Decreased Chronic-Stage Cortical $^{11}$C-Flumazenil Binding After Focal Ischemia-Reperfusion in Baboons
A Marker of Selective Neuronal Loss?

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Background and Purpose—Although the penumbra can be saved by early reperfusion, in the rat it is consistently affected by selective neuronal loss. Mapping selective neuronal loss in the living primate would be desirable.

Methods—Five young adult baboons underwent $^{15}$O positron emission tomography for cerebral blood flow, cerebral oxygen consumption, and oxygen extraction fraction mapping at baseline and serially during and after 20-hour temporary middle cerebral artery occlusion. At approximately day 30, $^{11}$C-flumazenil (FMZ), a potential positron emission tomography marker of selective neuronal loss, and structural magnetic resonance-based infarct mapping were obtained, and the brain was perfused-fixed. Reduced FMZ binding in noninfarcted cortical middle cerebral artery areas was searched voxel-wise, and specific binding was assessed using compartmental modeling of FMZ time-activity curves.

Results—Visual inspection revealed reduced late FMZ uptake in the affected cortical territory, extending well beyond the infarct. Accordingly, the incidence of selected voxels was greater than chance, documenting mildly but significantly reduced FMZ uptake and specific binding. Serial $^{15}$O positron emission tomography revealed moderately severe acute ischemia followed by reperfusion. Histopathology documented only mild neuronal changes in or near the affected areas.

Conclusions—We document moderate but definite late FMZ binding decrements in noninfarcted cortical areas in the baboon, consistent with previous rat and human studies. These were acutely characterized by moderate ischemia followed by reperfusion, consistent with neuronal damage from ischemic or reperfusion injury in the salvaged at-risk tissue. Only mild histopathological changes subtended these FMZ alterations suggesting subtle processes such as isolated dendrite or synapse loss. Whether these changes impact on clinical outcome deserves studying because they may be targeted by specific neuroprotection. (Stroke. 2008;39:991-999.)

Key Words: acute stroke, brain ischemia, cerebral blood flow, focal ischemia, imaging, metabolism, neuronal death, neuropathology, positron emission tomography, stroke

Although timely reperfusion can salvage the penumbra from infarction, ie, pan-necrosis, selective neuronal death (SNL) may develop from ischemic or reperfusion injuries. Because spontaneous reperfusion can also rescue the penumbra, SNL has implications beyond thrombolysis. SNL is important because it may affect functional outcome and may be preventable.

Few postmortem reports on SNL in humans are available. Lassen reported in abstract form extensive areas of “incomplete infarction” in 2 patients, but formal neuropathology was not presented. Three detailed studies found SNL to be a rare occurrence at best, but they stand in sharp contrast with experimental evidence consistently showing SNL/death after temporary focal ischemia in the rat, cat, and nonhuman primate. Rather than species-related, this discrepancy may reflect bias, ie, human postmortem samples may not include patients with early recanalization, most at risk for SNL. In addition, SNL is difficult to evidence on conventional histology of gyrencephalic brains. To be able to detect SNL in vivo using imaging would therefore be desirable.

In baboons subjected to temporary middle cerebral artery occlusion (tMCAo), $^{11}$C-flumazenil (FMZ), a ligand of the central benzodiazepine receptor (a component of the GABA$_A$ complex), was shown to be a suitable positron emission tomography (PET) marker of early ischemic neuronal death, and suppressed hyperacute FMZ uptake reliably predicts final infarction in both cats and humans. The use of FMZ as marker of poststroke SNL is not well-established, however. Five SPECT studies have reported reduced $^{123}$I-Iomazenil (IMZ, an FMZ analogue) uptake in cortex overlying subcort-
tical stroke, in keeping with SNL, whereas 2 did not. However, reduced central benzodiazepine receptor binding may also reflect terminal degeneration or receptor downregulation from disconnection, rather than SNL. Furthermore, brain uptake of IMZ shows dependence on initial delivery, whereas delivery influences initial FMZ uptake only, and kinetic modeling reliably differentiates binding from delivery.

Although FMZ has not thus far been used to assess SNL in vivo, reduced in vitro $^3$H-FMZ and ex vivo $^{123}$I-IMZ binding, colocalizing with SNL, has been reported after temporary ischemia, and reduced FMZ binding correlates with neuronal loss in temporal epilepsy.

Here we obtained FMZ PET and postmortem in the chronic stage after prolonged tMCAo in the baboon. The time course of perfusion and oxygen metabolism from MCAo onwards was also obtained.

