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Research article

The inhibition of evoked excitatory postsynaptic potentials produced by ammonium chloride in rat hippocampal CA1 neurons

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ABSTRACT

The depression of evoked fast excitatory postsynaptic potentials (EPSPs) following superfusion with various concentrations (3 μM-5 mM) of ammonium chloride (NH_4Cl) were investigated in rat hippocampal CA1 neurons. The amplitude of the evoked fast EPSPs decreased by NH_4Cl in a concentration-dependent manner. The half-maximal inhibitory concentration for the inhibition of evoked fast EPSPs was 198 ± 125 μM (n = 8). The facilitation of a pair of field EPSPs elicited by paired-pulse stimulation (40-ms interval) (paired-pulse facilitation, PPF) was recorded following superfusion with NH_4Cl (200 μM and 3 mM). The PPF ratio increased to 180 ± 23% (n = 9) in the presence of 200 μM NH_4Cl compared with that in the absence of NH_4Cl (142 ± 24%, n = 9). In the presence of 3 mM NH_4Cl, the PPF ratio increased to 172 ± 30% (n = 7) compared with that in the absence of NH_4Cl (126 ± 13%, n = 7). This implies that NH_4Cl suppressed the presynaptic release of glutamate. Exogenous glutamate- or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-induced depolarization elicited by using pressure application did not reduce following superfusion with 200 μM or 5 mM NH_4Cl in the presence of 0.3 μM tetrodotoxin, suggesting that NH_4Cl did not affect the postsynaptic glutamate response. Action potentials elicited by rectangular outward current injection from CA3 neurons projecting to CA1 neurons were persistent at 200 μM NH_4Cl but disappeared at 5 mM NH_4Cl. The abolishment of action potentials in the presence of 5 mM NH_4Cl was released by increasing the amplitude of the injection current. These results suggest that NH_4Cl depresses evoked fast EPSPs mainly via a presynaptic mechanism at low NH_4Cl concentrations, and the failure of action potential propagation through the excitatory nerve may also contribute to the depression of evoked fast EPSPs at high NH_4Cl concentrations.

1. Introduction

Hyperammonemia is a condition characterized by excess ammonia (NH_3) in the blood. Enzymes involved in the urea cycle are only found in hepatocytes; thus, hepatic function impairment causes hyperammonemia. Hyperammonemia, under conditions of congenital urea cycle enzyme deficiency or hepatic function deterioration, impairs the central nervous system. As hepatic function impairment progresses, severe neurological and neuropsychiatric disorders appear, such as disturbances of consciousness, involuntary movements, myoclonus, and/or asterixis. Acute impairment of hepatic function rapidly leads to hepatic encephalopathy, which induces unusual behavior, delirium, and coma within a few days [1]. In coma patients, the concentration of NH_3 is 300–500 μM in the arterial blood [2]. In a rat model of fulminant liver failure, *in vivo* concentrations of NH_3 in the blood and brain were 1 mM and 1–5 mM, respectively [3, 4].

At a normal body temperature and pH (pH = 7.4), 98% of NH_3 exists as ammonium ion (NH_4^+) [4]. Under normal physiological conditions, the arterial NH_4^+ levels are kept low (50–100 μM) via the removal of gut-derived NH_3 from the blood by the liver [1]. Cell membranes permit the passage of NH_3 more easily than that of NH_4^+; however, up to 25% of NH_3 may enter the brain as NH_4^+ at physiological pH values [5]. Swain *et al.* [3] found that under coma conditions with acute liver failure, the concentration of NH_4^+ in the brain may be nearly five times higher than that in the arteries.

The central nervous system may become widely depressed under conditions of hyperammonemia; intravenous application of ammonium acetate (3.5 mM/kg corresponding to the concentration observed during the onset of encephalopathy) suppressed inhibitory postsynaptic

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potentials (IPSPs) and changed pattern of electrocorticogram (sharp positive waves dominated and low-amplitude background activity decreased pattern) in cat spinal cord (gastrocnemius motoneurons) and cerebral cortex, respectively [6]. Furthermore, in an *in vitro* study, the frequencies of spontaneous action potentials were suppressed by 2 mM ammonium chloride (NH_4Cl) in rodents [7]. In the rat hippocampus, the release of glutamate (Glu) evoked by the application of KCl (56 mM) was reduced when tissue slices were exposed to 5 mM NH_4Cl [8]. In another study, the excitatory postsynaptic potentials (EPSPs) and presynaptic fiber potentials were depressed by NH_4Cl (8 mM) in rat hippocampal pyramidal neurons [9]. In addition, NH_4Cl (5–20 mM) was shown to suppress field EPSPs in rat hippocampal pyramidal neurons [10, 11]. However, in cat spinal motoneurons, EPSPs, input resistance, and action potentials did not change despite the intravenous application of ammonium acetate [12]. The effects of high concentrations of NH_4Cl (2–8 mM) on evoked EPSPs or field EPSPs have been studied [9, 11, 13, 14, 15]; however, there are no known studies on the effect of low concentrations of NH_4Cl on evoked EPSPs. The purpose of this study was to investigate the concentration of NH_4Cl that causes suppression of presynaptic Glu release following a rapid increase in NH_4Cl by examining the effect of various concentrations of NH_4Cl (3 μM-5 mM) on evoked EPSPs in rat hippocampal CA1 neurons.

