PET, we used another set of 12 healthy subjects (six males and six females, age 61.0 ± 7.9 years), using the same exclusion criteria as above.

This study was approved by the Cambridgeshire Regional Ethics Committee. All participants gave written informed consent to participate in the study.

Imaging protocols

All seven patients included in this study underwent a brain non-contrast CT and perfusion CT (on admission as part of local standard of care. FMZ-PET and MRI (including structural magnetic resonance, magnetic resonance angiography and functional MRI) were performed at follow-up, 1–3 months after stroke onset, on the same day or within a few days of each other. The control subjects underwent either functional MRI or FMZ-PET, both in association with structural MRI.

Computed tomography perfusion

Non-contrast CT and perfusion CT were acquired in succession using a helical scanner (Siemens Sensation 4 model, 120KVp, 258 mA). Perfusion CT was acquired after injecting 50 ml of iodinated contrast (iopamidon 300) into the cubital vein with flow rate of 8 ml/s using an injection pump. For three patients the protocol consisted of a 40 s cine acquisition with a repetition time of 1 s on two contiguous 12 or 14.4 mm axial slices (voxel dimensions: 0.43 × 0.43 × 12.0 mm or 0.43 × 0.43 × 14.4 mm) manually placed on plain CT at the level of the basal ganglia and the plane above. For the other four patients, due to upgrading of the CT system, the protocol consisted of 16 or 23 10 mm slices with 3 mm slice overlap (voxel dimensions: 0.38 × 0.38 × 4 mm or 0.42 × 0.42 × 4 mm) providing axial coverage of 64 or 92 mm, respectively. All data was acquired with a matrix size of 512 × 512.

Transcranial doppler

All patients underwent transcranial doppler to assess vasomotor reactivity to 30 s of breath holding according to established protocols (Markus and Harrison, 1992).

Structural magnetic resonance imaging

T1-weighted MRI was acquired at the Wolfson Brain Imaging Centre, University of Cambridge, UK, using a Siemens Magnetom Trio Tim scanner operating at 3 T (www.medical.siemens.com) and a MPRAGE sequence (176 slices of 1 mm thickness, repetition time = 2300 ms, echo time = 2.98 ms, inversion time = 900 ms, flip angle = 9°, field of view = 240 × 256). FLAIR images were acquired with 27 slices of 4 mm thickness and 1 mm interslice spacing, repetition time = 7840 ms, echo time = 95 ms, inversion time = 2500 ms, flip angle 150°, matrix = 320 × 256, field of view = 224 × 179 mm.

Magnetic resonance angiography

The magnetic resonance angiography sequence was a flow-compensated 3D time-of-flight of the circle of Willis. Scan parameters were as follows: flip angle = 20°, repetition time/echo time = 34 ms/minimum, number of excitations = 1, field of view = 240 mm, rectangular field of view = 75%, slice thickness = 1 mm interpolated to 0.5 mm, number of slices 116, acquisition matrix = 512 × 128 interpolated to 512 × 512, receiver bandwidth = ±32 kHz. To increase blood–tissue contrast enhancement, the time-of-flight sequence was enhanced by magnetization transfer prepulses. Further blood–tissue contrast enhancement by consideration of saturation effects from slow-flowing blood and multiple radiofrequency excitations was achieved by breaking the 3D slab into 2 smaller overlapping volumes (12 slices) and using a ramped excitation pulse with increasing flip angle in a distal direction.

Functional magnetic resonance imaging

Each functional MRI run consisted of varying repetitions of T2*-weighted echo-planar images (34 slices of 3-mm thickness with a 0.75 mm slice gap, repetition time = 2000 ms, echo time = 25 ms, flip angle = 75°, matrix of 64 × 64 with field of view = 192 × 192 giving 3 × 3 mm resolution). A task commenced after the collection of eight echo planar image volumes that were discarded to ensure T1 equilibrium.

Given the specific purpose of the present investigation, namely to test whether the NIP is capable of activation in patients with variable location and extent of initial penumbra and final infarct within the middle cerebral artery territory on either the right or left hemisphere, a set of six previously validated block-design functional MRI tasks was selected a priori aiming to examine as extensively as possible the left and right hemispheres, while at the same time keeping the duration of

Figure 1 Cartoon illustrating the different tissue compartments to be studied, and our two working hypotheses, namely, (i) functional MRI activation (orange areas) does occur in the non-infarcted penumbra, but (ii) it occurs only within areas of non-infarcted penumbra with normal 11C-FMZ binding, i.e. not affected by selective neuronal loss (see insert).
the functional MRI session as short as possible in order to limit the risk of non-compliance and head motion in stroke subjects. Three tasks probed the left hemisphere (a motor task using the right hand, a language task and a somatosensory task using somesthetic stimulation of the right hand), and three the right hemisphere (the same motor task but using the left hand, a vigilance task and the same somatosensory task, stimulating the left hand). These two subsets of tasks were applied to each particular patient depending on the side of their stroke. The control subjects performed all six tasks. All tasks were presented using E-Prime 1.2 software (Psychology Software Tools).

Motor task

The motor functional MRI block-design consisted of blocks of auditory-paced (1 Hz) finger-thumb opposition sequence (2, 3, 4, 5; 2, 3, . . .), alternating with rest, as previously applied in our laboratory (Sharma et al., 2008). For each hand in controls (affected hand only in patients), a block design was used with two conditions of 16 s duration, each replicated six times: (i) sequential finger-thumb tapping, auditory cued at a frequency of 1 Hz; and (ii) rest, with the auditory tones delivered at the same frequency. A total of 96 echo planar image volumes were acquired. Brief instructions to ‘Move’ or ‘Rest’ were aurally given just before the start and end of the ‘move’ block. Subjects were instructed to keep their eyes closed throughout the session. Finger movements during scanning were monitored by means of individually calibrated, highly sensitive fibre-optic gloves (Fifth Dimension Technologies) worn throughout the session (Sharma et al., 2008, 2009); the signal was continuously monitored in the control room for non-compliance. Before the experiment, subjects were instructed about the task and trained using the same stimulation software until they were able to adequately follow the aurally given cues and instructions, which took up to 10 min.

