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·Method·

# Preserving GABAergic interneurons in acute brain slices of mice using the N-methyl-*D*-glucamine-based artificial cerebrospinal fluid method

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## ABSTRACT

Defects in the function and development of GABAergic interneurons have been linked to psychiatric disorders, so preservation of these interneurons in brain slices is important for successful electrophysiological recording in various *ex vivo* methods. However, it is difficult to maintain the activity and morphology of neurons in slices from mice of >30 days old. Here we evaluated the N-methyl-*D*-glucamine (NMDG)-based artificial cerebrospinal fluid (aCSF) method for the preservation of interneurons in slices from mice of up to ~6 months old and discussed the steps that may affect their quality during slicing. We found that the NMDG-aCSF method rescued more cells than sucrose-aCSF and successfully preserved different types of interneurons including parvalbumin- and somatostatin-positive interneurons. In addition, both the chemical and electrical synaptic signaling of interneurons were maintained. These results demonstrate that the NMDG-aCSF method is suitable for the preservation of interneurons, especially in studies of gap junctions.

**Keywords:** artificial cerebrospinal fluid; acute brain slice; electrophysiology; N-methyl-*D*-glucamine; parvalbumin; somatostatin

## INTRODUCTION

GABAergic interneurons inhibit neuronal depolarization by releasing γ-aminobutyric acid (GABA). Dysfunction of these interneurons may be responsible for some psychiatric/neurological disorders, such as schizophrenia, autism, and epilepsy, so their electrophysiological properties have been intensively studied. One of the major research methods is patch-clamp recording in brain slices *in vitro*. The efficiency of this method mainly relies on the quality of the slices. To obtain good-quality slices, $Na^+$ in the artificial cerebrospinal fluid (aCSF) was initially replaced with equimolar sucrose[1]. Later, other $Na^+$ substitutes were introduced, including N-methyl-*D*-glucamine (NMDG)[2-4], glycerol[5], Tris, and choline. However, apart from the NMDG and choline substitutes, they are only adequate for mice younger than ~30 days. Choline substitution in aCSF results in the morphological preservation of slices from mice >3 months old and up to years of age, but the electrophysiological properties of neurons in these slices are lost. In contrast, the NMDG-based aCSF method not only enables morphological preservation of brain slices of mice over a lifespan similar to the choline-based method, but also preserves the function of chemical synapses[3,4]. The NMDG-aCSF method was initially described for studying glial cells in the spinal cord[2]. Later, it was introduced for the preparation of brain slices. Recently, additional supplements such as N-acetyl-L-cysteine (NAC) and glutathione ethyl-ester have been incorporated into the NMDG-aCSF method, resulting in the enhanced maintenance of slices *in vitro* for up to 12 h[3,4,6].

To facilitate studies of GABAergic interneurons, in this study we attempted to preserve their cellular morphology and electrophysiological properties using the NMDG-

aCSF method. We evaluated the reproducibility of this method, focusing on its effect on parvalbumin (PV)- and somatostatin (SST)-positive interneurons, which account for ~70% of all interneurons.

## MATERIALS AND METHODS

### Ethical Statement

All animal experiments were approved and reviewed by the Animal Advisory Committee at Zhejiang University in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

### Mice

The following mice were used throughout the experiments: GIN mice (#003718, The Jackson Laboratory, Bar Harbor, ME) expressing enhanced green fluorescent protein (eGFP) in a group of SST interneurons under the control of the *Gad1* promoter, and G42 mice (#007677, The Jackson Laboratory) expressing eGFP in a subclass of PV-positive interneurons under the control of the *Gad1* promoter. A CCK-Cre-ER$^{T2}$ (#012710, The Jackson Laboratory) mouse was crossed with an Ai9 mouse (#007909, The Jackson Laboratory) to produce mice expressing the fluorescent protein tdTomato under the control of the *Cck* promoter in cortical neurons (both interneurons and pyramidal neurons). These offspring are referred to as "CCK-TOM".