2. Materials and methods

2.1. Ethics

All experiments were conducted in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Science of the Physiological Society of Japan, and with the approval of the Institutional Animal Use and Care Committee of Kurume University.

2.2. Slice preparation

Male Wistar rats (7–10 weeks old, 250–300 g body weight) were quickly decapitated under ether anesthesia, and their forebrains were harvested and placed in chilled (4–6 °C) artificial cerebrospinal fluid (ACSF) aerated with 95% O_2 and 5% CO_2. The composition of the ACSF was (in mM): 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$ and 11 glucose. Transverse slices (350-μm thick) of the rats forebrains were made using a Vibrating microtome 7000 (model 7000smz-212; Campden Instruments Ltd., Loughborough, Leics, England). A single slice containing the hippocampus was placed on a nylon net in a chamber (volume, 500 μL) and held in place with a titanium grid placed on the upper surface of the slice. The brain slices were then completely submerged in the superfused medium (pH 7.4, temperature 36.5 ± 0.5 °C, flow at 4–5 ml/min) and used for all recordings in this study.

2.3. Electrophysiology

Intracellular recordings were made from the CA1 (Figures 1, 2, and 4) and CA3 (Figure 5) neurons with 2 M potassium (K) acetate-containing electrodes (resistance, 60–90 MΩ). The membrane potential of the recorded neurons was changed by passing a current through the recording electrode using an active bridge circuit (Axoclamp-2A; Molecular devices, San Jose, CA, USA). The apparent input resistance of the neurons was monitored by passing small hyperpolarizing pulses (0.1–0.2 nA, 200 ms) through the recording electrode. Fast EPSPs were elicited by focal stimulation of the Schaffer collaterals through monopolar tungsten electrodes (100-μm diameter, insulated except at the tip) placed on the stratum radiatum of the CA1-CA2 border in hippocampal slice preparations. Electrical stimuli (20–50 μA, 200 μs) were applied at 20-s intervals.

Extracellular recordings were made from the stratum radiatum of the CA1 region (Figure 3) with ACSF-containing electrodes (resistance, 5–10 MΩ). Extracellular direct current (DC) potentials were recorded in the proximal stratum radiatum (200–300 μm away from the stratum pyramidale) of the CA1 region.

Signals from the amplifier (Axoclamp-2A) were recorded with the thermal pen recorder (DC-1, Nihon Kohden, Tokyo, Japan). The data were stored and subsequently analyzed using pCLAMP 10 (Axon Instruments) data acquisition and analysis program on a personal computer (PC). The electrophysiological recordings were performed at 36.5 ± 0.5 °C.

2.4. Osmolarity measurements

The osmotic pressure of each solution was measured using a vapor osmometer (Wescor Vapor Pressure Osmometer VAPRO model-5520, Xlem. Japan, Kanagawa, Japan), and was derived by averaging the measurement results of three samples taken from the same solution. The osmotic pressure of each solution used in the study was obtained by averaging all values in each experiment.

2.5. Reagents

The drugs used were bicuculline, DL-2-amino-5-phosphonopentanoic acid (DL-AP5), and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (all from Sigma-Aldrich, St Louis, MO, USA), sodium glutamate monohydrate (Glu), NH_4Cl, sucrose, and tetrodotoxin (TTX) (all from FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

2.6. Drug application

All drugs except Glu and AMPA were dissolved in perfusate and applied by bath application. Owing to the volume of the connecting tubing, a delay of 15–20 s occurred before the new medium reached the chamber when the perfusate was switched. The chamber was filled with the test solution approximately 30 s after starting the perfusion. All antagonists were applied 10 min before the start of the recordings.

Glu and AMPA were dissolved in the perfusate and applied by pressure application. Glu- or AMPA-induced membrane depolarization was induced by pressure application of Glu (0.25 M) or AMPA (0.001 M), respectively, in the presence of TTX (0.3 μM) to block secondary neurotransmitter release. A glass micropipette with a tip diameter of 10–20 μm was filled with either Glu solution (Glu dissolved in water at 0.25 M) or AMPA solution (AMPA dissolved in ACSF at 0.001 M). Nitrogen gas (100%) was directed to either the Glu- or AMPA-filled pipette, and the Glu or AMPA solution was ejected via a gas pressure (2 kg/cm^2) of 20-ms duration. The micropipette used for the pressure application of Glu or AMPA was placed at least 100 μm away from the recording neuron of the slice. When the amplitude of Glu- or AMPA-induced response was less than 10 mV following pressure application of Glu and AMPA, the position of the Glu- or AMPA-filled micropipette was precisely adjusted to the position where amplitudes of Glu- and AMPA-induced depolarization were recorded from higher than 10 mV up to 50 mV.

2.7. Statistical analysis

All recordings were obtained from one neuron of each slice. The number of neurons (slices) examined is given in parentheses in each figure. All quantitative results except the dose-dependent curve (Figure 1B) are presented as mean ± standard deviation (SD). A repeated-measures analysis of variance with Dunnett's post hoc test was used to compare the data (Figures 1, 2, 3, and 4). Statistical significance was determined at $^*P < 0.05$ and $^{**}P < 0.01$, unless otherwise indicated.