**Methods**

**Experimental Protocol**

Five adolescent male Papio anubis baboons (7 to 19 kg) were subjected to MCAo and serial $^{15}$O-PET, as detailed elsewhere, with the only addition here being an FMZ study obtained after the final $^{15}$O-PET session. The anesthetic protocol avoided interference with FMZ binding.

After surgery, neurological deficit was scored at regular intervals using a 5-point stroke scale.

**PET Procedures**

We used the ECAT HR+ device (Siemens; intrinsic spatial resolution, 4.5×4.5×4.6 mm; xyz, 63 slices; voxel size, 1.47×1.47×2.42 mm). The procedures are detailed elsewhere.

Using the steady-state $^{15}$O method, the cerebral blood flow (CBF), cerebral oxygen consumption (CMRO$_2$), and oxygen extraction fraction (OEF) were measured 2 weeks before surgery (pre-MCAo), at ~1 hour (MCAo1) and 17.5 hours (MCAo2) after MCAo, and at ~1.5 hours after reperfusion (Rep1; performed at ~20 hours after occlusion). A final PET session (Rep2) took place at approximately day 30 (range, 21 to 47).

The FMZ study was performed ~30 minutes after the Rep1 $^{15}$O study. $[^1]$CFMZ was produced with specific activities of 271±71 $\mu$Ci/µmol at time of injection; tracer amounts were administered (5.16±2.51 mCi). Acquisition lasted for 60 minutes according to 12 frames (2×30 sec; 4×60 sec; 1×300 sec; 5×600 sec). Arterial samples were taken each 4 sec from 0 to 60 sec, then at 3-, 5-, and finally 10-minute intervals until 60 minutes. The percentage of $[^1]$C-labeled metabolites in plasma was determined at 6 time-points. T1- and T2-weighted MRI scans were obtained at ~25 days after reperfusion.

**Image Processing**

To obtain high count rate FMZ images that represent binding as independent of perfusion as possible, the last 4 frames (ie, 20 to 60 min) were summed, generating an image set termed FMZ20 to 60.

Coregistration of PET and T1-MR was performed using FMZ20 to 60 as reference, using MRI-Tool (Multi-Purpose Imaging Tool; Advance Tomo Vision GmbH). All remaining PET and MRI data sets were registered to this reference.

Because we had no a priori hypothesis about the topography of FMZ changes within the occluded MCA territory, we designed an objective voxel-based analysis to automatically identify voxels with reduced FMZ uptake, as follows.

Infarct masks were delineated on coregistered MRI. An MCA cortical territory mask was drawn on the nonoccluded hemisphere on the T1-weighted data set, on each plane that included the infarct plus 3 planes cranially and caudally, encompassing the gray matter gyri and interlaced white matter. This mask was mirror-copied onto the occluded hemisphere. The Infarct mask, plus a rim of 3 voxels to account for partial voluming, was subtracted from this mask, and this modified mask was mirrored back onto the nonoccluded hemisphere, making the definitive MCA masks.

The mean (and SD) FMZ20 to 60 voxel values for the healthy hemisphere MCA mask was obtained for each plane. Voxel-of-interest (VOIs) with FMZ20 to 60 uptake reduction were detected by applying the lower 95% confidence limit. The percentage of VOIs relative to the total voxel population in the MCA masks was computed. We predicted that this percentage would not differ from 5% on the nonoccluded hemisphere, ie, chance level, but would be significantly >5% on the occluded side.

The mean FMZ20 to 60% uptake for the VOIs and for the whole MCA masks, expressed as percentage of injected dose per 100 mL (% injected dose/100 mL), was then obtained and compared between the 2 sides.

We then used a validated kinetic model that provides K1 (min$^{-1}$), the transport of the ligand from blood to brain, and the ligand distribution volume (DV [mL/mL]), an index of specific binding. The arterial input function was corrected for labeled metabolites. To limit the potential effects of noise, modeling was performed on time-activity curves averaged across voxel clusters rather than voxel-wise. To this end, we extracted all clusters comprising at least 5 contiguous VOIs and generated their mirrors on the healthy hemisphere.

**Hemodynamic and Metabolic Data**

The hemodynamic and metabolic parameters for the selected clusters and their mirrors were obtained across all PET sessions. Occluded/healthy hemisphere ratios were then calculated for each subject and time point.

**Histopathology**

Perfusion-fixation was performed within days of Rep2. The brain was embedded in paraffin, cut in 9-µm coronal sections approximately orthogonal to the anterior commissure, and stained with hematoxylin-eosin (and glial fibrillary acidic protein [GFAP] for a subset of sections).

