Age-Dependent Exacerbation of White Matter Stroke Outcomes
A Role for Oxidative Damage and Inflammatory Mediators

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Background and Purpose—Subcortical white matter stroke (WMS) constitutes up to 30% of all stroke subtypes. Mechanisms of oligodendrocyte and axon injury and repair play a central role in the damage and recovery after this type of stroke, and a comprehensive study of these processes requires a specialized experimental model that is different from common large artery, gray matter stroke models. Diminished recovery from stroke in aged patients implies that damage and repair processes are affected by advanced age, but such effects have not been studied in WMS.

Methods—WMS was produced with focal microinjection of the vasoconstrictor N5-(1-iminoethyl)-L-ornithine into the subcortical white matter ventral to the mouse forelimb motor cortex in young adult (2 months), middle-aged (15 months), and aged mice (24 months).

Results—WMS produced localized oligodendrocyte cell death with higher numbers of apoptotic cells and greater oxidative damage in aged brains than in young-adult brains. Increased expression of monocyte chemotactic protein-1 and tumor necrosis factor-α in motor cortex neurons correlated with a more distributed microglial activation in aged brains 7 days after WMS. At 2 months, aged mice displayed increased white matter atrophy and greater loss of corticostriatal connections compared with young-adult mice. Behavioral testing revealed an age-dependent exacerbation of forelimb motor deficits caused by the stroke, with decreased long-term functional recovery in aged animals.

Conclusions—Age has a profound effect on the outcome of WMS, with more prolonged cell death and oxidative damage, increased inflammation, greater secondary white matter atrophy, and a worse behavioral effect in aged versus young-adult mice. (Stroke. 2013;44:2579-2586.)

Key Words: aging ■ axonal degeneration ■ inflammation ■ oligodendroglia ■ oxidative stress ■ stroke ■ white matter

Subcortical white matter stroke (WMS) constitutes up to 30% of all stroke subtypes,1,2 and has devastating clinical consequences. These infarcts produce focal deficits with incomplete recovery and are the second leading cause of dementia.3,4 Despite an abundance of preclinical literature featuring animal models of large artery, gray matter stroke, there have been few studies of white matter repair and recovery, a disparity which can be attributed to a lack of an effective animal model of WMS.

In addition, most studies using models of ischemic stroke have been performed on young animals. Ischemic stroke occurs mostly in older individuals, and recovery in aged patients is diminished compared with young patients.5,6 Aged animals differ from young animals in their neurophysiology, and pathophysiologic features of brain ischemia differ across the lifespan.7,8 Furthermore, remyelination efficiency in white matter lesions decreases with age.9 Thus, studies using young animals to model ischemic stroke may have not fully estimated the effects of ischemia on brain tissue in aged subjects.

We have recently developed a model of subcortical stroke in white matter below the mouse forelimb motor cortex that models many aspects of this disease in humans. The model includes an induction of focal ischemia resulting in cell death and white matter destruction at the infarct core, and a peri-infarct zone of partial myelin, axon, and oligodendrocyte loss.10,11 This model has been adapted here to study how the damage and functional consequences after white matter ischemia differ between the young and aged brain.

Methods

Mice

All experiments were performed in accordance with National Institutes of Health animal protection guidelines and were approved by the University of California at Los Angeles Animal Research Committee. Thirty-two 3-month-old C57/BL6 mice (Jackson), twenty-four...
15-month-old, and twenty-eight 24-month-old C57/BL mice (National Institute on Aging) were used in this study. Cohorts of animals (n=6–8 per group) survived to 1, 2, 7, and 56 days after stroke or sham procedure.

White Matter Stroke
White matter stroke was produced as described\textsuperscript{10} with modifications. The vasoconstrictor N5-(1-iminoethyl)-L-ornithine (27 mg/mL in sterile physiological saline; Millipore) was injected via micropipette through the cortex at an angle of 45° with a dorso-posterior to ventro-anterior injection path into the white matter underlying the forelimb motor cortex. This procedure promotes intense vasoconstriction at the site of injection, leading to focal ischemia.\textsuperscript{10,12} Three injections (each of 200 nL N5-(1-iminoethyl)-L-ornithine solution) were made in the following coordinates: A/P: +0.5, 0, −0.5; M/L: −0.96 (for all 3 injections); and D/V: −1.2, −1.15, −1.1. The pipette was left in situ for 5 minutes after injection to allow proper diffusion. Control animals underwent a sham procedure during which a craniotomy was done similarly to stroke animals, with no subsequent injections.

