Video Article

Excitotoxic Stimulation of Brain Microslices as an *In vitro* Model of Stroke

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Abstract

Examining molecular mechanisms involved in neuropathological conditions, such as ischemic stroke, can be difficult when using whole animal systems. As such, primary or 'neuronal-like' cell culture systems are commonly utilized. While these systems are relatively easy to work with, and are useful model systems in which various functional outcomes (such as cell death) can be readily quantified, the examined outcomes and pathways in cultured immature neurons (such as excitotoxicity-mediated cell death pathways) are not necessarily the same as those observed in mature brain, or in intact tissue. Therefore, there is the need to develop models in which cellular mechanisms in mature neural tissue can be examined. We have developed an *in vitro* technique that can be used to investigate a variety of molecular pathways in intact nervous tissue. The technique described herein utilizes rat cortical tissue, but this technique can be adapted to use tissue from a variety of species (such as mouse, rabbit, guinea pig, and chicken) or brain regions (for example, hippocampus, striatum, *etc.*). Additionally, a variety of stimulations/treatments can be used (for example, excitotoxic, administration of inhibitors, *etc.*). In conclusion, the brain slice model described herein can be used to examine a variety of molecular mechanisms involved in excitotoxicity-mediated brain injury.

Video Link

The video component of this article can be found at http://www.jove.com/video/51291/

Introduction

The most common form of stroke is ischemic stroke, which occurs when a cerebral blood vessel becomes occluded. The tissue ischemia which results from cessation of blood flow causes widespread depolarization of membranes, release of excitatory neurotransmitters, and sustained elevation of intracellular calcium, which leads to the activation of cell death pathways [1]. This process has been termed 'excitotoxicity', and is a common pathway involved in neuronal death produced by a variety of pathologies, including stroke [2]. Inhibition of the signaling pathways involved in excitotoxicity and other neuronal cell death cascades is an appealing approach to limit neuronal damage following stroke.

Identifying the precise molecular mechanisms involved in excitotoxicity and ischemic stroke can be difficult when using whole animal systems. As such, primary embryonic and 'neuronal-like' (*e.g.* neuroblastoma and adenocarcinoma immortalized lines) cell culture systems are often used. The main advantages of these models are that they are easy to manipulate, relatively cost-effective, and cell death can be readily measured and quantitated. However, signaling pathways can be altered by the culturing conditions used [3,4], and immature neurons and immortalized lines can express different receptors and signaling molecules when compared to mature brain [5-8]. Furthermore, cultured neurons only allow the examination of one cell type (or two, if a coculture system is used), whereas intact brain tissue is heterogeneous, containing a variety of cell types that interact with each other. Organotypic slice culture systems (thin explants of brain tissue) are also used, and these models allow the study of heterogeneous populations of cells as they are found *in vivo*. However, only a limited amount of tissue can be obtained from each animal when using this technique, slices cannot be cultured for as long as immortalized cell lines, and medium to long-term culture can result in alterations in signaling pathways and receptors in the slices. Whilst mature brain can be used to generate organotypic slices, slices from immature brain are more amenable to culture, and are more commonly utilized. There is therefore the need to develop models which mimic or represent intact mature brain, that are easy to use, in which neuronal signaling pathways can be examined.

Herein, an *in vitro* technique involving intact nervous tissue that can be used to elucidate molecular mechanisms involved in cell death following an excitotoxic or ischemic insult is described. This technique reduces the number of animals required to perform an experiment, is reproducible, and generates viable tissue that behaves in a metabolically similar fashion to larger organotypic slices. Additionally, the neuronal circuitry, cellular interactions, and postsynaptic compartment remains partly intact. The physiological buffer used allows the cell membranes to 'reseal', and enables cells to recover their original membrane resistance [9]. This brain slice model is able to faithfully mimic responses observed following excitotoxicity mediated brain injuries [10], and can be used to examine the molecular mechanisms involved in stroke.

