

## Author



# The Aged Cortex is Capable of Sensory-Induced Recovery from Ischemic Stroke

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*Psychology*

In her freshman year, Nina Butingan was inspired by a seminar, “Biology of the Mind,” that was offered by Dr. Frostig. As a result, she started working in Dr. Frostig’s lab the next year and eventually expressed interest in conducting her own project. The lab’s stroke protection studies are unique in that the neuro-protection provided from their treatment is complete—their animals behave and appear neurologically as intact as if they had not suffered a stroke. Nina’s project further questions the significance of the lab’s previous findings in a more clinically-relevant aged population of rats. Nina has continued to work in Dr. Frostig’s lab after her graduation in June 2012, and hopes to attend medical school.

## Key Terms

- ♦ Aged
- ♦ Ischemic Stroke
- ♦ Neuroplasticity
- ♦ Neuroprotection
- ♦ Permanent Middle Cerebral Artery Occlusion
- ♦ Rodent Model

## Abstract

Our lab has demonstrated that single-whisker stimulation is completely protective of the young adult rodent cortex when administered within 0 to 2 hours following a permanent middle cerebral artery occlusion (pMCAO). The results from this study, however, have yet to be tested in a more clinically-relevant aged group of animals. Research has suggested that the aged cortex is more vulnerable to stroke and less resilient in recovering from a vascular accident. In this study, single-whisker stimulation stroke treatment was assessed in the aged rodent cortex. Aged rats (21–24 months of age) were subject to a behavioral assessment a week prior to pMCAO and then divided into treated experimental and untreated control groups. A week following pMCAO, animals were again assessed behaviorally. According to behavioral and histological analysis, animals that received the treatment were equivalent to healthy control animals, while untreated controls showed impaired sensorimotor behavior following pMCAO and sustained cortical infarct. This suggests that mild sensory stimulation, in the form of a single-whisker treatment, is neuroprotective in the aged rodent model. Moreover, this suggests that translation of non-invasive treatments, such as cortical stimulation that could redirect blood flow to the appropriate brain areas, into the appropriate age-equivalent human population may indeed be possible.

## Faculty Mentor

The aged brain suffers from a bad reputation, especially when it comes to devastating problems like stroke. We have previously showed that mild sensory stimulation could completely protect the young-adult cortex of rats from an impending stroke. However, the major population vulnerable to stroke is the aged population. Using several techniques, the paper by Nina Buttingen describes how the same mild sensory stimulation can also completely protect the aged cortex from impending stroke, exactly like in young adults, despite its reputation. Taken together, this new non-pharmacological, non-invasive and no side-effects treatment holds much promise in helping stroke victims anywhere by first responders, even before the ambulance arrives. In my opinion, this paper is a great example of mentored research supported by UROP.

**Ron D. Frostig**

*School of Biological Sciences*

## Introduction

Stroke is a leading cause of death and long-term disability in the United States, trailing only cancer and cardiovascular disease, and affecting nearly 800,000 Americans each year (Roger et al., 2011). An ischemic stroke, defined as damage to the brain resulting from an arterial blockage typically caused by a clot, is the most common type of stroke to occur, and the Middle Cerebral Artery (MCA) is the most commonly affected vessel. Areas of the brain that sustain deprivation for extended periods eventually die off, causing what is known as an infarct to the affected area. The extent of recovery is influenced by many factors such as the severity and location of the ischemia as well as access to medical intervention and treatment within the first few hours of onset. While it is possible to recover from an ischemic stroke, fewer than 5% of patients suffering from the ischemia receive treatment with tissue plasminogen activator (tPA), which is used to dissolve blood clots formed in arteries and is currently the only approved stroke treatment. The need for a more immediate and noninvasive form of treatment, capable of being delivered within minutes of stroke onset, is necessary for enhancing the recovery of victims following stroke.

In order to combat the problem, our lab models the most common type of stroke using a rodent model of MCA ischemia. Using functional imaging, neuronal recording, blood flow imaging, histology, and behavioral assessment, our lab has demonstrated that mild sensory stimulation in young adult Sprague Dawley rats (aged 3–4 months old) completely protects the brain from cortical infarct when treatment is provided within a critical period of 0–2 hours following permanent Middle Cerebral Artery Occlusion (pMCAO) (Lay et al., 2010; Davis et al., 2011). Stimulation was provided for 120 minutes to a single whisker. When assessed 24 hours later, the young adult Sprague Dawleys that received treatment within 0–2 hours following pMCAO were fully intact according to all the techniques aforementioned.