Auditory language task

For this task, we used the paradigm previously published by (Crinion and Price, 2005). Subjects listened to two conditions: stories or incomprehensible reversed versions of the same stories. Reversing the speech signal destroys intelligibility while retaining the overall spectrotemporal complexity of the original speech signal, and therefore serves as an appropriate reference to assess language-specific brain activity. The length of each story/reverse story was 64 words (24 s duration). As previously described (Crinion and Price, 2005), the waveform for each story was reversed using SoundEdit software. Block design functional MRI was obtained under two conditions, each replicated five times: (i) stories (different each time); and (ii) reverse stories (different each time) (see Crinion and Price, 2005 for examples). A period of 8 s of silence was interspersed between the two conditions. The same person narrated all the stories. The order of presentation of stories was similar between subjects. Presentation was binaural with the volume set at a comfortable level for each subject. The subjects were asked to simply listen and try to understand the story. They were aware that the reversed stories were unintelligible but were asked to pay attention to the sounds.

Before the experiment, all subjects received instructions and listened to a reduced version of the task. To ensure that they attended to the stimuli, a surprise story recognition test was presented to each subject at debriefing. In this post-scanning test, subjects were presented with 10 individual phrases and asked to indicate after each one whether they recognized it as being part of a story played to them in the scanner or not. All subjects and patients successfully passed this debriefing.

Vigilance task

We used an auditory vigilance task adapted from (Paus et al., 1997), which requires the detection of a small drop in tone intensity that might occur at the end of a 1 s auditory stimulus. The normal auditory stimuli were 1000 Hz tones displayed at an intensity of 72 dB and presented with 2 s interstimulus intervals. The target stimuli were similar tones showing a decrease of 6 dB in the last 100 ms of tones. The task was deliberately difficult in order to stimulate vigilance. The subjects were asked to press a button with their right hand when they detected the target. The baseline (control) task simply required the subjects to listen to non-target auditory tones; the subjects were told that no targets would be presented, and therefore, they did not expect to detect and respond to targets in this control task. Block design functional MRI was obtained under these two conditions, each replicated eight times: (i) vigilance (18 s); and (ii) baseline (18 s). A period of 6 s of silence was interspersed between the two conditions.

The subjects learned to discriminate the target from non-target stimuli in a 5 min training session before scanning. The duration and intensity of the stimuli, as well as their interstimulus intervals, were identical to those used in the scanning tasks. By informing the subjects about the absence of target stimuli, we attempted to minimize voluntary engagement of attention during the baseline scans. All subjects in this study exhibited adequate vigilance to tone changes.

Sensory stimulation

The somatosensory task consisted of blocks of stimulation of the median nerve at the wrist, alternating with rest, adapted from (Ibanez et al., 1995). The stimulus consisted of a 0.2 ms electrical square-wave pulse delivered through surface disk electrodes from a Digitimer DS7 High Voltage Stimulator and DG2A Train Delay Generator. The intensity was set individually for each subject at the sensory threshold, i.e. below the pain threshold to avoid discomfort. The frequency of the stimulation was 2 Hz. For each hand in controls (affected hand only in patients), block functional MRI was obtained under two conditions, each replicated eight times: (i) electrical stimulation (16 s); and (ii) rest (16 s). Subjects were instructed to keep their eyes closed throughout the session. Before the experiment, all subjects were instructed in the task and trained using the same stimulation software.

Positron emission tomography

An FMZ-PET study was obtained in patients 1–3 months after the index stroke, using the methodology described in (Guadagno et al., 2008). We used a GE Advance PET scanner, which has an intrinsic isofield resolution of 6.8 mm. A 15 min 68Ge transmission scan was first carried out to correct for photon attenuation. The compound was injected intravenously as a bolus (275–480 MBq) followed by 75 min dynamic acquisition (55 frames: 5 s × 18, 15 s × 6, 30 s × 10, 60 s × 7, 150 s × 4 and 300 s × 10). Images were reconstructed using 3D filtered backprojection into a 128 × 128 × 35 array with voxel dimensions of 2.34 × 2.34 × 4.25 mm (x, y, z). Using voxel-wise kinetic modelling with the pons as reference tissue, FMZ specific binding was expressed by non-displaceable binding potential (BPND) maps, as described in (Guadagno et al., 2008).
Image processing

Overall strategy
To address our first hypothesis (see ‘Introduction’ section), we determined whether or not each patient’s functional MRI activation clusters encroach onto same subject’s NIP, defined by comparing acute-stage perfusion CT to follow-up structural MRI (Fig. 1). To address the second hypothesis that selective neuronal loss interferes with functional MRI activation within the NIP, two independent analyses were performed, again carried out for each patient separately. In the first, we compared FMZ binding potential between the two subsets of the NIP, i.e. that showing and that not showing functional MRI activation. In the second analysis, we assessed whether or not functional MRI activation clusters overlap with clusters of significantly reduced FMZ $B_{\text{PND}}$ identified using voxel-based analysis. Because their very nature (namely, lack of whole brain coverage in most patients and very anisotropic voxels; see above) would make spatial normalization of the CT data in Montreal Neurological Institute (MNI) space prone to substantial errors (see Guadagno et al., 2008), all analyses involving perfusion CT were undertaken in native CT space, whereas all those not involving CT were carried out in standard MNI space. The imaging data were therefore processed along two distinct streams, one in CT space and the other in MNI space, as illustrated and summarized in flow-chart form in Supplementary Fig. 1. Below are listed the main steps followed in these analyses.

