Short Communication

High-Temperature, but Not High-Pressure, Conditions Alter Neuronal Activity

Mika Mizunuma[1], Naoya Takahashi[1], Atsushi Usami[1], Norio Matsuki[1], and Yuji Ikegaya[1,2,*]

[1]*Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

[2]*Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, 5 Sanbancho Chiyoda-ku, Tokyo 102-0075, Japan*

Abstract. We describe the effect of high pressure and high temperature on neuronal activity. Increased intracranial pressure is generally a pathological sign observed in intracerebral hemorrhage, brain edema, and brain tumor, yet little is known about how the hyperbaric pressure *per se* affects neuronal activity. Using a pressure/temperature-changeable perfusion chamber, we carried out functional multineuron calcium imaging to record spontaneous spiking activity simultaneously from about 100 neurons in hippocampal slice cultures. High-pressure conditions (up to 100 mmHg) did not alter the network excitability, whereas high-temperature conditions (up to 40°C) increased synchronized network activity. Thus, neurons are sensitive to feverish conditions, but the acute hyperbaric circumstance itself is unlikely to exert a detrimental effect on neuronal function.

Keywords: pressure, calcium imaging, neuronal network

Intracranial pressure is homeostatically maintained in a limited range, but pathologic conditions such as brain edema, brain tumor, and intracranial hemorrhage cause hyperbaric pressure, which produces various aberrations including headache, vomiting, and consciousness disorder (1).

Intracranial hypertension is generally considered to indirectly impair brain function through brain herniation and reduced cerebral blood flow (2), but it has yet to be clarified whether the pressure change *per se* directly influences neuronal activity. Although some in vivo studies examined the effect of artificially increased intracranial pressure on neuronal activity (3 – 5), these experiments cannot exclude the secondary effect of brain herniation or reduced cerebral blood flow.

To address the pure effect of pressure elevation, in vitro experiments with a hermetically sealed perfusion system that can regulate water pressure are indispensable, but the sealed chambers, of course, do not allow recording electrodes to access brain preparations; and

thus, it is almost impractical to electrophysiologically record neuronal activity under high pressure conditions. To overcome this problem, we decided to utilize functional multineuron calcium imaging (fMCI), a technique that optically probes action potentials of neuron populations *en masse* at the single cell resolution; because an action potential reliably elicits a rapid and transient calcium rise in the cell body, one can reconstruct the firing activity by monitoring the change in the calcium fluorescence intensity of the neuron (6). The fMCI can optically access neurons even in a glass-sealed recording chamber. With this imaging technique, we succeeded in recording neuronal activity under high pressure conditions.