While our results suggest that sensory stimulation is neuroprotective for the young adult rodent cortex, strokes in humans are most prevalent in older adults, with onset typically occurring around the age of 55 (Rogers et al., 2011). Therefore, it was important to determine if the same sensory stimulation treatment would be protective in the aged cortex. To address this question, aged rats (21–24 months of age; equivalent to 60–65 years of age in humans) were subject to the same surgical techniques of pMCAO as the young adult rats in our previous study (Quinn, 2005). Behavioral assessment was performed to assess senso-

rimotor capabilities a week prior to pMCAO using three tasks: the Bederson neurological scale, forepaw-guided exploration, and whisker-guided exploration. The rats were then divided into two groups: treated rats received whisker stimulation immediately following pMCAO, and untreated control rats never received stimulation. A week following pMCAO, the aged rats were again assessed behaviorally. After the post-pMCAO behavioral assessment, the brains were removed and stained to determine infarct volumes.

Untreated animals that received no stimulation after pMCAO were significantly impaired behaviorally and sustained cortical infarct. Unlike the untreated animals, animals that received single-whisker stimulation after pMCAO were behaviorally intact and had no cortical infarct. Because of the effectiveness of this treatment in the rodent model, the results from this study have the potential to be translated into a clinical model for stroke treatment, which could be delivered within minutes after the onset of stroke symptoms. Furthermore, the successful recovery of aged rats subjected to single whisker stimulation suggests that the more vulnerable, aged cortex is capable of recovery from impending ischemic injury.

## Materials and Methods

All experiments were carried out in accordance with the Institutional Animal Care and Use Committee at the University of California, Irvine, and were consistent with Federal guidelines.

A week prior to pMCAO, all animals underwent a behavioral assessment. Following pMCAO, animals were subdivided into two treatment types: a treated experimental group and an untreated control group. Animals from both groups were allowed to recover for seven days, after which they underwent another behavioral assessment. After behavioral analysis, the rats were euthanized and had their brains removed. Staining with 2% 2,3,5-triphenyltetrazolium chloride (TTC) was performed to assess for infarct volume. For a detailed outline, refer to Figure 1.

### *Rats & Surgical Preparation*

Male Fischer 344 rats (21–24 months of age) were kept under standard laboratory conditions. The rats, raised individually, had free access to food and water. Body weights ranged from 350–550 g. For surgical preparation, rats were injected with a Nembutal bolus (55 mg/kg b.w.) followed by an injection of atropine (0.05 mg/kg b.w.). Supplemental injections of Nembutal (27.5 mg/kg b.w.) were given as necessary. Breathing was unassisted.

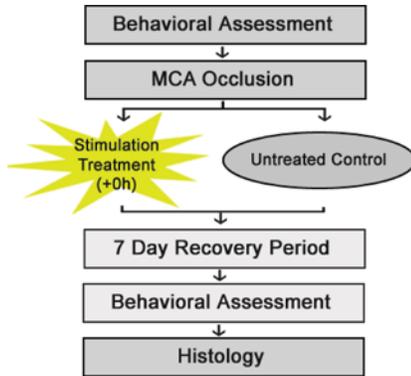


Figure 1

All animals (treated group  $n=10$ , untreated group  $n=8$ ) were behaviorally assessed seven days prior to pMCAO, and seven days following pMCAO. Occlusion surgery was performed on one animal every week for each animal, regardless of treatment type. Results from behavioral assessment were scored and recorded for analysis. Immediately following the post-treatment behavioral assessment, the animals were euthanized and their brains were removed for TTC staining. Brain infarct volumes were calculated from the obtained data.