Delineation of the infarct region of interest
Using MRicro (www.cabiatl.com/mricro/index.html), the final infarct was delineated on FLAIR images (with help from the T1-spoiled gradient images whenever appropriate) by an experienced neurologist (J.C.B.) blinded to all other data. Two sets of 3D-dilated infarct regions of interest were then generated: one dilated by 9 mm for perfusion CT-space analyses, and one dilated by 14 mm for the MNI-space analyses. These dilatations intended to account for both the effective PET Hann window applied) and the 12 mm isotropic smoothing subsequent to spatial normalisation for the analysis in MNI space, and thus aimed at excluding from all analyses any voxel affected by loss of brain tissue.

Delineation of the non-infarcted penumbra: computed tomography space
The procedures have been described in detail in (Alawneh et al., 2011) and will be only briefly summarized here. Images were processed using in-house software implemented with Matlab version 7.3 (The MathWorks, Inc.). In order to apply Wintermark et al.’s validated perfusion CT thresholds, the methodology closely followed that used by these authors (Wintermark et al., 2006). The arterial input function was derived from the anterior cerebral artery; if the signal from the anterior cerebral artery was poor, a branch of the middle cerebral artery was selected (Wintermark et al., 2007); the enhancement curve from the superior sagittal sinus was used as reference. The vascular mean transit time was derived for each voxel from the convolution of the arterial input function with a box-residue function. CSF voxels, based on non-contrast CT, and voxels representing blood pool, based on cerebral blood volume map (>8%) were excluded. In order to identify the ischaemic tissue, we then applied voxel by voxel onto the affected hemisphere a threshold of mean transit time ratio $\geq 1.45$ relative to the unaffected side (Wintermark et al., 2006), allowing a binary map representing the ischaemic tissue to be generated. The unaffected side mask used to calculate the mean transit time ratio included all voxels in the cerebral hemisphere contralateral to the stroke. As perfusion CT was acquired immediately after non-contrast CT, it was consistently in the same spatial orientation (no recruited patients showed movement artefacts). Coregistration of the structural magnetic resonance to the perfusion CT was carried out according to (Guadagno et al., 2008). The MPRAGE scan was co-registered to the whole-head acute non-contrast CT using SPM8 in the case of two-slice perfusion CT acquisitions. The NIP was defined as any voxel included in the mean transit time ratio $\geq 1.45$ mask but not part of the 9 mm-dilated infarct region of interest (see above).

Testing hypothesis 1: computed tomography space

Functional magnetic resonance imaging analysis
Assessed contrasts were (1) Motor versus Rest (to be referred to as motor task below); (2) Stories versus Reverse Stories (language task); (3) Vigilance versus Baseline (vigilance task); and (4) Sensory stimulation versus Rest (sensory task). As indicated above, contrasts 1, 2, and 4 were obtained for left-hemisphere strokes (using right hand motor and sensory stimulation), and contrasts 1, 3 and 4 for right-hemisphere strokes. Each task data set was processed separately using Statistical Parametric Mapping software (SPM8; www.fil.ion.ucl.ac.uk/spm). Images were realigned to the first volume and the movement parameters checked to verify that no subject moved $>2$ mm for any task. Additional preprocessing steps comprised slice acquisition time correction and high-pass filtering. The design was convolved by the canonical haemodynamic response function. In order to obtain individual activation maps for direct comparison to same-subject NIP mask and FMZ images, this analysis was run in native (i.e. CT) space. Accordingly, activation clusters were determined from fixed-effect single-subject analyses, using a $6 \times 6 \times 7.5$ mm smoothing (i.e. twice the voxel size) particularly aimed at improving the signal-to-noise ratio (Price et al., 2006; Manganotti et al., 2012). Given the specific purposes of the present investigation, brain activations were assessed according to two statistical cut-offs, namely default uncorrected $P<0.001$ and FWE $P<0.05$. The former was used as standard because the latter could be over-conservative for descriptive single-subject analyses, as opposed to adequate at the population level. However, to reduce the chance of spurious results, only clusters $>50$ voxels were considered as significant.

Assessment of functional magnetic resonance imaging-non-infarcted penumbral overlap
For each patient, the presence and, if any, volume of overlap between any significant clusters on functional MRI activation maps and the NIP mask were determined. To this end, the functional MRI clusters were inverse-warped into, and resliced to native CT-space. As explained earlier, the primary analysis used the functional MRI activation maps derived using the uncorrected $P<0.001$ cut-off, but the FWE 0.05 cut-off was applied for complementary analyses. The search for functional MRI-NIP overlap was constrained within a middle cerebral artery territory mask created using a combination of Damasio and Phan’s maps (Damasio, 1983; Phan et al., 2005), minus the 9 mm-dilated infarct region of interest. This was to focus on tissue within the initially ischaemic areas and avoid ‘remote’ areas potentially affected by diascisiss or Wallerian degeneration (Guadagno et al., 2008).
Testing hypothesis 2

Analysis 1: flumazenil binding in the functional magnetic resonance imaging-non-infarcted penumbra overlap (computed tomography space)

Coregistration between magnetic resonance, perfusion CT and FMZ: The magnetic resonance to CT matrix transformation file previously obtained was applied to the FMZ BP\textsubscript{ND} maps (already co-registered to the magnetic resonance), therefore placing the perfusion CT maps, outcome MRI and FMZ BP\textsubscript{ND} maps all in register. To allow this, the individual gantry tilt effect (only present in the two-slice perfusion CT) was first removed and the functional MRI and BP\textsubscript{ND} maps were smoothed over 9 mm using a box kernel in the z dimension to place all the images in a similar z resolution.