Tissue Processing, Microscopy, and Stereology
The tissue was processed as described previously\textsuperscript{10,11} (See detailed staining methods in the online-only Data Supplement).

Behavior
Gait and forelimb function were assessed in the grid-walking task and the pasta matrix task as described previously\textsuperscript{13–17} (See detailed behavior methods in the online-only Data Supplement).

Statistical Analysis
Mice were randomly allocated to treatment groups. All tests were analyzed blindly to stroke condition or age. The required number of animals per group was determined by a power analysis, with the expected variance and change in performance in the pasta matrix and grid-walking tasks predicted based on preliminary studies in

Figure 1. Apoptotic cell death and oxidative damage after white matter stroke (WMS). Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)–positive cells (green) localized to the infarct core 24 and 48 hours after stroke. Cell nuclei stained with propidium iodide (red). Scale bar=200 μm (A). 8-Hydroxy-2’-deoxyguanosine (8-OhdG)–positive cells (red) localized to the infarct core 24 and 48 hours after stroke. Cell nuclei stained with 4’,6-diamidino-2-phenylindole (DAPI; blue). Scale bar=100 μm (B). TUNEL–positive cells (green, arrows) express the oligodendrocyte marker Olig2 (red). Scale bar=50 μm (C). At 48 hours, there is a significant increase in the number of apoptotic cells and cells displaying oxidative damage in aged vs young-adult (D). *P<0.03; **P<0.01.
young-adult animals. A group size of n=8 was predicted to be sufficient to detect a statistically significant result in ANOVA with α=0.05 and power >0.8. All data are expressed as mean±SEM. For cell quantification, axonal degeneration, white matter measurements, and behavioral testing, differences between age and treatment groups were analyzed using 1-way or 2-way ANOVA, with level of significance set at P<0.05, with Tukey honestly significant difference post hoc analysis (Excel and SigmaStat).

Results

Infarct Volume, Oligodendrocyte Death, and Oxidative Damage After WMS

To assess the differences in initial tissue damage after WMS between the young and aged brain, animals from the 2 age groups were euthanized 24 and 48 hours after stroke. Infarct volume was similar 24 and 48 hours after WMS, and no difference was detected between young-adult and aged animals (Figure 1 in the online-only Data Supplement; P>0.3; n=8). Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining 24 hours after stroke revealed extensive cell death throughout the infarct core in young-adult and aged mice (Figure 1A). In both age groups, the apoptotic cells were localized to the white matter lesion and were not detected in surrounding cortical areas, confirming the focal nature of this stroke type. The number of apoptotic cells did not differ significantly between young and aged animals at the 24-hour time point (Figure 1A and 1D; P>0.1; n=8). However, 48 hours after WMS, the number of apoptotic cells was significantly higher in aged animals compared with young animals, resulting in a total higher number of apoptotic cells detected in aged animals (Figure 1A and 1D; P<0.01; n=8; terminal deoxynucleotidyl transferase dUTP nick-end labeling and the oligodendrocyte marker, Olig2, confirmed that cell death was primarily induced in oligodendrocytes (Figure 1C). Staining for 8-hydroxydeoxyguanosine revealed cells positive for this oxidative DNA adduct within the lesion. Similar to the observed pattern of apoptosis, there was no difference between the age groups in the number of cells displaying oxidative damage 24 hours after WMS, but the number was higher in aged animals 48 hours after stroke (Figure 1B and 1D; P<0.03; n=8).

Figure 2. Reactive microglia in the infarct core and motor cortex. White matter stroke (WMS) lesions are densely filled with Iba-1–positive microglia (red) 7 days after stroke. Cell nuclei stained with 4',6-diamidino-2-phenylindole (blue). Scale bar=100 µm (A). Iba-1–positive microglia (red) are more extensive in the motor cortex of aged animals. Scale bar=25 µm (B). There is no difference in Iba-1–positive cell number in white matter stroke lesions (C), but an increase in motor cortex of aged mice (D). *P<0.05, vs control young-adult (Y-A); **P<0.005, vs young-adult WMS; §P<0.01 aged control vs aged WMS.