3. Results

3.1. NH_4Cl inhibits evoked EPSPs in CA1 pyramidal neurons

We recorded EPSPs of hippocampal CA1 pyramidal neurons identified by histological position and electrophysiological properties [16].

These neurons were characterized by relatively hyperpolarized membrane potential, input resistance, and inward rectification of the cell membrane by hyperpolarizing current pulse injection. Hippocampal CA1 neurons with stable membrane potentials exceeding -60 mV were used for subsequent studies. The resting membrane potential and apparent input resistance in CA1 neurons were -68 ± 3 mV and 48 ± 16 MΩ (n = 41), respectively.

To evoke fast EPSPs, $GABA_A$ receptor antagonist, bicuculline (10 μM), was applied 10 min before and during the time at which recordings were obtained. In the presence of bicuculline, the amplitude of the evoked fast EPSPs increased, and their duration was prolonged. In several cases, large secondary EPSPs produced by the activation of *N*-methyl-D-aspartate (NMDA)-type Glu receptors (NMDARs), and sodium ion (Na^+) spikes were observed. Therefore, the NMDAR antagonist, AP-5 (100 μM), was applied with bicuculine 10 min before and during the recordings to elicit stable non-NMDA-type fast EPSPs.

Figure 1 shows the effects of various concentrations of NH_4Cl (3 μM-5 mM) on evoked non-NMDA-type fast EPSPs. The application of low concentrations of NH_4Cl (3–300 μM) did not change resting membrane potential and input resistance (-66 ± 3 mV, n = 18; 48 ± 16 MΩ, n = 18, respectively). Each neuron was alternately exposed to the entire series of NH_4Cl containing solutions. Continuous administration of NH_4Cl was carried with solutions in ascending order of NH_4Cl concentration; the application time of each concentration of NH_4Cl was fixed 10–15 min because it took ~15 min to obtain the maximal response at 300 μM NH_4Cl and ~10 min to obtain the same at 5 mM NH_4Cl. High concentrations of NH_4Cl (1–5 mM) depolarized or hyperpolarized the membrane potential in several CA1 neurons. The membrane potential was depolarized by 8 ± 3 mV (n = 15), and the input resistance increased to 65 ± 28 MΩ (n = 15) in 63% of all tested CA1 neurons (n = 24) within 5 min from the onset of 5 mM NH_4Cl application. The membrane potential was hyperpolarized by 7 ± 2 mV (n = 4), and the input resistance decreased to 29 ± 12 MΩ (n = 4) in 17% of the CA1 neurons within 5 min from the start of 5 mM NH_4Cl application. The remaining 20% of CA1 neurons (n = 5) did not show any marked changes in the membrane potential, and in the input resistance with the application of 5 mM NH_4Cl (-70 ± 1 mV, n = 5; 42 ± 7 MΩ, n = 5, respectively).

The mean amplitude of non-NMDA-type fast EPSPs in the absence of NH_4Cl was 7 ± 1 mV (n = 18). The mean amplitudes of non-NMDA-type fast EPSPs in the presence of 3 μM, 10 μM, 30 μM, 100 μM, 300 μM, 1 mM, 3 mM, and 5 mM NH_4Cl were reduced to 100 ± 3%, 79 ± 22%, 76 ± 27%, 65 ± 32%, 49 ± 35%, 27 ± 2%, 3 ± 4%, and 3 ± 1% (n = 8), respectively, compared with those in the absence of NH_4Cl (Figure 1). The mean amplitudes of non-NMDA-type fast EPSPs decreased in the presence of NH_4Cl in a concentration-dependent manner. The half-maximal inhibitory concentration (IC_{50}) value for the inhibition of non-NMDA-type fast EPSPs was 198 ± 125 μM (n = 8) (Figure 1B). Since the membrane potential depolarized by 8 ± 3 mV (n = 15) or hyperpolarized by 7 ± 2 mV (n = 4) in the presence of high concentrations of NH_4Cl (5 mM), we investigated the effect of a depolarized or hyperpolarized membrane potential on the amplitude of the evoked non-NMDA-type fast EPSPs. The amplitude of the evoked non-NMDA-type fast EPSPs was reduced when the depolarized or hyperpolarized membrane potential at high concentrations of NH_4Cl was restored to the resting membrane potential by the injection of anodal or cathodal DC current.