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Protocol

All procedures are performed with approval from the University of Newcastle Animal Care and Ethics Committee, as well as in accordance with the relevant guidelines and regulations, including the NSW Animal Research Act, the NSW Animal Research Regulation, and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

1. Dissection of Brain Tissue

1. Sacrifice twelve week old male rats by decapitation. Rats can either be sacrificed by stunning and decapitation, or can be first anesthetized with isoflurane (5% induction, 1.5-2% maintenance) in 70% N_2 and 30% O_2, and then decapitated.
2. Remove the brain from the skull as rapidly as possible (ideally, this should be performed in less than 2 min).
3. Remove the cortex by free-hand dissection. The dissection should be performed in the minimum time possible, with as little damage to the brain as possible so as to maintain the integrity of the slices.
4. In order to remove any residual skin, fur, blood, *etc.*, immerse the brain in a small amount (3-5 ml) of 37 °C Krebs buffer (118 mM sodium chloride, 4.7 mM potassium chloride, 1.3 mM calcium chloride, 1.2 mM potassium dihydrogen phosphate, 1.2 mM magnesium sulfate, 25 mM sodium hydrogen carbonate, 11.7 mM glucose, 0.03 mM disodium ethylenediaminetetraacetic acid (EDTA), equilibrated with 95% oxygen/5% carbon dioxide (carbogen), pH 7.4).

2. Preparation of Microslices

1. Place the brain on the stage of a McIlwain chopper on which two slices of filter paper have been placed and moistened with warm Krebs buffer. Keep the brain and filter paper moistened with warm Krebs buffer, but not so wet that there is free liquid on the surface of the paper that can cause the brain to slide around.
2. Slice the brain into 250 µm coronal sections. As nutrients, oxygen, and waste materials that are normally exchanged with the blood supply are exchanged with a fluid which surrounds the tissue in isolated neural tissue, the size of the slices should not be more than 400 µm in thickness to allow adequate oxygenation and nutrient exchange to occur.
3. Turn the McIlwain stage 90°.
4. Slice brains into 250 µm sagittal slices. Herein, these brain sections (250 µm x 250 µm slices of variable length depending on the thickness of the tissue) are referred to as microslices.
5. Place the microslices into a round bottom tube with 10-15 ml 37 °C Krebs buffer (the amount of Krebs buffer used will depend on the amount of tissue).

3. Equilibration of Microslices

1. Gently agitate until individual microslices are suspended, and then allow them to settle under gravity.
2. Carefully remove the supernatant, which will be cloudy due to suspended cell debris, and add 10 ml fresh 37 °C Krebs buffer to the tube.
3. Repeat this washing procedure four times, which will result in the removal of the majority of debris.
4. Following this, equilibrate the microslices at 37 °C with gentle shaking/inversion, and change the Krebs buffer every 15 min for 1 hr in total.
5. At this time, add 2 ml fresh 37 °C Krebs buffer, and transfer 15-20 microslices (150 µl of microslice suspension) to flat bottom polystyrene 5 ml tubes using an extra wide bore pipette tip with smoothed edges (which can be created by cutting ~2 mm from the end of a P1000 tip). Spread the microslices evenly over the base of the tube in a single layer, so that each microslice is not further than a few millimeters from the oxygenated atmosphere.

4. Excitotoxic Stimulation

1. Incubate the microslices at 37 °C, and continually pass carbogen over the surface of the 150 µl microslice suspension, using a gas line that is positioned just above the surface of the microslice suspension (to avoid mechanical disruption).
2. Treat microslices with either 150 µl 5 mM glutamate + 100 µM glycine in Krebs buffer (excitotoxic stimulus: final concentration 2.5 mM glutamate + 50 µM glycine) or 150 µl Krebs buffer alone (control nonstimulated). A variety of stimuli can be used at this step (including, but not limited to, AMPA, NMDA + glycine, KCl depolarization, and oxygen/glucose deprivation).
3. Remove the incubation solution at various times post-stimulation (0, 30, 60, 90, 120, and 300 sec), and replace it with 300 µl of ice-cold homogenization buffer (30 mM Tris, pH 7.4, 1 mM ethylene glycol tetraacetic acid, 4 mM EDTA, 100 µM ammonium molybdate, 5 mM sodium pyrophosphate, 25 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonylfluoride, complete protease inhibitor cocktail).
4. Homogenize microslices on ice, using a glass-Teflon Dounce Homogenizer (20 strokes; 700 rpm).
5. Store homogenates at -80 °C, and various signaling molecules, death molecules, *etc.* can be measured by western blot.