Inflammatory Mediators and Reactive Cells After White Matter Stroke

The inflammatory response to stroke was assessed at 24 hours and 7 days, the latter being a time of maximal microglia/macrophage response in young adults with a related WMS.11 Ly6G (neutrophil)/Iba-1 (microglia/macrophage) staining 24 hours after WMS showed neutrophil infiltration in the infarct core and reactive microglia surrounding the lesion (Figure II A in the online-only Data Supplement). The numbers of neutrophils and Iba-1–positive microglia were similar between the age groups (Figure II B in the online-only Data Supplement; Iba-1, P>0.3; neutrophils, P>0.3; n=6). Seven days after stroke, a greater infiltration of activated microglia densely filled and surrounded the infarct core (Figure 2A). The density of Iba-1–positive cells in the lesion did not differ significantly between the age groups (Figure 2C; P>0.2; n=8). Interestingly, although the increase in Iba-1 staining after stroke in young animals was limited to the infarct area, in aged animals, there was a higher density of Iba-1–positive cells throughout the motor cortex compared with aged controls and young stroke animals (Figure 2B and 2D; control young-adult versus aged, P<0.05; WMS young-adult versus aged, P<0.005; control aged versus WMS aged, P<0.01; n=8).

To test whether upregulation of inflammatory mediators could have contributed to microglial activation in the cortex, expression of monocye chemotactic protein-1 (MCP1) and the proinflammatory cytokine tumor necrosis factor-α (TNFα) was measured. Seven days after stroke, there was a robust MCP1 expression in reactive astrocytes in or around the infarct core (Figure IIIA in the online-only Data
The number of MCP1-positive astrocytes did not differ between age groups (Figure IIIB in the online-only Data Supplement; \(P > 0.3\); \(n=6\)). Interestingly, MCP1 could be transiently detected in a subset of motor cortex neurons only in aged brains 24 hours after stroke. Neuronal expression of MCP1 was not present in stroked young-adult animals or control animals of either age group, and was undetectable in stroked aged brains by day 7 (Figure 3C).

At day 7, TNF\(\alpha\) was expressed in neurons in cortical areas adjacent to the stroke lesion in both young-adult and aged mice (Figure 3A). When quantified in 3 regions at increasing distances from the infarct core, TNF\(\alpha\)-positive neurons were more numerous and extended further away from the infarct core in aged brains compared with young-adult (Y-A) brains (B). Monocyte chemotactic protein-1 (MCP1) expression (red) in motor cortex neurons (NeuN, green) 24 hours after white matter stroke (WMS; C). **\(P < 0.01\), vs young-adult WMS at same region.

**Long-term Tissue Outcome of White Matter Stroke: Myelin Loss and Axonal Degeneration**

Immunostaining for myelin basic protein revealed an area of myelin loss in the infarct core and a narrowing of the white matter medial to the stroke site at late time points after stroke (56 days; Figure 4). To quantify this volume change, the thickness of the white matter was measured at 10 equal increments from the midline (1) to the center of the infarct lesion (or equivalent point in controls: 10). A local decrease in the thickness of the white matter after stroke was observed in both young-adult and aged animals. However, it was markedly more pronounced in aged animals (Figure 4B; young-adult WMS versus control \(P < 0.04\) at interval #8; aged WMS versus control \(P < 0.005\) at intervals #6–9; WMS young-adult versus aged \(P < 0.009\) at intervals #6–9; \(n=8\)).

Immunostaining for the axonal marker NF200 revealed that the loss of myelin in the white matter was accompanied by loss of axonal projections from the cortex to the dorso-medial striatum after stroke. These axonal bundles include corticostriatal projections from the ipsilateral and contralateral cortex that play an important part in controlling motor programs and movement. Quantification of axonal staining in the region of corticostriatal projections (See detailed axonal measurement methods in the online-only Data Supplement) indicated a loss of axonal bundles at a late time point after stroke that was more pronounced in aged animals compared with young animals (Figure 5C; young-adult WMS, \(P < 0.04\) compared with control; aged WMS, \(P < 0.01\) compared with control; aged WMS, \(P < 0.03\) compared with young-adult WMS; \(n=8\)).