Determination of FMZ binding in the functional MRI-NIP overlap: For each task in each patient, the mean FMZ BP\textsubscript{ND} was computed across all voxels belonging to the overlap between the functional MRI clusters and the NIP mask, as well as for their mirror voxels on the opposite hemisphere, determined relative to the mid-sagittal axis. To assess whether FMZ binding was significantly reduced in the functional MRI-NIP overlap, we first obtained the weighted mean FMZ BP\textsubscript{ND} within the overlap across all tasks for each patient, and then expressed this as a ratio relative to its counterpart weighted mean BP\textsubscript{ND} in the mirror voxels. We then compared these ratios across all patients against neutral (i.e. unity), using paired t-tests. An equivalent analysis was performed for the NIP outside the functional MRI clusters. Finally, BP\textsubscript{ND} ratios in the functional MRI-NIP overlap were compared to BP\textsubscript{ND} ratios in the NIP outside the functional MRI clusters, again using paired t-tests. In order to restrict this analysis to meaningful tissue volumes, clusters <1 ml were excluded. Again, primary analyses used the functional MRI activation maps derived using the uncorrected P < 0.001 cut-off, but the FWE 0.05 cut-off was also applied for complementary analyses.

Analysis 2: Overlap between flumazenil and functional magnetic resonance imaging clusters (MNI space)

Functional MRI analysis: Here we again proceeded with single-subject analysis, using the same contrasts and cut-offs as in the native-space analysis above. Each task data set was processed separately using SPM8, applying the same preprocessing steps described above. The MPRAGE images were spatially normalized to the MNI standard space, which is in Talairach and Tournoux space, using the unified segmentation and warping method. In the case of the patients, this was carried out using lesion masking (Brett et al., 2001) to reduce incorrect warping around the lesion. The mean echo planar image from each session was coregistered to the MPRAGE and the transformation parameters from warping were applied to all echo planar image volumes to bring them into MNI space. The images were then smoothed using an $8 \times 8 \times 8$ mm Gaussian filter.

This single-subject analysis in MNI space was carried out both for each patient and for each control subject (see Supplementary Fig. 1). In addition, random-effect (Task versus Reference) group analyses of the 12 controls were carried out for each functional MRI task, but only for the sake of completeness as they were not used for any of the planned analyses (see Supplementary Fig. 2).

FMZ analysis in MNI space: This individual SPM analysis used the FMZ BP\textsubscript{ND} maps, according to the procedure detailed in (Guadagno et al., 2008). The BP\textsubscript{ND} maps from both the patients and the 12 controls were coregistered to each participant’s MPRAGE image, and the MNI warping transformation parameters applied to the BP\textsubscript{ND} maps to also bring them into MNI space. To improve sensitivity, the BP\textsubscript{ND} maps were then proportionally scaled to the mean of the unaffected hemisphere, obtaining the 0.8 global mean threshold in SPM. Before statistical analysis, the FMZ data were smoothed by a 12 mm Gaussian kernel. As with the CT space analyses, this analysis was applied within the middle cerebral artery territory mask, minus the 14 mm-dilated infarct region of interest. The analysis was performed within the final mask using the Wake Forest University Pick Atlas (WFU PickAtlas; Maldjian et al., 2003; Guadagno et al., 2008) applying small volume correction.

Single-subject analysis was performed as described in (Guadagno et al., 2008). In order to identify abnormal FMZ binding in each patient, a statistical threshold was identified from a permutation analysis carried out in the sample of 12 controls described above (Signorini et al., 1999). Each individual control was compared in turn with the others using a two-sample t-test in SPM8 and an uncorrected threshold of $P < 0.001$ with a cluster extent of 5. A small volume correction was applied to the results within the predominantly grey matter of the middle cerebral artery mask, defined as that part of the middle cerebral artery mask which has grey-matter probability >0.3 in the SPM average a priori grey matter template. This permutation analysis showed that no significant voxels were identified in any control with a minimum corrected threshold of $P < 0.1$ FDR. The same threshold was then applied to each patient’s FMZ maps. For this analysis, the individual’s infarct region of interest (dilated by a 14 mm sphere) was subtracted from the middle cerebral artery mask so that the analysis would be performed only on areas of grey matter sufficiently distant from the infarct borders.

Determination of FMZ-functional MRI overlap: Using the coregistered FMZ SPM maps and functional MRI activation maps, both in MNI space, the volume of overlap between these two types of clusters was determined for each patient within the middle cerebral artery mask. In order to assess the statistical significance of these overlap volumes, they were compared with the corresponding overlap volumes between each patient’s FMZ clusters and the individual functional MRI activation clusters from the 12 control subjects, again within the middle cerebral artery mask. To this end, the activation clusters for each task from each control were projected onto each patient’s FMZ maps in MNI space. This in turn allowed calculation of the volume of overlap between each patient’s FMZ cluster and the individual functional MRI clusters from the 12 controls. In order to err on the conservative side given our hypothesis that activations would be hampered by selective neuronal loss, i.e. that there would be only small or no functional MRI/FMZ overlap (see ‘Introduction’ section), we elected to use only the functional MRI default $P < 0.001$ cut-off for this analysis.

Results

Patients

We recruited seven consecutive patients (five males and two females, mean age 74 ± 10 years) (Table 1). No patient had significant carotid artery disease. Median NIHSS was 6 (range 6–13) at acute stage and 0 (range 0–1) at follow up ($P = 0.0014$), with complete or near complete recovery in five and two patients, respectively; the residual deficits in the latter did not interfere with the functional MRI tasks. The median time interval from stroke onset to perfusion CT was 193 min (range 73–312). As per protocol, both the functional MRI and the PET sessions took place on the same day in four patients (30–92 days after stroke onset),
Table 1 Patients’ characteristics