3.2. *Hyperosmolarity and a high concentration of NH_4Cl inhibit evoked EPSPs*

Synaptic transmission was inhibited following superfusion with hypertonic ACSF; the slope of the field EPSPs was reduced to 57% when the tonicity of the solution (300 mOsm/kgH_2O) was increased to 360 mOsm/kgH_2O [17]. To separate the effect of hyperosmolarity and that of high concentrations of NH_4Cl on evoked non-NMDA-type fast EPSPs, the amplitude of the evoked non-NMDA-type fast EPSPs was investigated in the same tonicity solution of ACSF containing 5 mM NH_4Cl or 10 mM sucrose. The osmolarity of normal ACSF was 290 ± 3 mOsm/kgH_2O (n = 5). The osmolarities of ACSF containing 10 mM sucrose and ACSF containing 5 mM NH_4Cl were 301 ± 1 mOsm/kgH_2O (n = 4) and 298 ± 5 mOsm/kgH_2O (n = 4), respectively, with no significant difference. The effects of ACSF containing 10 mM sucrose and ACSF containing 5 mM NH_4Cl on the amplitude of non-NMDA-type fast EPSPs were investigated (Figure 2). The amplitude of non-NMDA-type fast EPSPs in ACSF containing 10 mM sucrose (7 ± 6 mV, n = 4) tended to decrease, but there was no significant difference compared with the amplitude in normal ACSF (13 ± 5 mV, n = 4). After superfusion with ACSF containing 5 mM NH_4Cl, the amplitude of non-NMDA-type fast EPSPs was significantly reduced to 2 ± 1 mV (n = 4) compared with that in normal ACSF ($P < 0.01$) and in ACSF containing 10 mM sucrose ($P < 0.05$) (Figure 2B). These results indicate that the evoked non-NMDA-type fast EPSPs were unaffected by 10 mOsm/kgH_2O hypertonic ACSF. However, the same hypertonic ACSF containing 5 mM NH_4Cl instead of 10 mM sucrose showed significant inhibitory action on the evoked non-NMDA-type fast

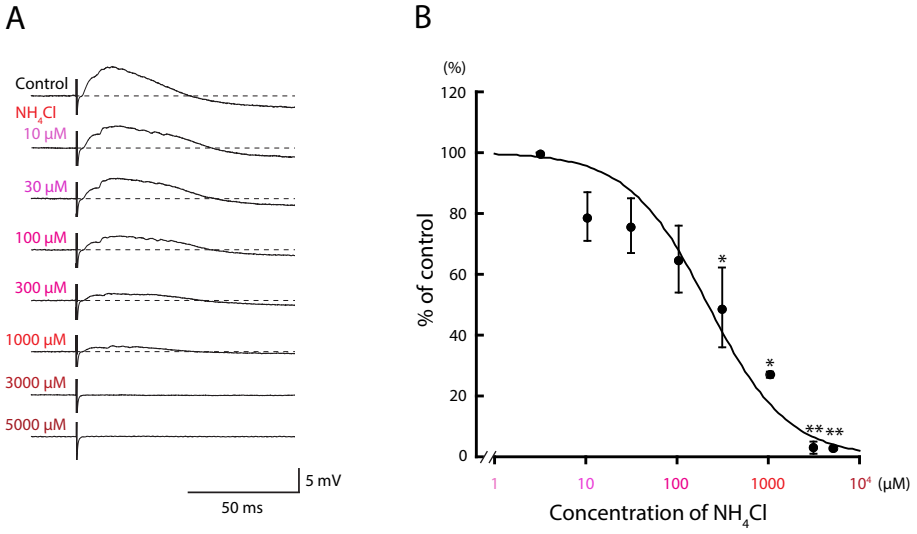


Figure 1. Effects of NH_4Cl on evoked fast EPSPs in CA1 neurons. A: Effects of various concentrations (10, 30, 100, 300, 1000, 3000, and 5000 μM) of ammonium chloride (NH_4Cl) on evoked fast excitatory postsynaptic potentials (EPSPs) elicited by electrical stimulation (100 μs in duration and 30 μA in intensity) at the Schaffer collaterals. NH_4Cl decreased the amplitude of evoked fast EPSPs in a concentration-dependent manner. B: The dose-response dependence of the amplitude of the evoked fast EPSPs on the NH_4Cl concentration. The average half-maximal inhibitory concentration was 198 μM. The vertical line shows the standard error of the mean. The amplitude of evoked fast EPSPs was significantly suppressed at concentrations of NH_4Cl above 300 μM (*$P < 0.05$, **$P < 0.01$, respectively, repeated measures analysis of variance with Dunnett's post hoc test).