Representative Results

Microslices generated using this procedure are viable, and a variety of species (for example, rat, mouse, and chicken) can be used to produce microslices. Three independent measures of viability have been utilized: respiration rate (**Figure 1**), adenine nucleotide ratios (**Figure 2**), and tissue potassium content (**Figure 3**). Using these measures, it has been demonstrated that microslices remain viable for at least 2 hr post-generation.

Brain microslices can be used to examine various signaling molecules, following a variety of stimuli (*e.g.* KCl depolarization [**Figure 4**], AMPA, NMDA, glutamate stimulation[10]), and can also be used to compare responses of cell signaling molecules between multiple brain regions (**Figure 4**). Additionally, the effect of various inhibitors/drug treatments on these molecules can also be examined (such as kinase inhibitors[10], glutamate receptor agonists[11], quisqualic acid[12]).

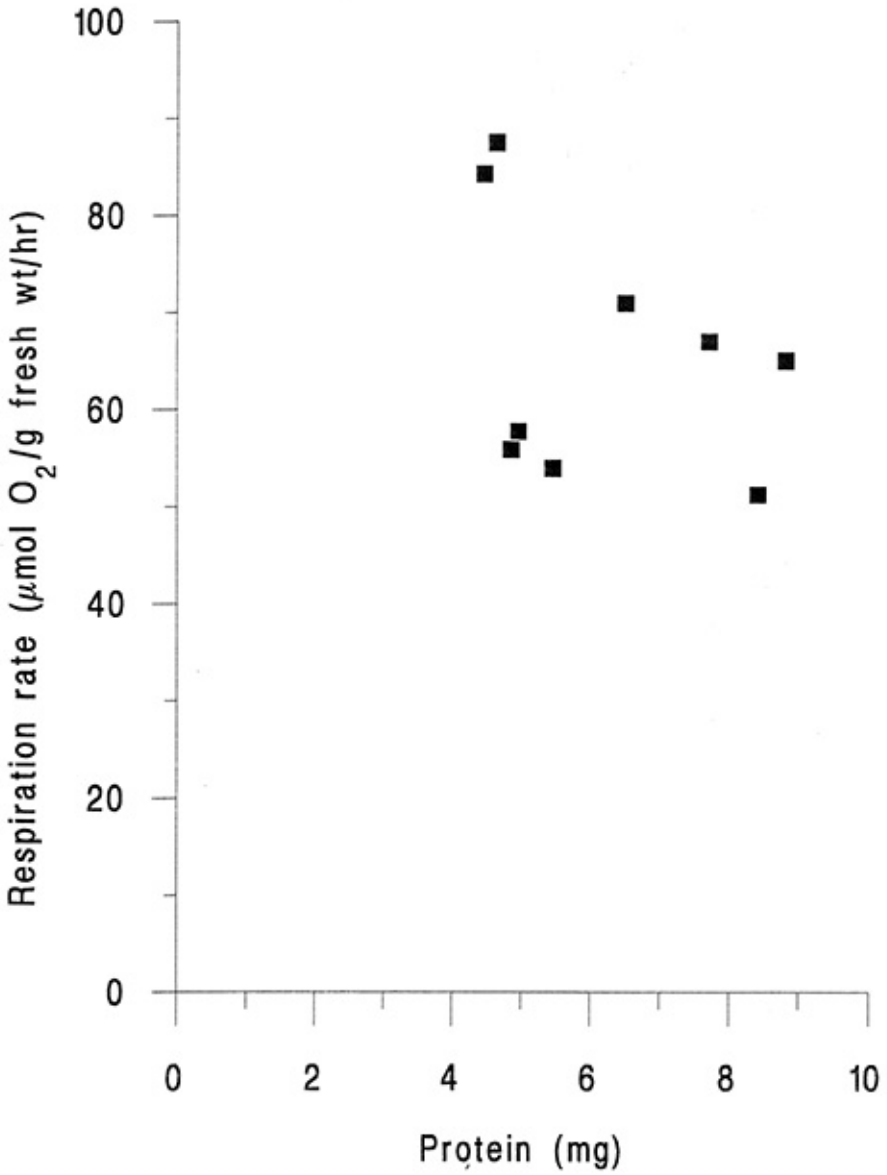

Figure 1. Respiration rates of microslices from chicken forebrain. The respiration rates of brain tissue were calculated as follows: the amount of oxygen consumed (difference between the oxygen electrode readings at the beginning and end of the experiment) multiplied by the moles of oxygen in solution, divided by the incubation time (hours) and amount of protein (mg). Typical respiration rates of isolated mammalian cerebral cortex are between 55-80 μmol O_2/g fresh wt/hr under ordinary conditions[9]. n = 9.