**Functional Impairments After White Matter Stroke**

To test whether the greater degenerative loss of white matter tracts and corticostriatal projections in aged WMS correlated with functional impairments, the baseline performance of young-adult and aged mice was assessed before and after stroke in 2 behavioral tasks that rely on forelimb motor control. A third, middle-aged group of animals was similarly tested in these tasks, to better evaluate whether the effect of age on functional impairment is graded with advancing age.
The pasta matrix reach task is specifically aimed at evaluating strength, reach capacity, and dexterity of the mouse forelimb. Baseline performance in the pasta matrix reach task was similar across the 3 age groups (Figure IV A in the online-only Data Supplement; \( P > 0.1; n = 8 \)). After WMS, there was a significant decrease in performance in all age groups, but middle-aged and aged mice performed significantly worse than young-adult mice (Figure 6A; week-1 poststroke performance was \( 62.2 \pm 11.3\% \) of baseline in young adults; \( 41.1 \pm 5.7\% \) in middle-aged and \( 48.2 \pm 5.7\% \) in aged; \( P < 0.02; n = 8 \)). Functional recovery and return to control performance levels were observed in young-adult animals starting 4 weeks after stroke (Figure 6A, left; weeks 1–3, \( P < 0.05 \); weeks 4–8, \( P > 0.1; n = 8 \)), and in middle-aged animals 6 weeks after stroke (Figure 6A, middle; weeks 1–5, \( P < 0.005 \); weeks 6–8, \( P > 0.06; n = 8 \)). In contrast, no improvement in performance was observed in aged animals throughout the 8 weeks of testing (Figure 6A, right; weeks 1–8, \( P < 0.001; n = 8 \)).

Similar to the pasta matrix reach task, there were no differences between age groups in baseline performance in the grid-walking task (Figure IVB in the online-only Data Supplement; \( P > 0.3; n = 8 \)). Analysis of forelimb foot faults revealed a significant increase in the number of foot faults recorded in middle-aged and aged, but not young-adult mice, after WMS (Figure 6B; 7 days after stroke, young adults, \( P > 0.3 \); middle-aged, \( P < 0.005 \); aged \( P < 0.01 \); \( n = 8 \)). Eight weeks after stroke, there was a functional recovery and return to control performance levels in middle-aged mice but not aged mice (Figure 6B, middle and right; 56 days after stroke, middle-aged, \( P > 0.1 \); aged, \( P < 0.01 \); \( n = 8 \)). These behavioral results indicate an age-related worsening of initial motor performance after stroke and a decline in recovery with age.

**Discussion**

In the aged brain, white matter stroke produces more prolonged oligodendrocyte cell death and oxidative damage, more distributed microglial activation and expression of cytokines MCP1 and TNFα, increased white matter atrophy, and greater loss of corticostriatal connections compared with the young-adult brain. When comparing young-adult, middle-aged, and aged animals, stroke has an age dose–response, with progressively greater functional impairment and a greater initial behavioral deficit from young-adult to middle-aged and then aged animals.

The greater oligodendrocyte death and oxidative damage in aged animals, despite a similar areal extent of the lesion across age groups, may be caused by the ischemic injury and oxidative stress that follow white matter stroke and are more severe in aged animals.21 The axons in the white matter contain abundant mitochondria, a main source of reactive oxygen species after ischemia. Mitochondrial activity and integrity decrease during aging, and endogenous antioxidant system activities exhibit an age-dependent decline.22,24 Both processes may contribute to an increased susceptibility to ischemia, making mitochondria-mediated oxidative stress a predominant pathway of white matter injury in the aged brain.7

The increase in oligodendrocyte cell death after stroke in aged animals likely contributes to axonal degeneration through secondary demyelination. Loss of normal myelination damages axonal integrity and leads to axonal dysfunction.25,26 In addition, the ischemic insult has a direct effect on axons that may be more severe or occur through distinct mechanisms in aged animals compared with young animals.21,27 The present data show that a greater sensitivity of myelinated axons to ischemia is not reflected in an initially larger infarct zone in WMS, but in a substantially greater secondary loss of these axons with age.