Hippocampal slice cultures were prepared from postnatal day 7 Wistar/ST rats (SLC, Shizuoka), as previously described (7). Briefly, rat pups were decapitated, and the brains were cut into horizontal 300-μm-thick slices using a DTK-1500 microslicer (Dosaka, Kyoto) in aerated, ice-cold Gey's balanced salt solution (Invitrogen, Gaithersburg, MD, USA) supplemented with 25 mM glucose. Entorhino-hippocampal stumps were cultivated for 7 – 10 days on Omnipore membrane filters (JHWP02500, ϕ 25 mm; Millipore, Bedford,
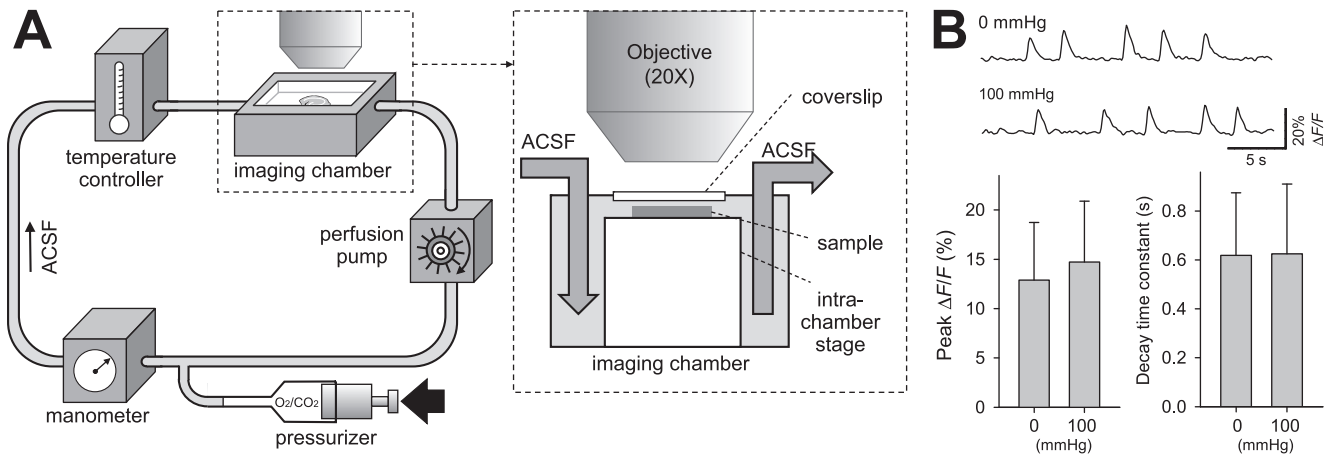
Fig. 1. Calcium imaging with a pressure/temperature-changeable chamber. A: Schematic illustrations of an imaging chamber connected to a hermetical circulation system (inset). An intra-chamber stage is placed in the center of the imaging chamber so that the working distance of a 20× water-immersion objective lens reaches brain slice preparations. The perfusion system consists of a peristaltic pump, an automatic pressurizer, a digital manometer, and a temperature controller. B: No effect of pressure on the dynamics of calcium signals *per se*. Top traces are an example obtained from the same neuron under normal (0 mmHg)- and high (100 mmHg)-pressure conditions. The size and decay constant of calcium transients were compared under both conditions (bottom).

MA, USA). Cultures were fed with 1 ml of 50% minimal essential medium, 25% Hanks' balanced salt solution (Invitrogen), and 25% horse serum (Cell Culture Laboratory, Cleveland, OH, USA) in a humidified incubator at 37°C in 5% CO_2. The medium was changed every 3.5 days.

For fMCI, slices were incubated with 0.0005% Oregon Green 488 BAPTA-1AM (Invitrogen) for 1 h at 37°C and then with artificial cerebrospinal fluid (ACSF) bubbled with 95% O_2 and 5% CO_2, at 36°C for >30 min (6). ACSF consisted of 126 mM NaCl, 26 mM $NaHCO_3$, 3.5 mM KCl, 1.24 mM NaH_2PO_4, 1.3 mM $MgSO_4$, 2.0 mM $CaCl_2$, and 10 mM glucose. A slice was transferred to a hermetically sealed imaging chamber (Fig. 1A), in which ACSF was warmed to 32°C with a TC-344B temperature controller (Warner Instruments, Hamden, CT, USA) and circulated at a rate of $1.5 - 2$ ml/min with a peristaltic pump (SJ-1215; Mitsumi Scientific, Tokyo). The intra-chamber pressure was increased by an electric pressurizer (A-99; Razel Scientific Instruments, Stamford, CT, USA) and continuously recorded by a digital manometer (PM015D; World Precision Instruments, Sarasota, FL, USA). The O_2/CO_2 air was filled in a tube immediately before the pressurizer to maintain the dissolved oxygen level and pH in ACSF. During our experiment period, the dissolved oxygen level was maintained at more than 20 mg/ml, and no significant change in pH was observed (data not shown). The stratum pyramidale was illuminated with a 488-nm laser and imaged at 10 frames/s with a CSU-X1 Nipkow-disk confocal unit (Yokogawa

Electric, Tokyo), a CCD camera (iXon-EM DV887; Andor Technology, Belfast, UK), and a 20× objective lens (UM Plan Fl; Olympus, Tokyo). Spike-triggered calcium signals were detected with custom-written software (8).