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Gender</th>
<th>HTN</th>
<th>CS</th>
<th>DM</th>
<th>HC</th>
<th>NIHSS admission</th>
<th>Infarct side</th>
<th>Vascular territory</th>
<th>Cause</th>
<th>tPA</th>
<th>NIHSS follow-up</th>
<th>Infarct volume (ml)</th>
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<td>65</td>
<td>M</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>11</td>
<td>R</td>
<td>Middle cerebral artery</td>
<td>Large artery disease</td>
<td>+</td>
<td>0</td>
<td>–</td>
<td>9.7</td>
</tr>
<tr>
<td>2</td>
<td>59</td>
<td>M</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>6</td>
<td>R</td>
<td>Middle cerebral artery</td>
<td>Cardioembolic</td>
<td>+</td>
<td>0</td>
<td>29.4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>74</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>6</td>
<td>R</td>
<td>Middle cerebral artery</td>
<td>Cardioembolic</td>
<td>–</td>
<td>0</td>
<td>65.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>87</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>13</td>
<td>L</td>
<td>Middle cerebral artery</td>
<td>Cardioembolic</td>
<td>–</td>
<td>1</td>
<td>55.7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>68</td>
<td>M</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>6</td>
<td>L</td>
<td>Middle cerebral artery</td>
<td>Cardioembolic</td>
<td>–</td>
<td>0</td>
<td>25.8</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>76</td>
<td>F</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>6</td>
<td>L</td>
<td>Anterior cerebral artery</td>
<td>Cardioembolic</td>
<td>–</td>
<td>0</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>83</td>
<td>F</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>7</td>
<td>L</td>
<td>Middle cerebral artery</td>
<td>Undetermined</td>
<td>+</td>
<td>1</td>
<td>55.1</td>
<td></td>
</tr>
</tbody>
</table>

M = male; F = female; HTN = hypertension; CS = cigarette smoking; DM = diabetes mellitus; HC = hypercholesterolaemia; R = right; L = left; tPA = intravenous thrombolysis using recombinant tissue plasminogen activator; *this patient had moderately stenotic (<50%) carotid bifurcation atherosclerotic plaque and no intracranial disease. Given the lack of other potential cause for his stroke despite extensive work up, he was adjudicated as having large artery disease.

whereas due to technical problems the PET study was delayed 25–30 days after the functional MRI in three patients (functional MRI date: 47–91 days after stroke). Vasoreactivity on transcranial doppler was within the normal range in all patients.

**Imaging**

Figure 2 illustrates for one brain section the ischaemic tissue as identified on admission perfusion CT, the final infarct on follow-up FLAIR, and the FMZ BP_{ND} map for the seven patients. All patients had penumbra acutely and ultimately exhibited an infarct. Final infarct volumes are shown in Table 1.

Group SPM maps in the control subjects showed the activation patterns expected for the studied tasks (see Supplementary Fig. 2).

**Functional magnetic resonance imaging-non-infarcted penumbra overlap (computed tomography space)**

Out of the 21 tasks studied across the seven patients, there was significant individual activation in 19 of 21 tasks. Some functional MRI/NIP overlap was present in all seven patients (in 17 of 19 tasks with activation). The overlap volumes (aggregated across all three tasks per patient) are shown in Table 2. They ranged from 0.02 to 25.9 ml (mean 9.3 ± 10.7; >2 ml in four patients). The results were not substantially different using the stringent FWE P < 0.05 cut-off (see Supplementary material).

Figure 3 illustrates the functional MRI/NIP overlap in Patients 2, 3, 4 and 6.

**Flumazenil BP_{ND} in the functional magnetic resonance imaging-non-infarcted penumbra overlap (computed tomography space)**

As shown also in Table 2, five of seven patients had summed clusters of functional MRI/NIP overlap >1 ml. Across the seven patients, the weighted mean BP_{ND} affected/unaffected ratio in the NIP outside functional MRI clusters was 0.90 ± 0.06 (P = 0.007 as compared to neutral, paired t-test), indicating reduced FMZ binding (Table 2). However, within the functional MRI/NIP overlap, the BP_{ND} was not reduced (affected/unaffected ratio = 0.98 ± 0.06; n = 5 patients, P = 0.5). In this subset of five patients, the BP_{ND} affected/unaffected ratio in the NIP outside the functional MRI/NIP overlap was 0.93 ± 0.05 (P = 0.03), significantly lower than the BP_{ND} ratio within the functional MRI/NIP overlap (P = 0.017, paired t-test). Despite smaller activation clusters, the results using the FWE P < 0.05 cut-off were entirely consistent (Supplementary material).

**Flumazenil /functional magnetic resonance imaging overlap (MNI space)**

The SPM analysis revealed clusters of significantly reduced FMZ binding in three patients, involving the affected hemisphere in two (Patients 3 and 4) and the unaffected hemisphere in one. Only the former patients will be further dealt with since the functional MRI tasks were designed to activate the affected hemisphere (see ‘Materials and methods’ section). Patient 3 had two FMZ clusters, located in the frontal and parietal convexity areas (Fig. 4A). Patient 4 had three FMZ clusters (Fig. 4B), an extensive one located in the left tempo-parietal cortex and two smaller in the frontal cortex. Remarkably, there was very little or no overlap between these FMZ clusters and the functional MRI clusters, for any task in any patient. The volumes of FMZ/functional MRI overlap for these two patients and the 12 controls (see ‘Materials and methods’ section) are shown in Table 3. Across the three functional MRI tasks, the volumes of overlap for the two patients were either zero, below the lowest control value, or at the lower range of controls. Across the six tasks, this difference in overlap volumes between patients and controls was significant (P = 0.04, Mann-Whitney U-test) (Fig. 5).