### Preparation of Acute Brain Slices

Generally, for the sucrose aCSF method, mice were anesthetized by ether inhalation and then were perfused with ice-cold sucrose-aCSF containing (in mmol/L): 185 sucrose, 2.5 KCl, 25 *D*-glucose, 25 $NaHCO_3$, 1.2 $NaH_2PO_4$, 0.5 $CaCl_2$, and 10 $MgCl_2$, pH 7.35 by NaOH or HCl. The brain was cut with a microtome (Leica VT1200S with vibrocheck, Nussloch, Germany) and slices (300 μm) were maintained in oxygenated standard recording aCSF at 34°C for 20 min and subsequently kept in standard recording aCSF at 22°C for at least 30 min until the experiment. The standard recording aCSF contained (in mmol/L): 124 NaCl, 2.5 KCl, 13 *D*-glucose, 24 $NaHCO_3$, 1.2 $NaH_2PO_4$, 5 HEPES, 2 $CaCl_2$, and 2 $MgSO_4$, pH 7.35 by NaOH or HCl.

For the NMDG aCSF method, acute slices were prepared based on the description by Guoping Feng at MIT[3,4]. Briefly, mice were deeply anesthetized by ether inhalation and then cardiovascular perfusion was performed using ice-cold NMDG aCSF consisting of (in mmol/L): 93 NMDG, 93 HCl, 2.5 KCl, 1.2 $NaH_2PO_4$, 30 $NaHCO_3$, 25 *D*-glucose, 20 HEPES, 5 Na-ascorbate, 2 thiourea, 3 Na-pyruvate, 10 $MgSO_4$, and 0.5 $CaCl_2$, pH 7.35 with NMDG or HCl. The brain was removed and placed into NMDG aCSF (0–2°C) bubbled with 95% $O_2$/5% $CO_2$. Slices were cut followed by primary recovery in oxygenated NMDG aCSF at 34°C for 10–12 min and secondary recovery in oxygenated HEPES aCSF at 22°C for >60 min. The HEPES aCSF contained (in mmol/L): 92 NaCl, 30 $NaHCO_3$, 25 *D*-glucose, 2.5 KCl, 1.2 $NaH_2PO_4$, 20 HEPES, 5 Na-ascorbate, 2 thiourea, 3 Na-pyruvate, 10 $MgSO_4$, 0.5 $CaCl_2$, and 12 NAC, pH 7.35 by NaOH or HCl. Slices from the prefrontal cortex, hippocampus, and midbrain were used for electrophysiological and imaging experiments. The osmolality of each aCSF was ~310 mOsm.

### Electrophysiology

Individual slices were maintained in a recording chamber perfused with oxygenated standard recording aCSF (1–2 mL/min) throughout the experiment. Whole-cell paired patch-clamp recording was performed using an intracellular solution containing (in mmol/L): 110 K-gluconate, 40 KCl, 10 HEPES, 3 Mg-ATP, 0.5 $Na_2$-GTP, and 0.2 EGTA, pH 7.25 with KOH or HCl. Signals were acquired using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA) with a Digidata 1440A (Molecular Devices) controlled by Clampex 10.4. Signals were filtered at 2 kHz for voltage clamp and 3 kHz for current clamp, and digitized at 10 kHz. Electrodes were made from borosilicate glass (with filament, Sutter, Novato, CA) with a resistance of 3.5–4.5 MΩ. Whole-cell patch-clamp was formed after a gigaohm seal was achieved with series resistance <20 MΩ. Chemical synaptic events were evoked by alternate field stimulation (square-wave pulses of 100 mV, 1000 Hz, duration 1 ms, the stimulating electrode was >50 μm apart from the recording pipette) with a pencil-shaped concentric bipolar electrode (outer pole 125 μm, inner pole 25 μm, rounded tip, #CBARC75, FHC, Bowdoin, ME). With the same pipette solution as above, both the inhibitory and excitatory postsynaptic currents were inward. Cells were held at −70 mV to detect unitary postsynaptic currents or miniature postsynaptic currents. For electrical coupling, we simultaneously patched two eGFP-positive cells (<20 μm apart). Cells were held under the current-clamp mode and resting membrane potential was maintained at −70 mV

with continuous current injection. Electrical coupling was evoked by 500 ms of hyperpolarizing current injection (−100 pA) into each cell alternately. The threshold for electrical coupling confirmation was 1% (ratio of the steady-state voltage deflection of coupling cell $\Delta V_2$ and injected cell $\Delta V_1$).

### Imaging

During the electrophysiology experiments, neurons were identified with either a Zeiss Achroplan 10×/0.25 Ph1 lens or a Zeiss Achroplan IR 40×/0.80 W lens mounted on an upright Zeiss Axioskop 2 FS mot platform with a mercury lamp. Images were captured with either a DAGE-MTI IR-1000 Monochrome camera or a Zeiss LSM 5 Exciter system.