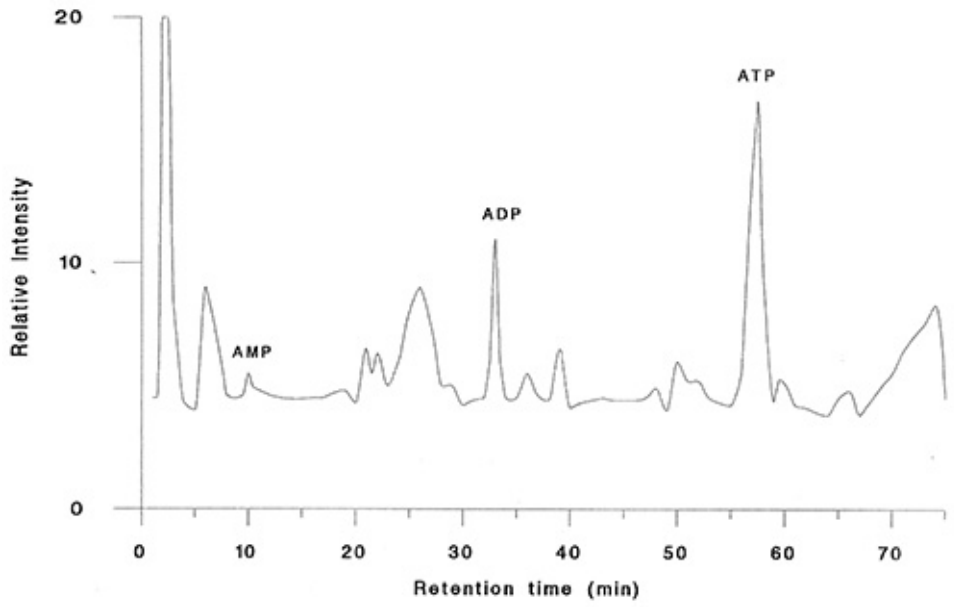

Figure 2. Presence of adenine nucleotides in microslices from chicken forebrain. Representative HPLC trace measuring ATP:ADP ratios, performed as previously described[13]. High ATP and low ADP levels are usually found in viable cells, whereas decreased levels of ATP and increased levels of ADP are characteristic for apoptotic and necrotic cells[14]. Typical ATP:ADP ratios of microslices varied between 3:1 and 6:1, and were maintained at these levels for greater than 2 hr.