**Discussion**

The main findings from this clinical study of a sample of anterior circulation stroke patients prospectively selected for both excellent clinical recovery and substantial amounts of surviving penumbra support our two a priori hypotheses, namely: (i) 1–3 months after stroke onset, functional MRI clusters were present within the surviving penumbra, indicating the latter is capable of neuronal activation; but (ii) functional MRI activation tended to occur within those subregions of the surviving penumbra with preserved FMZ.
Figure 2. Illustration, for each of the seven patients and for one selected axial section, of the acutely ischaemic tissue (i.e. mean transit time > 145%) on admission CT perfusion (green; left column), final infarct on follow-up FLAIR (middle, contours underlined in red) and $^{11}$C-FMZ BP$_{ND}$ map (right; areas of lower FMZ binding are darker, see pseudocolour scale in BP$_{ND}$ units next to each image). Arrows point to non-infarcted cortical areas showing apparently reduced FMZ binding as compared to contralateral side, based on visual inspection. Note that, according to the methodology employed in this study, the ischaemic tissue is shown only within the middle cerebral artery territory mask (see ‘Materials and methods’ section for details). Also, the choice of axial slice was inherently limited due to CT perfusion not covering the whole brain in most patients, and in some subjects different slices were more demonstrative of reduced FMZ binding in the non-infarcted penumbra than shown here.
binding, suggesting that selective neuronal loss significantly interferes with large-scale task-dependent neuronal activation.

The finding that the NIP is capable of activation in the early chronic stage is entirely consistent with, and likely underpins, the well-established pivotal role of penumbral salvage in early as well as long-term post-stroke neurological recovery (Furlan et al., 1996; Heiss et al., 1998; Hillis et al., 2002; Alawneh et al., 2011). It is also consistent with previous reports documenting the presence of activation in the immediate peri-infarct cortical areas in both rodents (Dijkhuizen et al., 2003) and stroke patients (Cao et al., 1999; Warburton et al., 1999; Cramer et al., 2000; Rosen et al., 2000; Jaillard et al., 2005). In a longitudinal median nerve stimulation functional MRI study in rats following temporary middle cerebral artery occlusion, Dijkhuizen et al. (2003) reported a gradual return over time of activation within the primary somatosensory cortex (S1), located at the immediate infarct borders in the stroke model used. Entirely consistent with these findings in rodents, a longitudinal functional MRI study using a finger tapping task in four patients with infarcts partially affecting the primary cortex (M1) disclosed an initially reduced ipsilesional M1 activation, with subsequent emergence of activation at the superior border of the infarct, in parallel with motor recovery (Jaillard et al., 2005). These findings in rodents and stroke patients could represent plasticity gradually building up in the NIP (Furlan et al., 1996) although that this tissue was initially penumbral was not directly assessed. A number of additional clinical studies also reported activations involving the rim of cortical infarcts in well-recovered patients, whereas patients with poor recovery tended not to show this pattern (Cao et al., 1999; Heiss et al., 1999; Warburton et al., 1999; Cramer et al., 2000). Altogether, these observations have been interpreted as reflecting disinhibition/unmasking of pre-existing latent representations and/or enhanced involvement of non-infarcted parts of the functional area.

Only two investigations before ours studied both acute-stage brain perfusion and brain activation at follow-up. In a rat study, Sicard et al. (2006a, b) reported a return of forepaw stimulation-induced S1 activation at 24 h following 20 min temporary middle cerebral artery occlusion; this cortical area had a 42% decrease in cerebral blood flow together with a mild but significant reduction in the apparent diffusion coefficient during occlusion, and was therefore probably penumbral. The findings from our present study are entirely consistent with this experimental study. In a preliminary report of two patients, Kleiser et al. (2005) compared functional MRI activations at follow-up to acute-stage magnetic resonance-based diffusion/perfusion mismatch. In one patient, however, diffusion/perfusion-weighted imaging was in fact carried out 2 weeks after stroke onset, and by that time spontaneous reperfusion had already occurred as documented by high cortical cerebral blood flow, making any identification of initially penumbral areas uncertain at best. In the second patient, survival of acutely hypoperfused tissue with perfusion characteristics compatible with penumbra (namely, time-to-peak > 4 s) was documented, and in this peri-infarct tissue, activation during an overt language task (generating sentences from a verb) was absent 3 weeks after stroke (note, however, that the patient was unable to speak at that stage), but present 6 months later in parallel with partial speech recovery. Our findings from a substantial patient sample that the NIP is capable of activation 1–3 months post-stroke are therefore consistent with this earlier case report.

Our study does not exclude the possibility that initially the NIP is not capable of activation, a scenario supported by the above-mentioned rodent studies (Dijkhuizen et al., 2003; Sicard et al., 2006b) and by a previous experimental study following severe global ischaemia (Dietrich et al., 1986). Although the NIP may be functionally impaired in the subacute phase, the above findings may be confounded by the haemodynamic disruption that characterizes the acutely reperfused penumbra (Marchal et al., 1999, 1999), which may cause loss of blood oxygen level-dependent response despite normal neuronal activation. Note that in the present study we set out to address neuronal function in the NIP in the early chronic phase, and accordingly did not envision carrying out functional MRI at earlier stages. It is expected that 1–3 months after stroke any haemodynamic disturbances would have resolved, and furthermore we specifically took care to exclude patients with significant carotid stenosis or persistent middle cerebral artery occlusion on magnetic resonance angiography or showing abnormal vasoreactivity on transcranial doppler. Longitudinal functional MRI studies studying the temporal dynamics of activations within the NIP would nonetheless be of interest, albeit challenging.