### Permanent Middle Cerebral Artery Occlusion (pMCAO)

Ischemic conditions were achieved via surgical occlusion and transection of the M1 segment (just distal to lenticulostriate branching) of the left middle cerebral artery such that only MCA cortical branches would be affected, and thus only cortical infarct would be expected (Tamura et al., 1981, Brint et al., 1988, Wang-Fischer, 2009). The skull and dura were carefully removed from a 2x2mm surgical window over the occlusion location; the M1 segment of the MCA just distal to its lenticulostriate branching and a half-curve reverse cutting suture needle and thread (4-0 silk) was passed through the pial layer of the meninges, below the MCA and above the cortical surface. A double ligature was tied and tightened around the MCA and the vessel was then transected (completely severed) between the two knots. Experiments were terminated if there was any sign of bleeding from the MCA, or if there were obvious arterial abnormalities or malformations (Fox et al., 1993, Niiro et al., 1996).

### Stimulation Treatment

Rats were divided into either treated experimental or untreated control groups. Treated rats were administered intermittent whisker stimulation treatment to a single whisker once every 21 seconds, ranging randomly from 16–26 seconds, over the course of two hours, with each stimulation occurrence consisting of 1s of deflections at 5 Hz

A total of 28 aged (21–24 months) Fischer 344 rats were used for this study. Eight aged rats had to be excluded from the study due to hemorrhage/mortality during or soon after the surgical procedure. Additionally, two aged rats were disqualified due to too little exploratory activity during behavioral testing. Of the remaining eighteen aged rats, ten were used in the treated group and eight in the untreated group.

immediately following pMCAO. Control rats underwent the same experimental protocol but did not receive stimulation.

### Sensorimotor Behavior

To evaluate changes in neurological function associated with ischemia, sensorimotor behavior was assessed seven days prior to, and seven days post-pMCAO on three tasks sensitive to stroke behavioral deficit: the Bederson Neurological scale (Bederson et al., 1986), forepaw guided exploration (Schallert et al., 2000), and whisker guided exploration (Chen-Bee and Frostig, 1996, Luhmann et al., 2005). Observers blind to the experimental condition performed testing and analysis of behavioral data.

The Bederson Neurological Scale was used to assess the general mobility of all rats. Rats were first held by the base of the tail and suspended 2.5 meters above the floor for 10 seconds in order to determine whether the rat withdrew his affected limb at the shoulder, and/or elbow joint, and/or wrist. This withdrawing behavior is known as limb flexion, and is recognized as a sign of ischemic injury. The score “0” was designated for animals that demonstrated no limb flexion or reached with both paws toward the floor symmetrically, and the score “1” was designated for limb flexion characterized by the withdrawal of the affected limb and flexion of the unaffected limb toward the floor.

Next, the subject was placed directly into a large cylindrical chamber and allowed to roam freely for five minutes. Scorers looked for signs of spontaneous circling behavior, difficulty with gait, and difficulty remaining upright. Results of the observation period (suspension and spontaneous movement) were scored on a 0–4 scale as shown in Table 1.

Table 1

Observation Scoring Key (from Wang-Fischer, 2009)

Score	Definition
0	Normal movement
1	Failure to extend forepaw contralateral to infarct; this level of impairment is typically seen in rats with a mild focal lesion
2	Circling locomotive behavior; suggestive of a moderate focal lesion
3	Falling to side of infarct; occurs in rats with a severe, but focal, lesion
4	Lack of spontaneous movement or stupor; associated with very severe cortical and sub-cortical lesions

Forepaw-guided exploration was assessed by placing each rat in a testing cylinder. Wall touch following rearing, using

the left forepaw, right forepaw, or both paws together, were recorded for five minutes. Forepaw use was calculated as an asymmetry score (right paw touches minus left paw touches divided by total touches), with a negative score signifying a subject's preference to explore with the left forepaw. In healthy rats, roughly symmetric use of left and right paws is observed, while unilateral damage to the somatosensory cortex results in asymmetric use (Schallert et al., 2000).

Whisker-guided exploration was evaluated by placing each rat in a 25-cm-wide rectangular track (120 x 80 cm, outer diameter). Rats were allowed 10 seconds to acclimate before the start of the 5-minute testing session. Whisker scanning was defined as the time spent by the subject touching the walls of the rectangular track with one set of whiskers while locomoting and was measured in seconds spent using either the left or right whisker pad (Chen-Bee and Frostig, 1996). Each subject was then assigned a whisker asymmetry score (right minus left score divided by total scanning time), with a negative score signifying a subject's preference to scan with the left set of whiskers. On average, healthy rats do not exhibit a preference for one whisker set over the other. Rats with unilateral damage to the somatosensory cortex, however, show a preference for the unaffected whisker set (Luhmann et al, 2005).