## RESULTS AND DISCUSSION

To evaluate and compare the NMDG-aCSF method with other methods, we first examined morphological preservation. In NMDG-aCSF, cells in the prefrontal cortex, hippocampus, and dorsal raphe nucleus of the midbrain displayed clear and plump cell bodies while in sucrose-aCSF the cells swelled and died (Fig. 1A). In addition, confocal images clearly showed the morphology of different types of neurons in CCK-TOM, G42, and GIN mice (Fig. 1B and C). The improved morphological preservation using the NMDG aCSF method was most likely due to relief from oxidative stress. Such stress can induce edema[7-9] and activate glutamate receptors resulting in acidosis[10]. These events lead to irreversible membrane damage such as lipid peroxidation, while replenishing the endogenous antioxidants can relieve these processes. Regular antioxidants include sodium pyruvate[11], sodium ascorbate[7], thiourea[12] and HEPES[13]. However, in aging mice the effect is not strong enough to preserve the health of slices, so the very powerful endogenous antioxidant, glutathione, was introduced[6,14]. This antioxidant rescues neurons from degeneration while simultaneously maintaining synaptic plasticity[15]. As glutathione is not membrane-permeable, NAC is introduced. NAC is membrane-permeable and is converted into glutathione

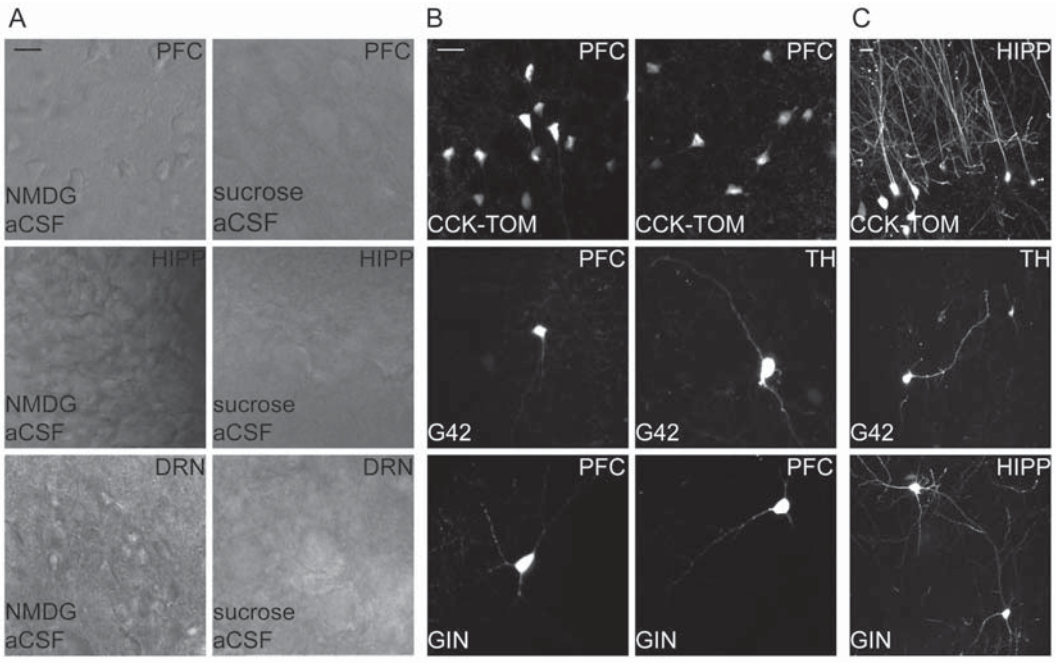


**Fig. 1. Morphological preservation of neurons in brain slices. (A) Direct comparison of neurons (2–3 months old) from the prefrontal cortex (PFC), hippocampus (HIPP) and dorsal raphe nucleus (DRN) using the NMDG and sucrose aCSF methods. More neurons swell and die when prepared with sucrose aCSF. (B, C) Confocal microscopic images of neurons from the PFC, hippocampus, and thalamus (TH) from 3 transgenic mice (3–6 months old). Neurons display clear, healthy, and branched projections. Scale bars, 20 μm.**