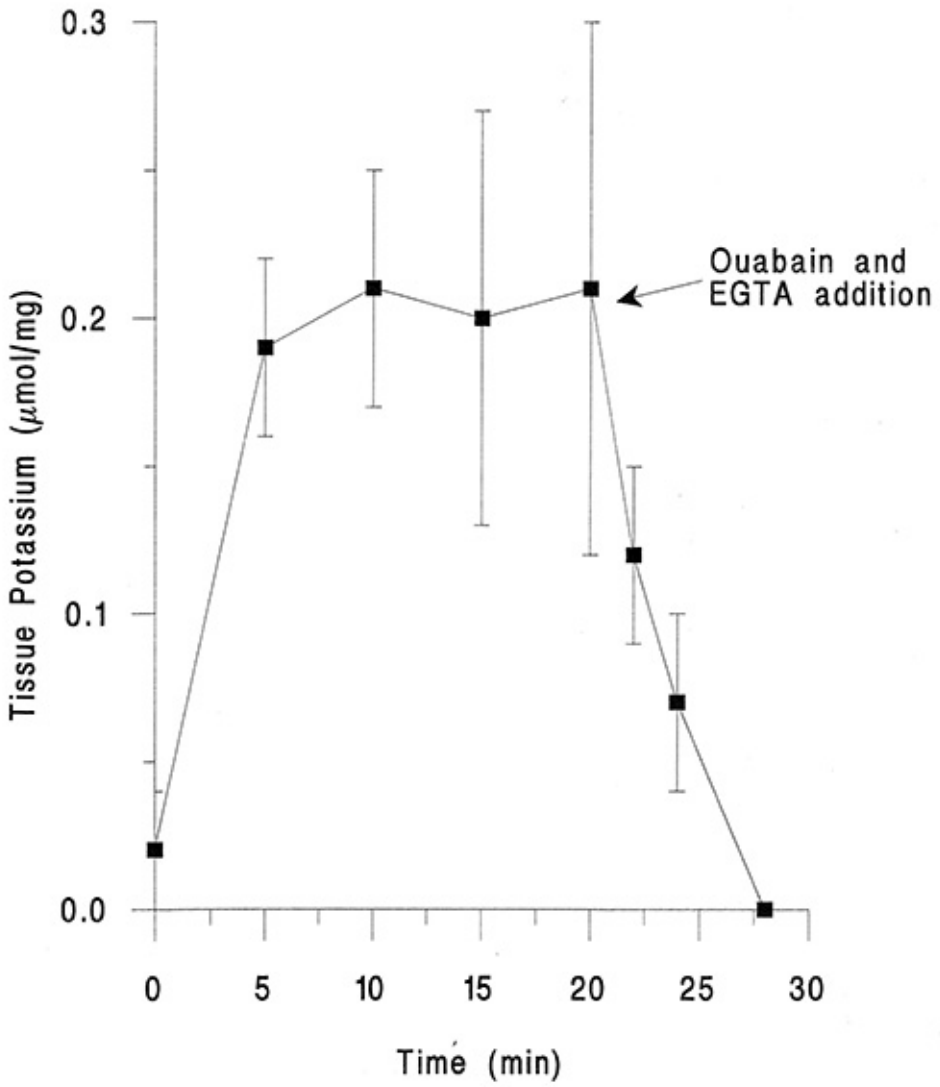

Figure 3. Tissue K^+ content of brain microslices from immature chicken forebrain. The determination of tissue potassium is a useful measure of viability, as many of the chemical and physiological activities of a tissue are dependent upon membrane potential, which requires a normal intracellular potassium content[9]. When microslices were washed in K^+-free buffer immediately after chopping, tissue K^+ was depleted to levels which were just above background (time 0). However, on subsequent incubation with Krebs buffer, the microslices rapidly accumulated K^+ from the surrounding medium, and within 5 min they had reached a steady state level similar to that for untreated microslices (*i.e.* microslices washed and incubated in normal buffer). The addition of ouabain (0.2 mM) and EGTA (2 mM) after 20 min incubation caused tissue K^+ content to decline to 0 within 8 min. This demonstrates that the microslices normally actively pump K^+, and that the tissue K^+ levels are not due to K^+ trapped in dead tissue or interstitial spaces. n = 4.

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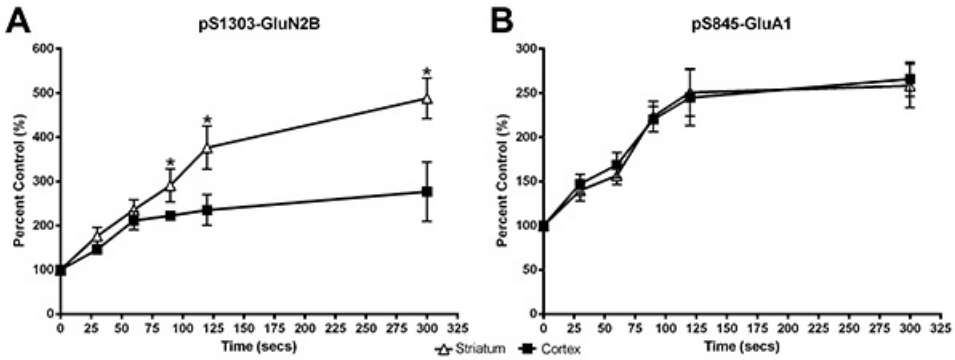