There is an age-specific induction of several interacting arms of neuroinflammation after WMS. In the aged brain, WMS induces MCP1 and TNFα in neurons distant to the infarct, but the axons of which project through the ischemic area. Such a remote neuronal induction of MCP1 to axonal...
injury has been reported in nerve axotomy. These 2 cytokines attract and activate microglia and macrophages and may underlie the larger numbers of microglia/macrophages found in the cortical regions above a WMS in the aged brain. TNFα induces a more injurious response in tissue macrophages, greater damage to myelin and oligodendrocytes.

Figure 5. Loss of corticostriatal projections. Neurofilament 200 (NF200) staining shows loss of axons descending from the cortex into the dorso-medial striatum. The intensity was quantified in a 0.5×0.7-mm area (outlined; A). The corresponding plot profiles (B) show staining intensity (gray values) in each column of pixels from medial (left of graph) to lateral (right of graph) in the region outlined in (A). The cumulative intensity of NF200 in the regions of interest shows a significant decline in aged animals after WMS (C). *P<0.04; **P<0.01 compared with age-matched controls. #P<0.03 aged white matter stroke (WMS) vs young-adult WMS.

Figure 6. Behavioral deficits after white matter stroke (WMS). Performance in the pasta matrix reach task was impaired after WMS, with greater impairment in middle-aged (middle) and aged (right) mice and no functional recovery in aged mice (A). Performance in the grid-walking task was impaired after WMS only in middle-aged (middle) and aged (right) mice. There was no recovery in aged mice (B). *P<0.05; **P<0.01; ***P<0.005.
and can result in greater injury in hypoxic central and peripheral insults. A more widespread and damaging inflammatory response in the cortex of aged animals has been reported in other models of injury, such as chronic stress or systemic LPS injection.

The observed loss of corticostriatal projections likely underlies, at least in part, the motor impairment observed in this WMS model. The greater impairment observed in middle-aged and aged mice in the first week after stroke, and the diminished functional recovery in aged mice at longer time points, can be attributed respectively to greater initial damage and inefficient repair processes that result in lasting white matter atrophy. In addition to a more efficient white matter repair process in young brains, the behavioral recovery observed 4 weeks after stroke in young-adult mice, and 6 to 8 weeks after stroke in middle-aged mice, may be achieved through axonal sprouting and formation of new patterns of neuronal connections in adjacent brain regions. The gene observed 4 weeks after stroke in young-adult mice, and 6 to 8 weeks after stroke in middle-aged mice, may be achieved through axonal sprouting and formation of new patterns of neuronal connections in adjacent brain regions. The gene expression profile that drives this reorganization differs significantly between the young and aged brain, and this may further contribute to the diminished recovery after WMS in aged animals.

In summary, white matter stroke causes increased inflammation, oxidative tissue injury, and delayed white matter atrophy in aged animals, which translate into an age-dependent exacerbation of behavioral motor deficits. The use of a clinically relevant model is likely crucial in translational research aimed at developing therapeutic interventions for WMS. The present studies use an extensive and long-duration multimodal analysis of the cellular and behavioral phenotypes of WMS in the aged brain, setting the stage for future pharmacological approaches to mitigate this progressive damage.

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Disclosures

None.

References


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SUPPLEMENTAL MATERIAL

Age-dependent exacerbation of white matter stroke outcomes: a role for oxidative damage and inflammatory mediators

The Supplementary Material includes the following:

1. Methods
   I. Tissue Processing for TUNEL and Immunohistochemistry
   II. Quantification of NF200-positive axons
   III. Behavior

2. Figures
   I. Infarct volume 24 hours after WMS
   II. Reactive microglia and neutrophil infiltration 24 hours after WMS
   III. MCP1 expression in infarct core 7 days after WMS
   IV. Baseline performance in behavioral tests