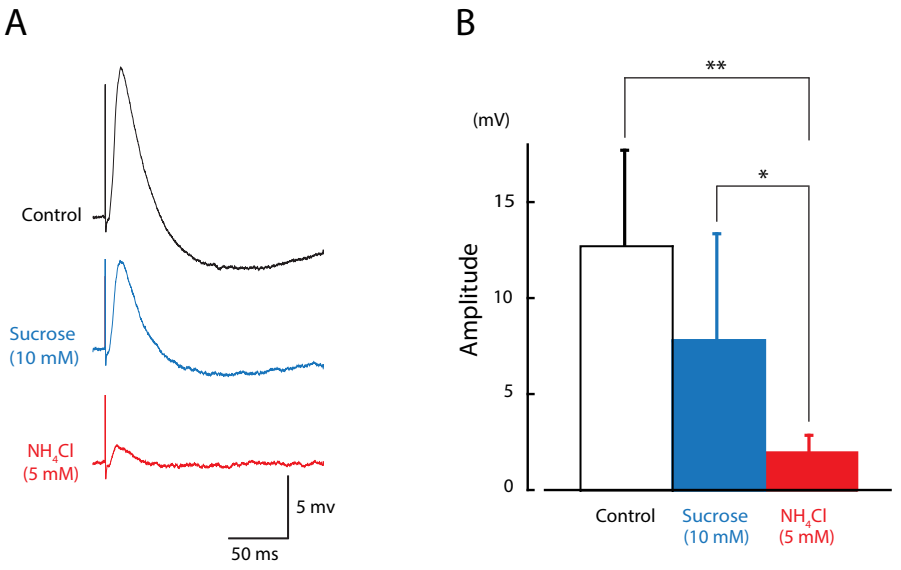


Figure 2. Effects of hyperosmolarity and high concentration of NH_4Cl on evoked fast EPSPs. Effects of a hypertonic solution (ACSF containing 10 mM sucrose) and ACSF containing 5 mM NH_4Cl on the amplitude of evoked fast EPSPs. The osmotic pressure of ACSF containing 10 mM sucrose, and ACSF containing 5 mM NH_4Cl was not significantly different (301 ± 1 mOsm/kgH_2O, n = 4; 298 ± 5 mOsm/kgH_2O, n = 4, respectively). A: Typical recordings of the non-NMDA-type fast EPSPs inhibited by the continuous application of ACSF containing 10 mM sucrose and ACSF containing 5 mM NH_4Cl. B: The amplitude of non-NMDA-type fast EPSPs in ACSF containing 10 mM sucrose was 7 ± 6 mV (n = 4), which was not significantly different from that in normal ACSF (13 ± 5 mV, n = 4). After superfusion with ACSF containing 5 mM NH_4Cl, the amplitude of non-NMDA-type fast EPSPs was significantly reduced to 2 ± 1 mV (n = 4), compared with the amplitude in normal ACSF and ACSF containing 5 mM NH_4Cl (*P < 0.05, **P < 0.01, respectively, repeated measures analysis of variance with Dunnett's post hoc test).

EPSPs. The inhibition of the evoked non-NMDA-type fast EPSPs may be due to the high concentration of NH_4Cl, and not due to hyperosmolarity.

3.3. NH_4Cl reduces the release probability of Glu

When the Schaffer collaterals are stimulated twice in rapid succession, there is characteristic facilitation of the field EPSP elicited by the second impulse in the CA1 region [18]. The paired-pulse facilitation (PPF) of evoked EPSPs was inversely correlated with the release probability of Glu in hippocampal neurons [19].

To investigate the effect of NH_4Cl on the release probability of Glu, a stimulus intensity of 30–50 μA and an interstimulus interval of 40 ms were applied to evoke a pair of field EPSPs (Figure 3A and C), and the effect of NH_4Cl on the PPF ratio ([maximal slope of the 2nd field EPSP/maximal slope of the 1st field EPSP] × 100) was examined (Figure 3B and D). A pair of field EPSPs induced by an equal-intensity stimulus pair, and the PPF ratio before and after the application of 200

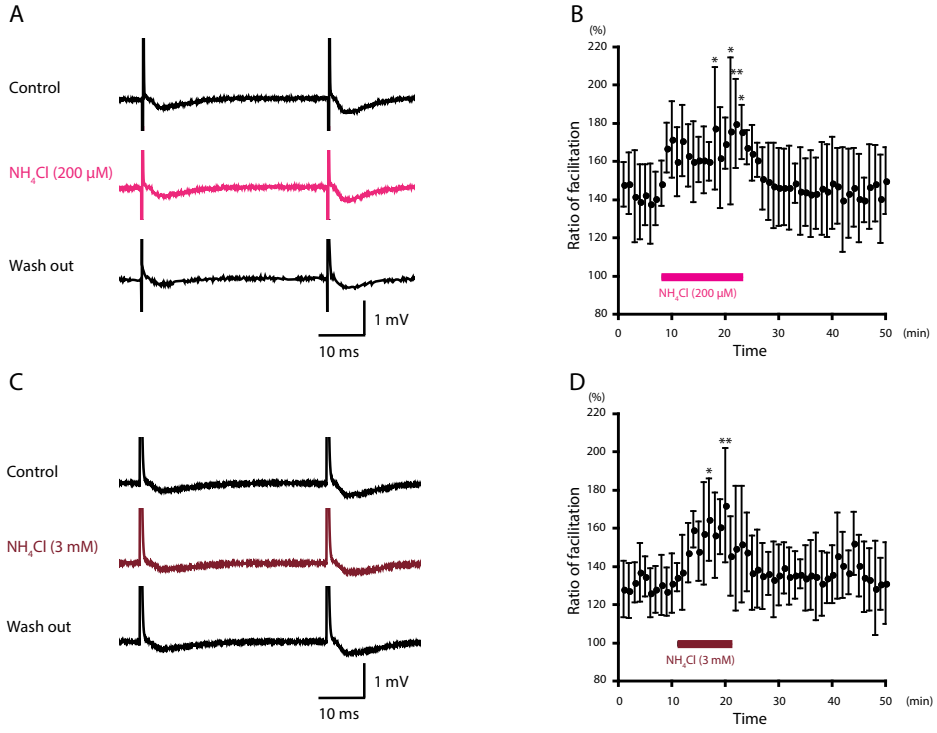
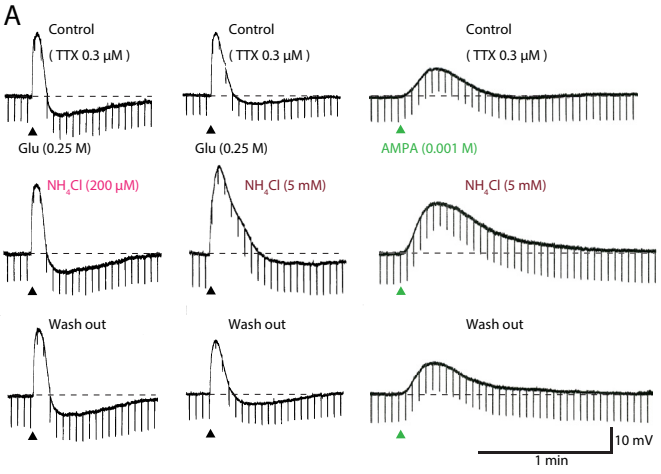