in the cytosol, providing on-line *de novo* glutathione synthesis[3,4,14]. As indicated by Jonathan Ting at the lab of Guoping Feng, NAC treatment is able to preserve the morphology and functionality of neurons for up to 12 h (www.brainslicemethods.com)[3,4]. We also found that NAC stabilized cellular health and prolonged survival time. Although it has been suggested that NAC should be applied throughout the slice preparation process, limiting NAC to the second HEPES aCSF recovery step was sufficient to keep slices alive and healthy for at least ~6 h. Once slices were transferred to the recording chamber, they could stay alive for another 3–4 h. Another more effective but expensive alternative to NAC is glutathione ethyl-ester, which is a membrane-permeable glutathione that is able to directly replenish endogenous glutathione depletion. The explanation for the strong beneficial effects of NMDG on slices is not directly evident. Based on previous reports, NMDG is less membrane-permeable than $Na^+$ and $K^+$[16]. Thus, replacement of either $Na^+$ or $K^+$ with extracellular NMDG hyperpolarizes the resting membrane potential[17]. The hyperpolarized resting membrane potential can keep neurons in a more “silent” mode, which might explain the protective effect of NDMG aCSF.

Besides morphological preservation of neurons in slices with the NMDG aCSF method, we also investigated the preservation of electrophysiological properties. Neurons of different types (Fig. 2A) and at different locations (Fig. 2B) were able to generate regular firing patterns. In contrast to the choline aCSF preservation method, in which electrical activity is lost, neurons prepared with the NMDG aCSF method displayed normal $Na^+$, $Ca^{2+}$, and $K^+$ currents (Fig. 2C). Moreover, we applied field stimulation to the Schaffer collaterals and detected postsynaptic currents in the CA1 region (Fig. 2D upper panel). Together with the miniature postsynaptic current recordings (Fig. 2D lower panel), these results demonstrated the presence of chemical coupling. Besides their chemical connections, we determined whether gap junctions were also preserved. We performed whole-cell patches on two SST interneurons simultaneously (see Material and Methods) and recorded electrical coupling (Fig. 2E). These results showed that electrical synapses are preserved by the NMDG aCSF method and extended the application of NMDG aCSF to the study of gap junctions in slices.

Besides the preservation method, to obtain good-quality brain slices, care must also be taken with the slicing operation. Vertical vibration of the blade, the horizontal amplitude, friction of the blade surface, cutting speed, and the blocking of the brain are all important factors contributing to the quality of slices[18]. In our experiments, we used a Leica VT1200S with vibrocheck which minimizes the vertical vibration of the blade at a customized horizontal amplitude. Generally, razors with low friction are recommended. In the experiments described here, we used razors from Schick (Germany). There are also other options for blades, such as homemade glass, sapphire, or self-customized zirconium ceramic, but they are either difficult to produce or have a low cost-effectiveness. For coronal sections from the prefrontal cortex, we preferred to use a speed of 0.2 mm/s, a horizontal amplitude of 1–1.5 mm, and a 15° blade angle, producing good morphological and functional preservation of cells. For coronal slices from the midbrain, we preferred a speed of 0.07–0.08 mm/s due to the presence of highly myelinated fiber tracts. However, there are no universal settings for the configuration of a vibratome since they vary depending on the specific type of machine and the specific brain region. For example, Guoping Feng and colleagues prefer the VF-200 vibratome from Precisionary Instruments, which has a fixed horizontal amplitude of 2 mm and a 13° blade angle[3,4]. Generally, it is suggested to use a large horizontal amplitude with the vertical vibration at its minimum value (0 mm is best, if possible)[18]. As indicated by Jonathan Ting, if NMDG aCSF is applied during the cardiovascular perfusion, there is no need to worry about the slicing speed and the transfer of slices to the recovery chamber (www.brainslicemethods.com). However, there are still challenges. For example, it is unclear whether the NMDG-based aCSF method is applicable to slices from damaged brain tissue. In addition, the NMDG aCSF method has a limitation on the age of mice, as it is not suitable for mice younger than 30 days. For these mice, the sucrose aCSF or the Tris -based aCSF method is suggested.