3. References
1. Supplementary Methods

I. Tissue Processing for TUNEL and Immunohistochemistry

Animals were perfused transcardially with 0.1 M phosphate buffered saline followed by 4% paraformaldehyde. Following cryoprotection in 30% sucrose brains were frozen and sectioned using a cryostat (Leica CM 0530) into 20-35µm sections. For Iba-1, NF200, MBP and Olig2 immunostaining, the sections were blocked in 5% donkey serum, incubated with a primary antibody over night at 4°C, incubated with a secondary antibody for 1-2 hours at room temperature, and coverslipped with DPX. Primary antibodies were: rabbit anti Iba-1 (1:1000, Wako Chemicals) rabbit anti-NF200 (1:1000, Sigma) rat anti-myelin basic protein (MBP, 1:1000, Millipore), rabbit anti Olig2 (1:500, Millipore), mouse anti-8-OHdG (1:100, Abcam), rat anti-neutrophil (1:100, Abcam), rabbit anti-MCP1 (1:500, Novus Biologicals), goat anti-TNFα (1:500, R&D Systems), rat anti-GFAP (1:500, Millipore). All secondary antibodies were donkey F(ab)2 fragments conjugated to cy2 or cy3 (Jackson Immunoresearch) and were used at a dilution of 1:500. TUNEL assay was performed using ApopTag® In Situ Apoptosis Detection Kit (S7110, Millipore). Briefly, sections were incubated with terminal deoxynucleotidyl transferase which catalyzes the addition of nucleotide triphosphates labeled with digoxigenin to the 3’-OH ends of double-stranded or single-stranded DNA (which localize in apoptotic bodies in high concentrations). The digoxigenin was then detected with an antibody conjugated to fluorescein. Nuclear stains were performed by incubating the sections for 5 minutes in phosphate-buffered saline containing either DAPI or propidium iodide before coverslipping.

II. Quantification of NF200-positive axons

Analysis of corticostriatal projections was performed in high-resolution confocal images and Z-stacks of sections stained for the axonal marker NF200. To quantify the axonal loss, intensity profiles were created for 0.5x0.7mm areas in the dorsomedial striatum (sample regions of interest outlined in Figure 5A with corresponding ImageJ plot profiles in Figure 5B). Peak grey values were added across plots to yield the cumulative NF200 intensity for the regions of interest (Figure 5C).

III. Behavior

The pasta matrix task was performed as described previously. In this task, mice reach through a window to retrieve small pieces (3.3 cm in height and 1 mm diameter) of vertically-oriented capellini pasta from a grid arrangement on a shelf in front of the animal. Mice were trained daily on the task for 5 weeks before stroke. The number of pasta breaks per 10-minute daily session was averaged across two consecutive days of training. Acquisition of the task was defined as a >4 average with a standard deviation <6 of breaks. Mice that failed to learn the task after 5 weeks of training were excluded from the study. Following the stroke the mice were tested weekly, on two consecutive days each week, for 8 weeks. Performance on the two days of testing
each week was averaged and compared to the pre-stroke performance (each mouse was compared to its baseline pre-stroke performance). The grid-walking task was performed as previously described 3-5. The total number of footfaults as well as the total number of correct, non-footfault steps were counted, and a correct step percentage was calculated. Mice were tested in the grid-walking task once before the stroke to establish baseline performance levels, and then re-tested 1 week, one month and two months following the stroke.

2. Supplementary Figures

**Supplementary Figure I.** Infarct volume 24 hours after WMS. The infarct core is defined in the white matter by surrounding microglia (red) expressing Iba-1. Scale bar = 50µm (A). Similar infarct volume between young adult and aged animals 24h after WMS (B).
Supplementary Figure II. Reactive microglia and neutrophil infiltration 24 hours after WMS. Iba-1-positive microglia (red) are present around the outline of the lesion, while Ly6G-positive neutrophils (green) can be detected throughout the lesion core. Scale bar = 20µm (A). Similar numbers of microglia and neutrophils in young-adult and aged mice 24h after WMS (B,C).
Supplementary Figure III. MCP1 expression in infarct core 7 days after WMS. GFAP-positive astrocytes (green) express MCP1(red). Similar numbers of MCP1-expressing astrocytes in young-adult and aged mice 7 days after WMS (B).
**Supplementary Figure IV.** Baseline performance in behavioral tests. Pasta matrix reach task: average number of pasta breaks for a 10-minute session before WMS was similar between age groups (A). Grid-walking task: percent of correct steps in a 10-minute session was similar between age groups.
3. References

1. Tennant KA, Jones TA. Sensorimotor behavioral effects of endothelin-1 induced small cortical infarcts in c57bl/6 mice. *Journal of neuroscience methods*. 2009;181:18-26