To match as precisely as possible the histological sections to the T1-MRI volume, we used as reference the center of the anterior commissure on coronal planes of both data sets. The location of each cluster was then reported on the axial, coronal, and sagittal MRI planes, using MIRcro. Using major anatomical landmarks such as large cortical gyri, easily identifiable on both MRI and histological sections, the clusters were then manually drawn on the histological sections, with the center of the anterior commissure as reference. The number of histological coronal sections finally retained for each cluster accounted for its axial extent on the FMZ20 to 60 images.

To assess SNL, we used a 5-point scale comparing each area to their mirror, as follows: 0=normal; 1=definite but mild; 2=moderate; 3=severe, but with preserved tissue structure; and 4=pan-necrosis. Two authors (C.G. and J.C.B.) independently scored all selected areas. Regions for which both observers scored >0 were further analyzed, and only definite changes by consensus were retained. Findings regarding the infarct have been reported elsewhere.

**Statistical Analysis**

Comparisons to target 5% used 2-tailed t tests. All comparisons between hemispheres used nonparametric tests to account for the small sample.

**Results**

**Neurological Scores**

Acutely, all animals displayed left hemiparesis, together with turning of head and eyes to the right, but regained normal leg mobility within 1 week. Variable recovery of upper limb took place during the second week. Before euthanasia all animals...
exhibited equal impairment of the left hand only (supplemental Table I, available online at http://stroke.ahajournals.org).

11C-Flumazenil

Figure 1 depicts clearly reduced FMZ uptake in noninfarcted cortical MCA areas in all 5 animals, particularly conspicuous in V1 (frontal and temporal cortex), V4 (frontal and temporal), and V5 (frontal, parietal and temporal).

Figure 2 depicts the VOIs in each animal. They were clearly more prevalent on the occluded hemisphere, appearing either as small scattered clusters or as extended rims mainly located over dorsal aspects of the frontal and parietal lobes, occasionally extending over orbitofrontal and temporal areas.

The percentage of VOIs in the MCA masks was 5.4±0.8% for the nonoccluded hemisphere, not different from 5%, and 13.6±0.9% for the occluded hemisphere, greater than 5% (P<0.001) and than the nonoccluded side (P<0.05).

FMZ uptake for the VOIs on the occluded and nonoccluded hemispheres was 1.839 (±0.50) and 1.874 (±0.50) percent injected dose/100 mL, and corresponding values for whole MCA masks were 2.60 (±0.67) and 2.80 (±0.70) percent injected dose/100 mL, respectively (P<0.05 for both).

Based on these results, VOI clusters were selected (N=27, 5, 2, 19, and 21 in baboons 1 to 5, respectively). FMZ time-activity curves were obtained for each cluster and mirror, and kinetic modeling was applied. Good quality fits were consistently obtained (supplemental Figure I). K1 was significantly reduced in 4 of 5 animals as well as across the group, while DV was significantly reduced in 3 of 5 animals, but the reduction across the group did not reach significance (Table 1). For whole MCA masks, both K1 and DV were significantly reduced on the occluded side in each animal as well as across the group. There was no evident relationship between the degree of FMZ binding reductions and neurological outcome.

| Table. Weighted-Mean K1 and DV across All Voxels Belonging to the Selected Clusters and the MCA Mask on the Occluded Hemisphere and Their Mirrors on the Nonoccluded Side |
|---|---|---|---|---|---|---|
| | Cortical Mask | | | Selected Clusters | | |
| | | | | | | |
| Baboon | | | | | | |
| V1 | 0.384† | 0.480 | 4.835† | 5.220 | 0.275† | 0.384 | 3.210† | 3.781 |
| V2 | 0.262† | 0.296 | 4.199† | 4.436 | 0.195† | 0.241 | 2.826† | 3.148 |
| V3 | 0.258† | 0.318 | 3.848† | 4.173 | 0.227 | 0.264 | 2.762 | 2.555 |
| V4 | 0.339† | 0.383 | 4.816† | 5.115 | 0.219† | 0.231 | 3.140 | 3.102 |
| V5 | 0.302† | 0.369 | 4.188† | 4.541 | 0.230† | 0.307 | 2.991† | 3.418 |
| Mean | 0.309* | 0.369 | 4.377* | 4.697 | 0.229* | 0.285 | 2.986 | 3.201 |
| SD | 0.053 | 0.071 | 0.433 | 0.452 | 0.029 | 0.062 | 0.193 | 0.451 |

*Significant difference between hemispheres across animals (P<0.05, Wilcoxon).
†Significant difference (P<0.05, Wilcoxon) between occluded and nonoccluded hemispheres, across the selected clusters in each animal.