We chose the hippocampus, because this brain region is known to be vulnerable to cerebral ischemia and energy deprivation (9). To address the effect of a change in external solution pressure, a cultured hippocampal slice was placed in the pressure-changeable sealed chamber, and its spontaneous network activity was monitored with fMCI. Of 15 slices recorded, 7 slices were used for control experiments, and 8 slices were used for high-pressure experiments. On average, 100 ± 45 neurons per slice (means ± S.D. of 15 slices), ranging from 30 to 226 neurons, were monitored from the stratum pyramidale. For each slice, a movie was taken for 10 min to avoid photobleaching and phototoxicity.

In control experiments, spontaneous activity was recorded without pressure elevation (Fig. 2A). To quantify the activity, we employed four parameters that are proven to sensitively reflect the changes in the global level and spatiotemporal pattern of network activity (10, 11), that is, i) the percentage of the number of active neurons that exhibited calcium activity to the total number of neurons monitored, ii) the event frequency per cell per minute, iii) normalized Shannon index for the time axis (NSI_{time}), and iv) normalized Shannon index for the cell number axis (NSI_{cell}). The Shannon index is usually used to measure the extent
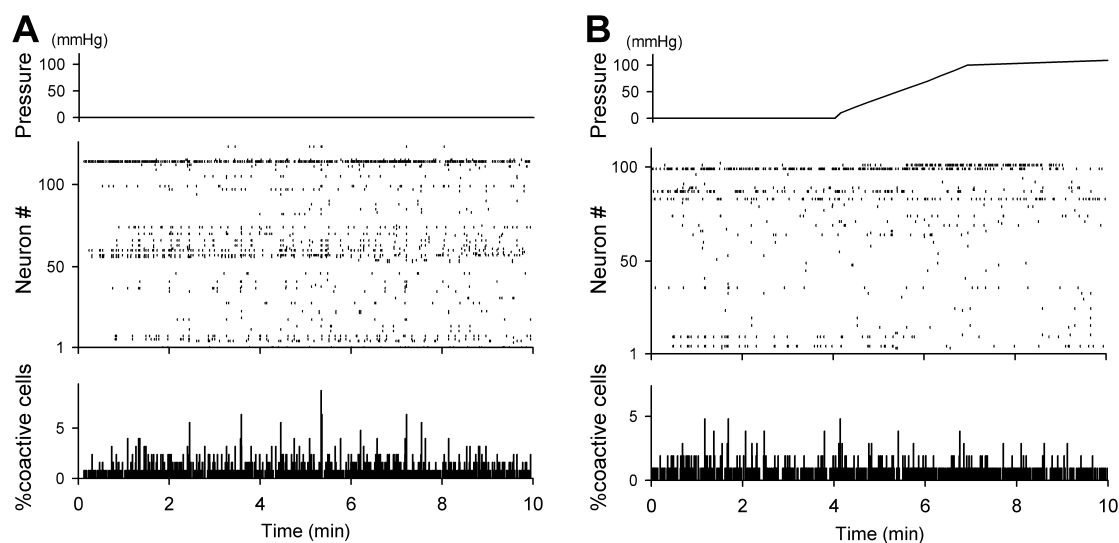
Fig. 2. No effect of pressure increases on spontaneous network activity in hippocampal slice cultures (representative data). During 4 – 7 min of the imaging period, the solution pressure was linearly increased from 0 to 100 mmHg (B, top), whereas in control experiments, it was maintained at 0 mmHg for 10 min (A, top). The middle rastergrams indicate the spatiotemporal patterns of hippocampal network activity. Each dot represents the timing of a single calcium-rise event that reflects an action potential of the corresponding neuron. The bottom histograms indicate the percentage of active neurons to the total at any given 100-ms time window.

of "diversity" in a focused region, and here, it was normalized to its possible range to ensure comparisons across different slice preparations (10). NSI_{cell} captures the spatial dispersion, that is, "evenness", of calcium events across neurons, whereas NSI_{time} captures the degree in temporal decorrelation, that is, "asynchronism", of calcium events. In control slices, none of these four parameters was changed with time (Fig. 3A). This indicates that slices were kept healthy at least for 10 min in our perfusion chamber.