Figure 4. Time course of GluN2B and GluA1 phosphorylation following depolarization with KCl. Microslices were generated from the striatum and sensory cortex of male rats (n = 8), depolarized using high K^+ Krebs Buffer, homogenized, and examined for (**A**) GluN2B phosphorylation (at Ser1303) and (**B**) GluA1 phosphorylation (at Ser845) by western blotting at various times post-stimulation (0-300 sec). Phosphorylation levels are normalized to total GluN2B and GluA1 expression (*i.e.* phosphorylation/total expression). * denotes statistical significance between brain regions ($p < 0.05$). Depolarization with KCl resulted in phosphorylation of GluN2B and GluA1 receptors at S1303 and S845, respectively. The rate of GluN2B phosphorylation at S1303 varied between cortex and striatum, whereas the rate of GluA1 phosphorylation at S845 was the same in cortex and striatum. In subsequent experiments, we have shown that this is due to differential regulation of the multifunctional kinase, calcium/calmodulin-stimulated protein kinase II (CaMKII), which phosphorylates GluN2B at S1303, but not GluA1 at S845[10]. Click here to view larger image.

Discussion

Herein, an *in vitro* technique for the generation of microslices that can be used to examine the molecular mechanisms involved in excitotoxicity and ischemia-mediated cell death in intact mature brain tissue is described. This technique produces viable tissue (**Figures 1-3**), that is metabolically similar to larger organotypic slices[15]. Furthermore, this microslice model closely corresponds to the response observed following excitotoxicity mediated brain injuries *in vivo*[10]. A 90 second *in vitro* stimulus of brain microslices with AMPA, NMDA, or glutamate induces the same responses in a variety of molecules (*e.g.* CaMKII, GluN2B, and GluA1) as a 90 second period of occlusion *in vivo*[10]. This provides further validation that the microslice model described herein is a valid method that can be used to investigate the molecular mechanisms involved in ischemia and excitotoxicity-mediated cell death.

As with any experimental technique, this procedure can be varied, and these variations may or may not affect experimental outcome. Based on experience, the protocol detailed herein represents the optimal conditions for generating the highest tissue yield and microslice viability. This protocol can be adapted for use in a variety of species (microslices from rats, mice, and chickens have previously been successfully generated), and from any brain region (**Figures 1-3** depict microslices derived from whole forebrain; **Figure 4** depicts microslices derived from cortex and striatum). Additionally, the microslice thickness can be altered from 150-350 µm, without any appreciable loss in viability of the slices. The optimal slice thickness is 250 µm, as this produces the highest yield of tissue, and more reproducible sampling than for larger slices. The statistical sampling of brain tissue produced by the aliquots of microslices needs to be representative of the tissue from which the slices were prepared. To do this, we need to use the smallest sized slices per aliquot to ensure a representative sampling of the tissue. The optimum conditions for this were found to be 250 µm thickness, and 1 mg of tissue protein, which corresponds to ~15-20 slices/aliquot. The temperature at which microslices should be chopped and washed was investigated by comparing the respiration rates of microslices generated with and without cooling to 4 °C. Although the effects of acidosis and anoxia may be reduced when tissue is placed at 4 °C, microtubule reassembly can be altered if tissue is chilled and then rewarmed[16]. This reorganization of the cytoskeleton may affect the functional activity of cells for some period after warming. Noncooled microslices have a higher viability and respiratory rate when compared to cooled slices. Furthermore, three washes (steps 3.1-3.3) are sufficient to remove most of the debris. The equilibration period (step 3.4) with periodic changes in buffer allows the microslices to 'recover' from chopping, and improves reproducibility in various functional assays (such as calcium accumulation).

The method is suitable to examine the effects following a variety of stimuli, and following treatment with drugs/inhibitors. Any drug/inhibitor that is membrane permeable has the potential to be examined *via* this technique, and any molecule that can be detected *via* western blot (or similar) can be investigated.

In summary, the manipulation of signaling cascades involved in excitotoxicity and ischemia-mediated cell death offer an attractive approach for the development of drugs that limit neuronal cell death following stroke. The described microslice method is an easily applicable model that allows the investigation of these signaling cascades in intact mature brain tissue. This technique may be used to help identify new treatments that limit neuronal cell death following stroke.

Disclosures

The authors declare that they have no conflict of interest regarding any of the work conducted within this manuscript.

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