Because the Bederson neurological scale is a categorical assessment, the nonparametric Mann-Whitney U test was used to compare between groups at baseline, and Wilcoxon Signed Ranks tests were used to compare within groups before and after pMCAO. For both forepaw and whisker guided tasks, two sample t tests were used to compare baseline performance between groups, and paired sample

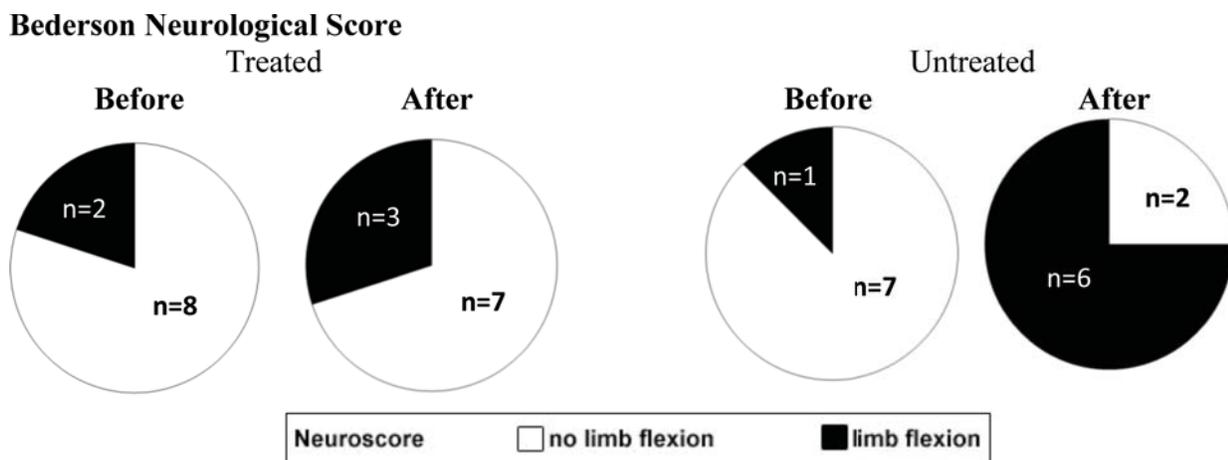
t-tests were used to compare within groups before and after pMCAO.

*Histology (TTC staining for infarct)*

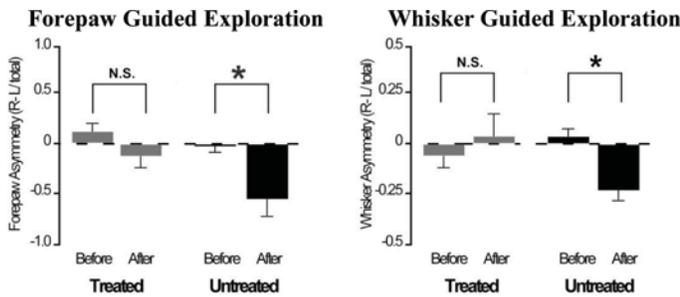
Rats were euthanized with 3.0 ml of Euthasol at the conclusion of each experiment. Their brains were then carefully removed and sectioned into 2mm slices along the coronal plane. The brain slices were incubated in TTC at 37°C for 20 minutes in the dark (Bederson et al., 1986). TTC was enzymatically reduced, producing formazan (a bright red byproduct), by dehydrogenases in active mitochondria. Red stain intensity correlates with the number and functional activity of the mitochondria, with unstained (white) areas being indicative of infarct (Goldlust et al., 1996, Lavie et al., 2001). The TTC-stained sections were photographed with a digital camera, and the total infarct volume was determined by multiplying the infarct area of each slice by the thickness of that slice and summing across slices. An observer blind to experimental condition performed this volume calculation.