Figure 3. Effects of 200 μM and 3 mM NH_4Cl on the paired-pulse facilitation of field EPSPs. A: Paired field excitatory postsynaptic potentials (field EPSPs) were elicited by a pair of stimuli with a constant interstimulus interval of 40 ms and a constant duration of 200 μs. Paired field EPSPs shown in the *top trace* is recorded in the absence of 200 μM NH_4Cl (control), those in the *middle trace* were recorded in the presence of 200 μM NH_4Cl, and those in the *bottom trace* were recorded 10 min after NH_4Cl wash-out. B: Time course of changes in the paired pulse facilitation (PPF) ratio obtained from recordings of nine neurons. The filled circles show the mean, and the vertical lines show the SD. The value of each ratio was calculated by averaging three consecutive ratios induced by each paired-pulse stimulus applied with a 20-s interval. C: Paired field EPSPs shown in the *top trace* were recorded in the absence of 3 mM NH_4Cl (control), those in the *middle trace* were recorded in the presence of 3 mM NH_4Cl, and those in the *bottom trace* were recorded 10 min after NH_4Cl wash-out. D: Time course of changes in the PPF ratio obtained from recordings of seven neurons. The filled circles show the mean, and the vertical lines show the SD. After superfusion with NH_4Cl (200 μM and 3 mM), the PPF ratio increased significantly compared with that in the absence of NH_4Cl (*P < 0.05, **P < 0.01, respectively, repeated measures analysis of variance with Dunnett's post hoc test).

μM NH_4Cl are shown in Figure 3A and B. At a low concentration of NH_4Cl (200 μM), the PPF ratio showed a reversible increase. The PPF ratio was 142 ± 24% (n = 9) in the absence of 200 μM NH_4Cl (at 5 min before superfusion with NH_4Cl), and 180 ± 23% (n = 9) in the presence of 200 μM NH_4Cl (at 14 min after superfusion with NH_4Cl). After a 10-min wash-out of 200 μM NH_4Cl using normal ACSF, the PPF ratio was 149 ± 10% (n = 9) (Figure 3B). The PPF ratio in the presence of 200 μM NH_4Cl was significantly higher than that in the absence of 200 μM NH_4Cl ($P < 0.01$). At a high concentration of NH_4Cl (3 mM), the PPF ratio showed a reversible increase as well. The PPF ratio was 126 ± 13% (n = 7) in the absence of 3 mM NH_4Cl (at 5 min before superfusion with NH_4Cl), and 172 ± 30% (n = 7) in the presence of 3 mM NH_4Cl (at 10 min after superfusion with NH_4Cl). After a 10-min wash-out of 3 mM NH_4Cl using normal ACSF, the PPF ratio was 140 ± 14% (n = 7) (Figure 3D). The PPF ratio in the presence of 3 mM NH_4Cl was significantly higher than that in the absence of 3 mM NH_4Cl ($P < 0.01$). These results suggest that the superfusion with NH_4Cl may lead to reduction in the probability of Glu release from excitatory nerve terminals.

3.4. Effect of NH_4Cl on Glu- and AMPA-induced responses

To determine whether superfusion with NH_4Cl inhibits postsynaptic Glu- and AMPA-induced responses, exogenous Glu (0.25 M) and AMPA (0.001M) were applied by pressure application (2 kg/cm^2, 20 ms) in the presence of 0.3 μM TTX. The left column of Figure 4A shows Glu-induced responses at a membrane potential of -62 mV. In the control condition, exogenous Glu applied by pressure application depolarized membrane potential (Glu-induced depolarization). The amplitude and half-width of the Glu-induced depolarization were 25 ± 7 mV (n = 4) and 7 ± 3 s (n = 4), respectively. Glu-induced depolarization was not affected by the application of 200 μM NH_4Cl (amplitude, 25 ± 6 mV, n = 4; half-width, 7 ± 3 s, n = 4) (Figure 4B). The middle column of Figure 4A shows the Glu-induced depolarization at a membrane potential of -64 mV. In the control condition, the amplitude and half-width of the Glu-induced depolarization were 24 ± 6 mV (n = 4) and 8 ± 4 s (n = 4), respectively. In the presence of 5 mM NH_4Cl, the amplitude and the half-width of the Glu-induced depolarization were significantly increased by 143 ± 19% (35 ± 14 mV, n = 4, $P < 0.05$) and 160 ± 45% (12 ± 4 s, n = 4, $P < 0.05$),