Hemodynamic and Metabolic Variables

The individual time course is illustrated in Figure 3 (data in supplemental Table II). The changes were characterized by: (1) decrease in CBF and increase in OEF, but no change in

Figure 1. 11C-Flumazenil20 to 60 summed PET images, displayed for each animal. Occluded hemisphere is shown on the left side.
CMRO$_2$ at MCAO$_1$; (2) moderate but significant increase in CBF and decrease in CMRO$_2$ at MCAO$_2$; (3) further CMRO$_2$ decline at Rep$_1$, despite return of CBF to normal (note the significant fall in OEF); and (4) matched moderate decrease in both CBF and CMRO$_2$ at Rep$_2$, with normalized OEF.

There was no significant correlation between the FMZ data and the CBF, OEF, or CMRO$_2$ ratios at any time point (N=11005).

For instance, although baboon 1 had the greatest decreases in DV and CBF at MCAO$_1$ (Figure 3), baboon 5 had the least CBF decrease but a substantial reduction in DV, and baboon 3 had the opposite pattern.

**Histopathology**

No definite changes were observed in the vast majority of areas matching the clusters. However, minor or questionable changes (all class 1, except 2 class 2) were observed in at least 1 cluster in each animal except V3. Overall, changes were observed in 7 of 74 clusters across the group, consisting of an apparent reduction in the number of large neurons, their replacement by dark shrunken neurons (Figure 4a,b), or a decreased density of all neuron types (Figure 4c,d). The areas concerned were the superior temporal and parietal cortices in V1; parietal cortex in V2; inferior frontal cortex in V4; and superior insular cortex in V5 (bordering a cluster; Figure 4e,f). A variable degree of increased glial density was observed in these regions, together with occasional mild increase in GFAP staining. No instance of laminar necrosis was observed.

**FMZ Parametric Maps**

Although not originally planned, voxel-based maps of K1 and DV$^*$ were generated post hoc to assess whether the reductions in DV$^*$ found with the time-activity curves could be visualized voxel-wise, in a descriptive way. These maps turned out to be of good quality, and clearly depicted DV$^*$ decreases in noninfarcted cortical areas (Figure 5). We manually drew on each plane an oval-shaped region of interest encompassing the area with most reduced DV$^*$ (N=11 to 13 region of interests per subject), avoiding the infarct itself (Figure 5), and mirrored them onto the nonoccluded side. Across animals, both K1 and DV$^*$ were significantly reduced in the occluded relative to nonoccluded side ($P<0.05$), with percentage reductions of 9.7±1.0% for DV$^*$ and 22.0±6.2% for K1 (no significant correlation). Histopathologically, these regions of interest did not exhibit more prominent changes than those found in the clusters, with dark neurones observed in 1 subject (V1).

**Discussion**

We document consistent FMZ binding decreases in noninfarcted MCA cortical areas after 20-hour tMCAo in the
baboon, though with substantial individual variations. Importantly, these areas exhibited ischemia acutely, suggesting neuronal damage developed from prolonged tissue hypoxia. Somewhat surprisingly, however, these FMZ alterations were subtended by only moderate and inconsistent histopathological changes.

To detect voxels with low FMZ uptake, we designed an objective and comprehensive voxel-based method, subjected to rigorous validation. There was greater-than-chance incidence of VOIs in the affected hemisphere, and a significant reduction in FMZ$_{20-60}$ uptake in the VOIs, consistent with simple visual inspection. Kinetic modeling confirmed significantly reduced FMZ binding in 3 of 5 animals for the clusters, and 5 of 5 for the cortical masks.

Here, perfusion-fixation was implemented to allow infarct volumetry, precluding formal neuron counting, as in all previous primate stroke studies. Detecting SNL in the gyrencephalic brain is notoriously difficult, particularly in the chronic stage when acutely dead neurons have vanished, leaving elusive “ghosts.” We used a conservative semi-
quantitative visual assessment, so more subtle changes may have been missed. Furthermore, comparing in vivo functional imaging data (mm-range resolution) to histological sections (μm-range resolution) is notoriously difficult. Because only a few histological sections within each PET-defined cluster were assessed, more severe changes in interleaved slices may have been missed; however, post hoc random search did not support this idea. More severe changes may affect other areas, but even post hoc analysis of the region of interests with maximal DV decrease did not reveal more conspicuous histopathology, although searching in the whole baboon brain would be impractical. Thus, the histopathological correlates of reduced cortical FMZ binding after tMCAo appeared mild at best.