The normal intracranial pressure in vivo ranges from 7 to 15 mmHg (12). Under normal conditions, a slight increase in the brain tissue volume produces few effects on the intracranial pressure, but a suprathreshold volume increase results in a marked rise in the pressure to tens of mmHg (12). We thus increased the fluid pressure up to 100 mmHg. There were no differences in the size and decay constant of calcium transients between normal (0 mmHg) and high (100 mmHg) pressure conditions (Fig. 1B), indicating that pressure does not affect the kinetic or properties of spike-evoked calcium events. Moreover, tetrodotoxin completely abolished calcium events under both conditions (n = 2 slices, data not shown). Thus, calcium signals arose from spiking activity even under high-pressure conditions, suggesting that we can directly compare the data between both conditions.

For each slice, a movie was taken for 10 min; four minutes after the imaging started, the pressure was gradually increased for 3 min at a rate of 33 mmHg/min and maintained at 100 mmHg for 3 min (Fig. 2B). The four activity parameters were compared between the 3-min periods before the pressure increased (0 – 3 min) and after the pressure reached 100 mmHg (7 – 10 min), but they did not show significant changes (Fig. 3B). When the pressure increase started from 5 – 15 mmHg (but not 0 mmHg), similar results were obtained (n = 5 slices, data not shown).

Finally we sought to confirm that the parameters adopted here actually work as good measurements to capture network activity by investigating the effect of different temperatures: 24°C, 28°C, 32°C, 36°C, and 40°C. All four parameters showed significant temperature dependence: % active cell: $P<0.001$, one-way ANOVA; events·cell^{-1}·min^{-1}: $P = 0.032$, Kruskal-Wallis test; NSI_{time}: $P = 0.037$, Kruskal-Wallis test; NSI_{cell}: $P<0.001$, one-way ANOVA. The trends indicate that higher temperatures were associated with higher degrees of neuronal activity and network synchronization (Fig. 3C).

In this study, we devised an imaging chamber with a hermetical circulation system to evaluate the effect of hyperbaric pressure on network activity of the hippocampus, a region known to be vulnerable to hypoxia, hypoglycemia, and stress. We found that the network activity was unchanged even under extremely high pressure conditions, as assessed by firing rates, active cell ratios, or diversity information.

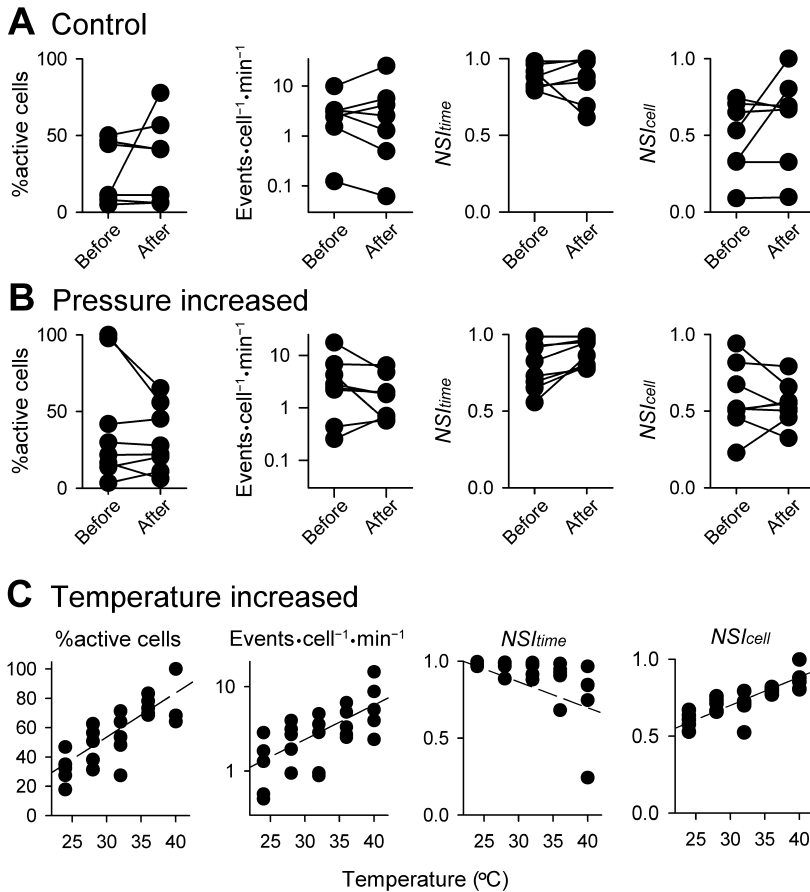Because the skull is made of rigid bones, the intra-