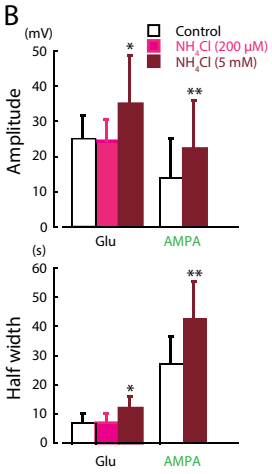


Figure 4. Effects of NH_4Cl on Glu- or AMPA-induced depolarization. A: Left column; Effect of 200 μM NH_4Cl on Glu-induced depolarization in the presence of 0.3 μM tetrodotoxin (TTX). The Glu-induced depolarization elicited by the pressure application of 0.25 M Glu (2 kg/cm^2, 20 ms) shown in the *top trace* was recorded in the absence of NH_4Cl (control), that in the *middle trace* was recorded in the presence of 200 μM NH_4Cl, and that in the *bottom trace* was recorded 10 min after NH_4Cl wash-out. The dotted lines in each trace show the pre-exposure membrane potential (-62 mV). Middle column; Effect of 5 mM NH_4Cl on Glu-induced depolarization in the presence of 0.3 μM TTX. The Glu-induced depolarization shown in the *top trace* was recorded in the absence of NH_4Cl (control), that in the *middle trace* was recorded in the presence of 5 mM NH_4Cl, and that in the *bottom trace* was recorded 10 min after NH_4Cl wash-out. The dotted lines in each trace show the pre-exposure membrane potential (-64 mV). Right column; Effect of 5 mM NH_4Cl on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-induced depolarization in the presence of 0.3 μM TTX. The AMPA-induced depolarization elicited by the pressure application of 0.001 M AMPA shown in the *top trace* was recorded in the absence of NH_4Cl (control), that in the *middle trace* was recorded in the presence of 5 mM NH_4Cl, and that in the *bottom trace* was recorded 10 min after NH_4Cl wash-out. The dotted lines in each trace show the pre-exposure membrane potential (-68 mV). B: Glu-induced depolarization was significantly increased following superfusion with a high concentration of NH_4Cl (5 mM, $*P < 0.05$), but was not affected by superfusion with a low concentration of NH_4Cl (200 μM). AMPA-induced depolarization was significantly increased following superfusion with a high concentration of NH_4Cl (5 mM, $**P < 0.01$).

respectively (Figure 4B). In Figure 4A, the right column shows AMPA-induced responses at a membrane potential of -68 mV. In the control condition, AMPA applied by pressure application depolarized membrane potential (AMPA-induced depolarization). The amplitude and the half-width of the AMPA-induced depolarization were 14 ± 11 mV (n = 4) and 27 ± 9 s (n = 4), respectively. In the presence of 5 mM NH_4Cl, the amplitude and the half-width of the AMPA-induced depolarization were significantly increased by 169 ± 28% (22 ± 14 mV, n = 4, $P < 0.01$) and 160 ± 30% (43 ± 12 s, n = 4, $P < 0.01$), respectively (Figure 4B). These results suggest that Glu-induced depolarization was not altered by superfusion with a low concentration of NH_4Cl (200 μM), but was increased by superfusion with a high concentration of NH_4Cl (5 mM). The AMPA-induced depolarization was also increased following superfusion with a high concentration of NH_4Cl (5 mM).