Our findings are largely consistent with available literature. Sette et al reported reduced chronic-stage FMZ binding in the “peri-infarct area,” defined as a circumferential rim around the CT-based infarct, but histopathological correlates were not formally reported. A subsequent similar baboon study, reported in abstract form, was entirely consistent with the present investigation. Reduced central benzodiazepine receptor binding has been reported in the striatum and
hippocampus after tMCAo in the rat, colocalizing with histopathological SNL/neuron damage. In tMCAo cats, Heiss et al reported reduced FMZ binding surrounding the infarct, with some postmortem evidence for SNL, but detail is lacking. Finally, clinical SPECT studies have reported decreased IMZ in neocortical areas overlying large striato-capsular infarcts, though not consistently. Taken together, reductions in FMZ/IMZ binding in the noninfarcted penumbra appear consistently mild to moderate, except perhaps nearer the infarct. Such moderate FMZ decreases would be expected to be underlain by subtle, rather than marked, neuronal damage.

In this study, average decreases in FMZ binding were of a few percent only, but reached 15% in the most affected baboon (V1), and up to 20% in individual clusters. Because it was impossible to define individual “mirror” voxels because of asymmetries in sulci anatomy, VOIs were defined relative to a contralateral whole cortical MCA territory mask. As a result, voxels at the borders rather than the center of the cortical ribbon were preferentially detected, hence the frequent crescent shapes (Figure 2), with attending underestimation of changes attributable to partial voluming. Furthermore, to facilitate interpretation, our method avoided the immediate infarct vicinity, where SNL likely predominates. Nevertheless, our results compare well with previously reported average IMZ reductions of 11%, 5%, 11%, and 5.5%.

Figure 5. Illustrative K1 and DV parametric maps in each baboon, also showing typical region of interests positioned over noninfarcted cortical areas exhibiting reduced DV.

The lack of clear correlation between acute-stage CBF and eventual FMZ reduction, consistent with 1 human study, suggests that complex relationships prevail. PET studies are only snapshots, and CBF may fluctuate; in addition, some degree of spontaneous reperfusion occurred here, which was unexpected and could have confounded this correlation. After clip removal, there was marked reperfusion, as expected for the eventually surviving penumbra. Thus, the observed FMZ changes likely reflect subtle neuronal damage in the reperfused at-risk tissue.

Apart from SNL per se, loss of synapses or dendritic spines with preserved neuron bodies is well-reported in the reperfused cortex of the rodent and may have contributed to the reduced FMZ binding observed here. An entirely different interpretation invokes receptor downregulation in disconnected areas. Reduced GABA receptor subunit (which bears the central benzodiazepine receptor) surrounding cortical lesions has been reported in rats. The matched reduction in CBF and CMRO in the areas affected by reduced FMZ here does not provide a useful clue because it could reflect deafferentation just as well as ischemic neuronal damage. Against the deafferentation hypothesis, however, areas of reduced FMZ appeared confined to the MCA territory (Figure 1) rather than based on anatomical connections, and previous studies have consistently shown that areas exhibiting diaschisis have normal FMZ binding. A combination of neuronal damage and deafferentation, or even apoptosis, may, however, underlie some of the changes observed here.

Despite 20-hour MCAo, mainly striato-capsular infarcts ensued, which differs from usual human observations. However, cortical infarction was present in baboon V5 here, and also occurred after permanent MCAo. The limited infarcts in this baboon model are probably largely attributable to...
neuroprotective effects of anesthesia.\textsuperscript{30} Accordingly, much larger infarcts were reported in pioneer MCAo studies in the awake monkey.\textsuperscript{32} We elected 20-hour tMCAo because this seemed optimal to elicit SNL because it causes prolonged cortical ischemia but small infarcts. However, unexpected partial spontaneous reperfusion might have prevented full-blown SNL. Although pMCAo might produce more severe SNL, it may confound measurement of specific binding attributable to persistently reduced perfusion, and would not be relevant to clinical thrombolysis.

In this study, only cortical areas were searched for reduced FMZ uptake because central benzodiazepine receptor are scarce in the basal ganglia.\textsuperscript{20,28} However, the basal ganglia appear particularly vulnerable to SNL,\textsuperscript{1,7,8} so using radioligands with high affinity there would be of interest.

The behavioral correlates of our findings remain elusive. All our baboons made an excellent recovery although the affected hand remained impaired, probably from direct damage to the cortico-spinal tract. Because formal cognitive assessment was not performed, subtle “cortical” signs may have been missed. Plasticity might, however, compensate any effects of SNL, although would tap the “brain reserve.” Detailed behavioral studies with correlation to FMZ binding would be of interest.

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Disclosures
None.

References


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