**Results**

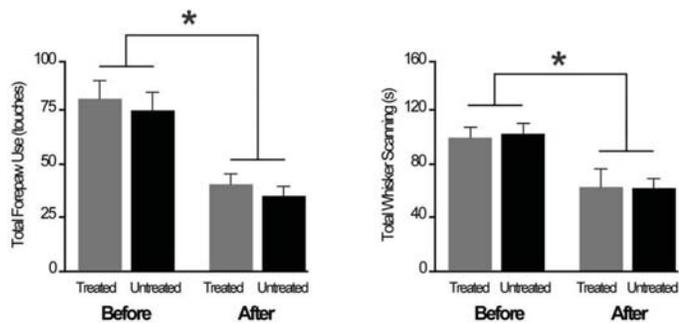
When assessed using the Bederson Neurological scale, the treated animals behaved similarly to healthy animals (Figure 2). Healthy animals demonstrate symmetrical extension of both limbs, as witnessed with the treated animals both before and after pMCAO (Wilcoxon Z = 0.58, p = 0.56). The untreated animals were significantly impaired after pMCAO (Wilcoxon Z = 2.24, p = 0.03), showing preference for the extension of their unaffected limb while their affected limb was held close to the base of their bodies.



**Figure 2**  
Results from the Bederson Neurological scale, used to assess the general mobility of aged rats. The score 0 was designated for animals that demonstrated no limb flexion and the score 1 was designated for limb flexion. Pie charts represent the number of rats with the corresponding neurological score.



**Figure 3**  
Forepaw guided (left) and whisker guided (right) asymmetry scores before and after pMCAO. Horizontal line indicates 0, or no asymmetry. In the forepaw guided exploration task, treated animals showed no significant difference before and after pMCAO. Untreated animals showed a significant difference in limb usage after pMCAO, indicating preference for their unaffected limb. Likewise, untreated animals showed significant asymmetry in whisker usage in the whisker exploration task. Asterisks indicate a significant difference from baseline, \* $p < 0.05$ .



**Figure 4**  
Total amount of forepaw guided exploration before and after pMCAO (left) and total amount of whisker guided exploration scanning before and after pMCAO (right). No difference was found between subjects of different group types; however, a significant difference was found in behavior before and after pMCAO for both treated and untreated animals. Asterisks indicate a significant difference from baseline, \* $p < 0.05$ .

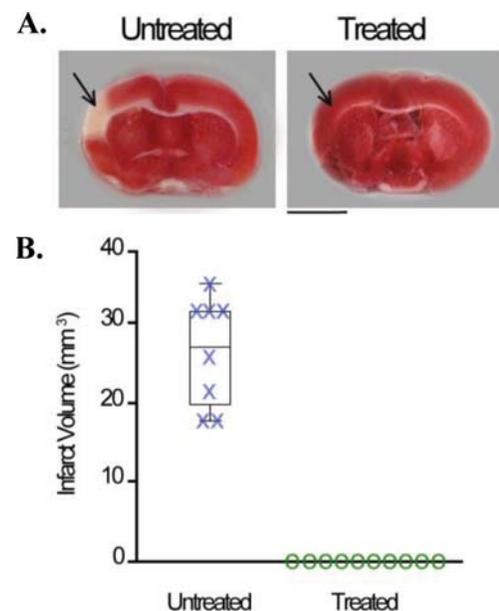
Behavioral impairment in the untreated aged rodents was also seen in the forepaw and whisker guided exploration tasks. As shown in Figure 3, untreated animals showed a significant impairment in both forepaw ( $t(7) = 5.42, p = 0.0009$ ) and whisker ( $t(7) = 2.67, p = 0.03$ ) use, while treated animals showed no significant difference in limb and whisker use following pMCAO. Animals that received treatment tended to use both paws ( $t(9) = 1.70, p = 0.12$ ) and both sides of whiskers ( $t(9) = -0.66, p = 0.53$ ) in an equal amount. Animals that did not receive treatment showed preference for forepaw and whisker exploration using the unaffected side.

To determine that there were no differences between individual animals in either treatment group, total forepaw use and total whisker scanning time prior to pMCAO were com-

pared to those after pMCAO (Figure 4). While there was no significant difference between groups of either treatment type before or after pMCAO for forepaw use ( $F(1,19) = 3.25, p = 0.09$ , ANOVA) or whisker use ( $F(1,19) = 0.01, p = 0.93$ , ANOVA), a noticeable decrease in both forepaw and whisker use was observed for both treated and untreated animals in the post-surgery behavioral assessment. This observed decrease is most likely due to the habituation of the animals to their exposed environment, where less exploration is likely to occur after a previous exposure.