The present finding that FMZ binding was mildly but significantly reduced in the whole NIP is consistent with previous

### Table 2: Volumes of the different tissue compartments, and corresponding $^{11}$C-FMZ BPND ratio values

<table>
<thead>
<tr>
<th>Patient (number of perfusion CT slices)</th>
<th>NIP (ml)</th>
<th>Functional MRI/non-infarcted penumbra overlap (ml)</th>
<th>FMZ BPND affected/unaffected ratio within functional MRI/NIP overlap</th>
<th>FMZ BPND affected/unaffected ratio outside functional MRI/NIP overlap</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (2)</td>
<td>13.3</td>
<td>1.8</td>
<td>1.07</td>
<td>0.98</td>
</tr>
<tr>
<td>2 (2)</td>
<td>23.7</td>
<td>21.3</td>
<td>0.97</td>
<td>0.94</td>
</tr>
<tr>
<td>3 (16)</td>
<td>21.4</td>
<td>12.3</td>
<td>1.01</td>
<td>0.97</td>
</tr>
<tr>
<td>4 (23)</td>
<td>10.9</td>
<td>0.42</td>
<td>N/A</td>
<td>0.86</td>
</tr>
<tr>
<td>5 (2)</td>
<td>21.4</td>
<td>3.35</td>
<td>0.91</td>
<td>0.89</td>
</tr>
<tr>
<td>6 (23)</td>
<td>40.3</td>
<td>25.9</td>
<td>0.95</td>
<td>0.87</td>
</tr>
<tr>
<td>7 (23)</td>
<td>5.3</td>
<td>0.02</td>
<td>N/A</td>
<td>0.81</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>19.5 ± 11.3</td>
<td>9.3 ± 10.7</td>
<td>0.98 ± 0.06$^5$</td>
<td>0.90 ± 0.06$^*$</td>
</tr>
</tbody>
</table>

$^5p = 0.5$ and $^*p = 0.007$ as compared to neutral, paired t-test. See ‘Results’ section for further statistical results.
Figure 3  Axial brain sections from four patients illustrating the overlap between non-infarcted penumbra (NIP) and functional MRI (fMRI) activation for selected tasks (two to four axial sections are shown per functional MRI task), projected onto each subject’s co-registered follow-up FLAIR images to show also the final infarct. The acutely ischaemic tissue is shown in green, the functional MRI activation clusters ($P < 0.001$, >50 voxels) outside the NIP in red, and areas with functional MRI/NIP overlap in yellow. Arrows point to particularly clear overlap in each patient. Note that for the sake of illustration, this figure shows the data for the entire affected hemisphere, not just for the middle cerebral artery territory mask. However, quantitative data were extracted only for the latter (see ‘Materials and methods’ section for details).
FMZ-PET studies from our group in two independent samples (Guadagno et al., 2008; Morris et al., 2012) as well as two 123I-Iomazenil SPECT studies (Nakagawara et al., 1997; Saur et al., 2006). Importantly, 11C-FMZ-PET has been validated against specific immunohistopathological neuronal markers as a sensitive in vivo surrogate for selective neuronal loss (Ejaz et al., 2013). Taken together with these earlier reports and consistent with a large body of experimental literature (see Baron, 2005 for a review), the present findings further document the presence of widespread, albeit of small magnitude, selective neuronal loss in the NIP in humans. It is, however, still unclear whether this scattered death of neurons in the NIP is primarily ischaemic from a cascade of biochemical events initiated during the acute ischaemic phase, or is secondary to subsequent microglial activation (Hughes et al., 2010).

The second novel finding from the present study is that selective neuronal loss appears to interfere with neuronal activations. This was shown with two separate but complementary analyses. In the first, we found that FMZ binding was preserved within the portion of the NIP exhibiting functional MRI activations, in contrast to the rest of the NIP where it was reduced. In the second, there was little or no overlap between functional MRI activation clusters and areas of significant FMZ binding decrease, which on volume analysis was statistically significant. These two analyses differed in that, in the latter, individually significant FMZ clusters were sought using SPM and a stringent statistical cut-off, whereas in

Figure 4 Illustrative sections from the functional MRI and FMZ SPM maps in Patients 3 and 4, projected onto each patient’s FLAIR images in MNI space (see ‘Materials and methods’ section), showing the final infarct (dilated by 14 mm) in red, the clusters of significantly reduced FMZ binding on the affected hemisphere (right and left hemisphere for Patients 3 and 4, respectively) in purple, and the significant functional MRI activation clusters (P < 0.001, clusters < 50 voxels excluded) in yellow for the motor and in blue for the language or vigilance tasks. These images illustrate the almost complete absence of overlap between FMZ and functional MRI clusters (see ‘Results’ section for details).
the former the FMZ BPND voxel values were collected across all patients within the tissue compartments of interest. Both analyses nevertheless concurred in suggesting that selective neuronal loss hampers the capacity for neuronal activation of the salvaged penumbra. In turn, preventing selective neuronal loss may speed up or enhance recovery, a notion with potential therapeutic implications.

Note that in order to avoid spurious findings due to loss of signal from partial volume effects around infarcts, infarct regions of interest were dilated and these dilated masks were excluded from all subsequent analyses. This is unfortunate as these areas are expected to have significant selective neuronal loss, but the methodology employed here dictated this precaution be taken.

The only previous study that included acute perfusion measurement, follow-up brain activation mapping and assessment of selective neuronal loss is the experimental investigation of Sicard et al. (2006b) already alluded to above. Following 20 min transient middle cerebral artery occlusion in rats, these authors found that forepaw stimulation-induced activation in S1 had returned to normal from 24 h post-stroke onwards, and this area showed mild but significant selective neuronal death at standard histopathological assessment performed 20 days later. In addition to species differences, the apparent discrepancy with our present results that selective neuronal loss tended to impede brain activation may be because in the Sicard study the relative degree of neuronal death in S1 was small (8%, compared with e.g. 18% in S2), and furthermore, in contrast to our study, no attempt was made to coregister in space the functional MRI response with histopathology. In addition, the median nerve stimulation paradigm used in rats under general anaesthesia may not compare to the physiological paradigm used here in humans.