A Control

B Pressure increased

C Temperature increased

Fig. 3. No effect of pressure increases on spontaneous network activity (summarized data). To quantify the pressure effect, four parameters were obtained from 7 control slices (A) and 8 slices were used for high-pressure experiments (B, for details, see text). For each parameter, the average value was calculated for the time periods of $0-3$ min (Before) and $7-10$ min (After). Each symbol indicates the data of a single slice. No significant changes between Before and After were found in any parameters (paired t-test). C: Positive control experiments with changes in temperature. The imaging system and the parameters used here could detect the changes in network activity. All parameters significantly responded to temperature (see text).

cranial space is virtually constant. Therefore, the intracranial pressure may elevate when the brain swells due to edema or tumor, but indeed, the pressure is homeostatically maintained. This is simply due to a passive compensation, that is, when the brain volume increases, brain blood and cerebrospinal fluid are reflectively extruded from the intracranial space. This compensation range, however, is confined because the available volume of the intracranial fluid is limited. Therefore, excessive brain swelling finally increases the intracranial pressure, resulting in a decrease in cerebral perfusion pressure (1, 2). The decreased perfusion pressure leads to a drop of cerebral blood flow and thereby a shortage of oxygen and energy in the brain, eventually incapacitating neuronal function.

As for the functional meaning of the intracranial pressure homeostasis, there are roughly two phenomenological interpretations, that is, active protective adaptation and passive collateral result. In the scope of the protective adaptation hypothesis, it is considered that the pressure 'has to' be maintained in order to protect neurons because high pressure renders severe damage to neurons. In the scope of the collateral result hypothesis, the intracranial pressure is constant merely through

physically 'passive' reflection, so the homeostasis *per se* has no significant function. These two interpretations differ in terms of the vulnerability of neurons, that is, which is more injurious to neurons, high pressure itself or resultant energy shortage?

Nobody can ever answer this question, because the hyperbaric effect on neural function has been assessed exclusively by in vivo experiments. In vivo studies demonstrated that pressure elevation led to aberrations in sensation $(3-5)$. These studies, however, cannot discriminate the direct and indirect effects of pressure because increased intracranial pressure is inevitably accompanied by energy deprivation via a reflective fall of cranial blood flow. Therefore, the pressure-induced aberrations, as observed in the previous studies, are not attributable to the direct action of hyperbaric pressure.

To resolve this problem, we developed a new in vitro experimental system with a pressure-changeable chamber and examined the behavior of network activity against pressure elevation. As a result, neuron population activity did not change in response to pressure elevation, suggesting that physical pressure itself is not a major determinant of neuronal activity. These data hence favors the collateral result hypothesis, rather than

the protective adaptation hypothesis. It is still possible, however, that there exist other pressure-sensitive parameters that cannot be captured by our imaging technique. For example, some mechanosensors might change cellular metabolism or gene expression (13, 14). In addition, our observation period was restricted to a relatively short time (10 min) because of experimental limitations such as photobleaching and phototoxicity. High pressure–induced aberrations in neuronal excitability could take place at longer time scales, such as hours and days. Taken together, we conclude that at least an acute increase in extracellular fluid pressure does not affect spontaneous spiking activity.

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