Finally, differences in the cortical structures of aged rodents were assessed using histological assay. As seen in the untreated rat’s brain, white staining indicates infarct or dead tissue (Figure 5A). Red staining indicates vital tissue, and is seen throughout the brain slices of treated animals. Untreated animals show extensive infarct volumes (average =  $27.6 \pm 2.5 \text{ mm}^3$ ) when compared to treated animals, which had no cortical infarct (Figure 5B).

Overall, the animals that received the single whisker stimulation treatment were behaviorally equivalent to their condition prior to pMCAO on all three sensorimotor tasks,



**Figure 5**  
Stimulation treatment protects cortical structure in aged rats. (A) Representative coronal sections from an untreated rat (left) and treated rat (right) using 2,3,5-Triphenyltetrazolium Chloride (TTC) assay for infarct. Red staining indicates healthy tissue, lack of staining indicates ischemic infarct. Arrows point towards MCA blood supply territory. Scale bar equals 5mm. Untreated animals sustained cortical infarct, whereas treated animals had no infarct. (B) Box-and-whisker plot of infarct volume ( $\text{mm}^3$ ), with individual subjects plotted for untreated (blue) aged subjects, and treated (green) aged subjects.

and they did not have any cortical infarct. Unlike treated animals, the animals that did not receive stimulation following pMCAO demonstrated impaired sensorimotor behavior on all three tasks and sustained cortical infarcts.

## Discussion

Although a majority of strokes occurs in people over the age of 55 (Roger et al., 2011), a compelling amount of stroke research has been conducted on younger animals. Cortical plasticity, the ability of the brain to reorganize and adapt its cortical architecture, has long been an area of great interest in our lab's research (Polley et al 1999, Frostig 2006). Through this experiment, we have demonstrated how the single whisker stimulation treatment was equally effective in the aged rodent model as it was in the young rodent model.

Our findings suggest that despite differences between the young rodent cortex and aged rodent cortex, the aged cortex is also capable of complete cortical neuroprotection following ischemic damage using the same technique of single-whisker stimulation. This project is unique because it shows complete, rather than partial, recovery from impending ischemic damage. Animals that undergo a stroke and receive the treatment behaved no differently than healthy control animals, and their brains showed no signs of cortical damage. Cortical reactivation using non-invasive, mild sensory stimulation immediately following an ischemic attack is vital for both old and young animals. By delivering blood to the deprived area of the brain through alternate vessels immediately following an ischemic stroke, the brain is amazingly capable of surviving impending cortical damage.

This study further suggests that the brain retains its ability to adapt to damage, even in old age. The human brain, like the rat brain, demonstrates an incredible capacity for cortical plasticity. The potential of applying these findings to a human correlate would have a profound impact on future stroke treatments, as the treatment is completely non-invasive and can be performed by almost anyone immediately following any signs of stroke. Although humans lack the same cortical representations that rats have for their whiskers, an alternative to whisker stimulation could be finger, visual, or auditory stimulation in humans. Our lab hopes to explore these alternatives, and progress has already been made to understand how auditory stimulation might affect the rodent cortex during an ischemic stroke.

As with any research project involving an animal model, we must assume some caveats when anticipating a connection to a human alternative. Although the warning signs of a stroke may be obvious, it is less obvious exactly where in the brain the stroke is occurring without access to appropriate medical imaging. Because our strokes were induced in the same area of the cortex every time in every animal, our stimulation treatment was successful because we already knew where the stimulation needed to be targeted. Another limitation might be any adverse effects of this treatment that might occur when stimulation is administered after the 0–2 hour critical window (Lay et al. 2010). Lastly, while the use of this stimulation treatment could ideally be performed on a human suffering from a stroke, more research needs to be done to better understand the molecular mechanism underlying the reactivation of the cortex before it can successfully be expanded into human stroke research. Despite these limitations, however, translation of this procedure into humans still provides a promising area of research for the recovery and rehabilitation of people following neurovascular diseases such as stroke.

## Acknowledgements

Thank you, Dr. Ron Frostig, for giving me the opportunity to work in your laboratory alongside my fellow researchers who have been equally passionate and supportive of this project, Christopher Lay and Melissa Davis for being my mentors and performing the pMCAO on the animals, Patricia Vu, Jon Low, and Quynh Vu for their assistance with the behavioral assessment, and UROP for funding my project and making this all possible.

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