Taken together, we showed that the improved NMDG aCSF method offers a better way to perform patch-clamp recordings on neurons compared with the traditional sucrose method. Our results further demonstrated that this method is suitable for studying gap junctions, providing

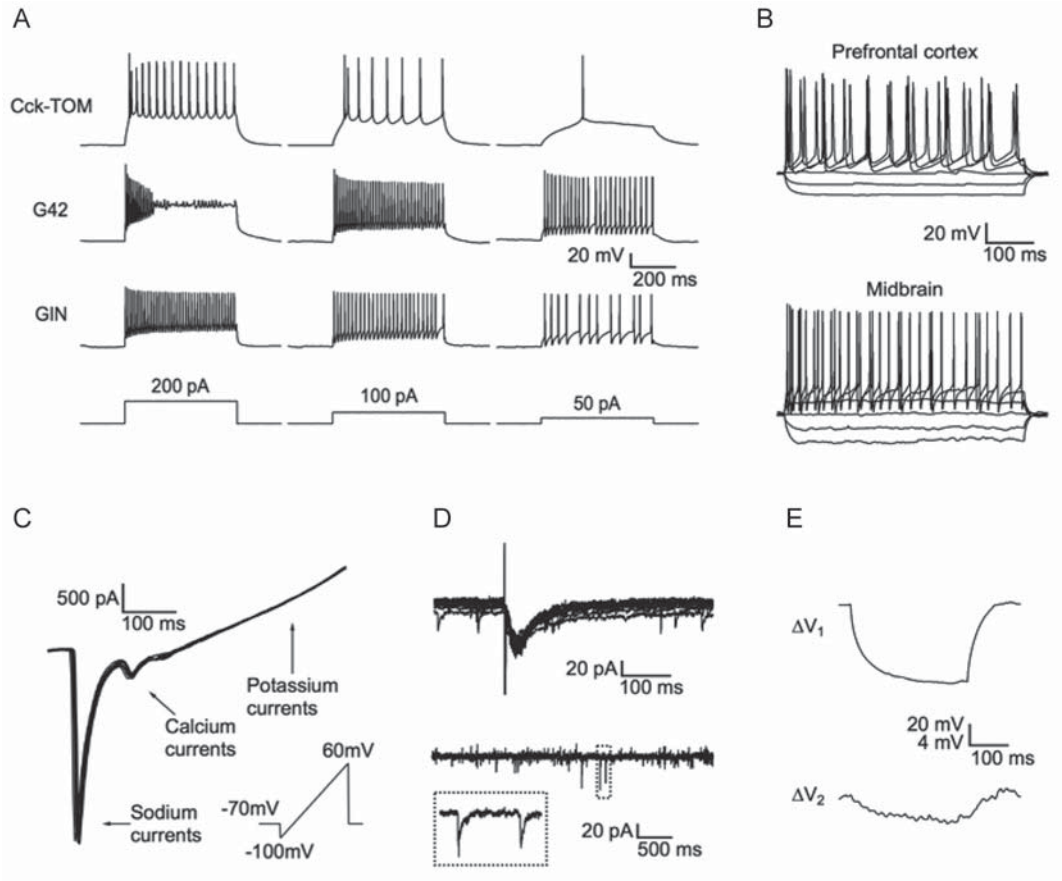


**Fig. 2. Electrophysiological properties of neurons in brain slices. Neurons prepared with the NMDG aCSF method from (A) three transgenic mice (3–6 months old) and (B) at two locations (prefrontal cortex and midbrain) all displayed normal firing patterns. (C) Neurons expressed $Na^+$, $Ca^{2+}$, and $K^+$ currents evoked by voltage ramp stimulation from −100 mV to +60 mV, indicating the preservation of these ion channels on the cell membrane. (D) Chemical synapse activity induced by field stimulation (upper panel): a neuron at CA1 was patched while Shaffer collaterals were stimulated by square-wave pulses (square-wave pulses of 100 mV, 1000 Hz, duration 1 ms, the stimulating electrode was >50 μm apart from the recording pipette); and by miniature postsynaptic currents (lower panel): a cell in the CA1 region was held at −70 mV and representative inward traces are shown in the dashed rectangle. (E) Electrical coupling between two somatostatin interneurons (<20 μm apart) in a slice from a mouse ~2 months old. The cells were held at −70 mV with current clamp. Hyperpolarization was generated in one cell ($\Delta V_1$) while voltage deflection was recorded from the other cell ($\Delta V_2$).**

an additional method for comparative research studies. However, this work only focused on the presence of chemical and electrical coupling. Whether and how the NMDG aCSF method is able to regulate the chemical and/or electrical coupling ratio remain to be determined.

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