One intriguing issue already raised in our previous report (Guadagno et al., 2008) is the contrast between the presence of selective neuronal loss and our patients’ excellent neurological recovery (final NIHSS range: 0–1), particularly given the impairment of neuronal activation within selective neuronal loss documented here. This apparent discrepancy may be caused by two main, mutually non-exclusive factors. First, plastic processes may develop over time so that neural functions impaired by selective neuronal loss are taken over by neighbouring cortical areas with similar functional roles, as is known to occur with small cortical infarcts (Jaillard et al., 2005). This would account for the observation in the rodent that initially impaired subtle motor tests have returned to baseline 3 weeks after temporary middle cerebral artery occlusion causing pure, mainly striatal selective neuronal loss (Sicard et al., 2006b). Second, it is also possible that neurological scales such as the NIHSS do not capture persisting but subtle cognitive or sensorimotor impairments, as suggested by recent studies after minor stroke or transient ischaemic attacks (Gold et al., 2007; Pendlebury et al., 2012). Along the same line of thinking, note that the functional MRI tasks used here involved much subtler behavioural functions than those tested with the NIHSS. These issues might be resolved by longitudinal studies of subtle behavioural tasks in relation to FMZ-PET and functional MRI.

### Table 3
Volumes of FMZ/functional MRI overlap for each functional MRI task in Patients 3 and 4, and corresponding values in healthy controls

<table>
<thead>
<tr>
<th>Functional MRI Task</th>
<th>FMZ/functional MRI overlap (ml)</th>
<th>FMZ/functional MRI overlap (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 3*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vigilance</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>Motor left</td>
<td>0.17</td>
<td>6.7</td>
</tr>
<tr>
<td>Sensory left</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>Language</td>
<td>0.25</td>
<td>0.7</td>
</tr>
<tr>
<td>Motor right</td>
<td>1.98</td>
<td>5.5</td>
</tr>
<tr>
<td>Sensory right</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>Controls (n = 12)*§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vigilance</td>
<td>0.81 (0.33–1.80)</td>
<td>32.4 (13.2–72.00)</td>
</tr>
<tr>
<td>Motor left</td>
<td>0.20 (0.12–0.85)</td>
<td>8.0 (4.8–34)</td>
</tr>
<tr>
<td>Sensory left</td>
<td>0.04 (0.00–0.10)</td>
<td>1.6 (0–4.0)</td>
</tr>
<tr>
<td>Language</td>
<td>2.25 (0.53–6.03)</td>
<td>6.23 (1.47–16.70)</td>
</tr>
<tr>
<td>Motor right</td>
<td>5.02 (2.36–11.35)</td>
<td>13.91 (6.54–31.44)</td>
</tr>
<tr>
<td>Sensory right</td>
<td>1.38 (0.38–5.28)</td>
<td>3.82 (1.05–14.63)</td>
</tr>
</tbody>
</table>

Across the six tasks, the difference in volumes between patients and controls was significant ($P = 0.04$, Mann-Whitney U-test).

*The total volume of FMZ clusters was 2.5 ml and 36.1 ml in Patients 3 and 4, respectively.

§Median and IQR.

### Figure 5
Box and whisker plot of the volumes of FMZ/functional MRI overlap across the six functional MRI tasks in patients (Patients 3 and 4 together) and in the 12 healthy control subjects, showing significantly smaller overlap in the patients ($P = 0.04$, Mann-Whitney U-test). See ‘Materials and methods’ section and Table 3 for details.
Our proof of principle study does have limitations. The first relates to the small sample of patients included. However, the multimodality imaging protocol required was demanding and inclusion/exclusion criteria were stringent in order to match the scientific questions, but on the other hand ensured sample homogeneity and hence improved power. Despite the small sample, the results were statistically significant, supporting our conclusions. Nevertheless, our findings will need to be replicated in larger samples. Second, there was a degree of variability in stroke size, location and side among patients, expected from the daily case-mix of admissions to an acute stroke unit. However, our goal here was not to determine a correlation between function and anatomy but merely whether activations occurred within the NIP. Thus, the functional MRI tasks were not selected individually according to each subject’s particular NIP topography, but as a set of tasks prospectively chosen to cover the cerebral hemispheres as widely as possible in order to increase the chance of detecting activations within the NIP. Furthermore, experience dictates that in a stroke population functional MRI tasks have to be simple and imaging sessions must not last too long. Third, although as per protocol all magnetic resonance and PET studies took place beyond 30 days after the stroke, in three patients a delay of ~1 month occurred between the functional MRI and PET studies, owing to unexpected technical PET failures after the functional MRI session. However, this is not expected to affect the findings in these three patients since decreases in central benzodiazepine receptor binding in non-infarcted cortex have been reported to stabilize from three weeks post-stroke onwards (Nakagawara et al., 1997). Fourth, co-registering imaging data from distinct modalities obtained at different spatial resolutions inevitably causes errors when estimating intersection or exclusion volumes. However, care was taken to reduce these errors when coregistering the magnetic resonance and PET data onto the CT data (see ‘Materials and methods’ section). Furthermore, to account for potential coregistration errors, two patients with small functional MRI/NIP overlap volumes were excluded from the analysis of $BP_{NO}$ within or outside this overlap (Table 2). Likewise, a post hoc analysis of the FMZ/functional MRI overlap volumes (Table 3) after considering any volume smaller than the spatial resolution of the lowest-resolution modality, namely PET (i.e. 6.8 mm$^3$ = 0.31 ml), found unchanged statistical significance ($P = 0.042$, Mann-Whitney). Finally, as we did not repeat the functional MRI study at later time-points we cannot exclude that activations may eventually return in that part of the NIP affected by selective neuronal loss. However, that they were still absent or weak by 1–3 months post-stroke suggests substantial damage to local neural circuits. Longitudinal studies with long term follow-up would be required to address this specific point.

In conclusion, the present study supports the idea that within the salvaged penumbra affected by selective neuronal loss, the neural circuits may not recover their normal functional abilities, at least not up to 3 months after the stroke. Although its clinical correlates are still elusive and will require further investigation, selective neuronal loss may represent a novel therapeutic target in the aftermath of stroke.

Acknowledgements

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Supplementary material

Supplementary material is available at Brain online.

References


Ejaz S, Williamson DJ, Ahmed T, Sitnikov S, Hong YT, Sawiak SJ, et al. Characterizing infarction and selective neuronal loss following...