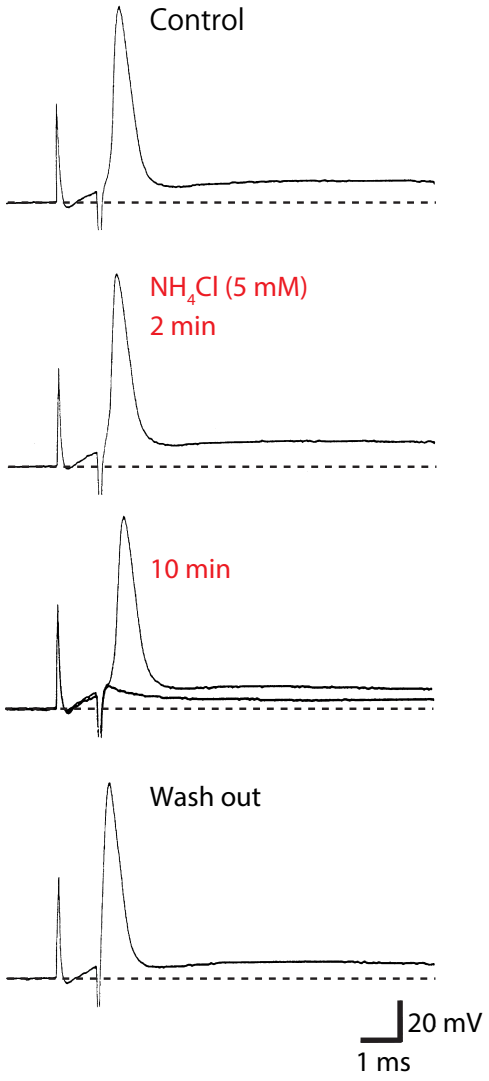


Figure 5. Effects of NH_4Cl on action potentials in CA3 neurons. Action potentials were elicited by depolarizing current pulse injections (0.21 nA, 1 ms) through recording electrodes. The membrane potentials were kept at -70 mV by direct current injection through the recording electrode. Traces from top to bottom: the rising phase of each action potential in the absence of NH_4Cl (control), 2 min after the application of 5 mM NH_4Cl, 10 min after the application of 5 mM NH_4Cl, and 10 min after NH_4Cl wash-out. Note that the action potentials disappeared after 10-min of 5 mM NH_4Cl application. At this moment, when the current injection was increased (0.24 nA), the action potentials reappeared.

3.5. NH_4Cl suppresses action potentials elicited from CA3 neurons

To investigate the effect of NH_4Cl on CA3 neurons, projecting to CA1 neurons, we elicited action potentials from CA3 neurons and observed the effect of NH_4Cl on these potentials. Hippocampal CA3 neurons with stable membrane potentials exceeding -60 mV were used for subsequent studies. The resting membrane potential and apparent input resistance in CA3 neurons were -67 ± 6 mV (n = 10) and 37 ± 12 MΩ (n = 10), respectively. Action potentials were recorded from CA3 neurons, which served as the origin of the Schaffer collaterals. An action potential was elicited by a rectangular outward current injection (0.2–0.3 nA, 1 ms), and abolished by 0.3 μM TTX; this suggests that TTX-sensitive Na^+-channels involved in generation of the recorded action potentials. The amplitude of action potentials was 80 ± 7 mV (n = 10) in the absence of NH_4Cl, 80 ± 8 mV (n = 4) in the presence of 200 μM NH_4Cl, 80 ± 6 mV (n = 6) in the presence of 5 mM NH_4Cl, and 80 ± 6 mV (n = 10) after 10 min of NH_4Cl wash-out. The action potentials were not significantly affected even at 2 min after superfusion with NH_4Cl (5 mM); however, at 10 min after superfusion, the action potentials disappeared (n = 6). At this point, when the current injection was increased (0.23–0.5 nA), the action potentials reappeared (Figure 5). The action potentials were not significantly affected even at 2 min after the application of NH_4Cl (200 μM and 5 mM), but were suppressed by superfusion with 5 mM NH_4Cl when the application time increased to 10 min.

4. Discussion

4.1. Effect of NH_4Cl on evoked EPSPs in the CA1 pyramidal neurons

4.1.1. Subtype of evoked EPSPs reduced by NH_4Cl

The fast EPSPs were separated from the IPSPs by using bicuculline, $GABA_A$ receptor antagonist. By superfusion with bicuculline alone, a single electrical stimulation simultaneously evoked a non-NMDA-type fast EPSP and a large secondary NMDA-type fast EPSP, followed by Na^+ spikes. AP-5, an NMDAR antagonist, was applied with bicuculline to obtain stable recordings of non-NMDA-type fast EPSPs; therefore, the effect of NH_4Cl on the NMDA-type fast EPSPs was not investigated in this study. The previous studies showed the presence of AMPA-type Glu receptors (AMPARs) in the postsynaptic density of excitatory synapses in rat Schaffer collaterals [20, 21]. Another study found AMPARs and NMDARs to be colocalized in many axodendritic asymmetric synapses within the CA1 subfield of rat cultured hippocampal pyramidal neurons and rat hippocampal tissue slices [22, 23]. In the presence of bicuculline and AP-5, the evoked non-NMDA-type fast EPSPs may be due to activation of the AMPARs on the postsynaptic density in axodendritic asymmetric synapses within the CA1 subfield. In this study, electrical stimulation of Schaffer collaterals induce fast EPSPs suggesting that AMPARs mainly contribute to evoked non-NMDA type fast EPSPs.

4.1.2. Effect of low concentrations of NH_4Cl on evoked EPSPs

The application of low concentrations of NH_4Cl (3–300 μM) did not change the resting membrane potential or input resistance, suggesting that NH_4Cl did not affect the properties of the postsynaptic membrane. Under treatment with 0.3 μM TTX, the Glu-induced depolarization obtained by pressure application was not affected by 200 μM NH_4Cl, suggesting that the low concentration of NH_4Cl had no effect on the postsynaptic Glu response. In contrast, the amplitudes of non-NMDA-type fast EPSPs were decreased by low concentrations of NH_4Cl (3–300 μM) in a concentration-dependent manner and were significantly decreased by 300 μM NH_4Cl, compared with the value in normal ACSF ($P < 0.05$, Figure 1 B). Under normal physiological conditions (arterial NH_4^+ concentration is 50–100 μM) [1], NH_4Cl gradually inhibited the presynaptic transmission of Glu without affecting the postsynaptic response. When the concentration of NH_4^+ was increased to 300 μM, which is the arterial blood concentration level of coma patients (300–500 μM) [2], NH_4Cl significantly reduced Glu release from excitatory nerve terminals. The

PPF ratio of the field EPSPs showed a reversible increase at 200 μM NH_4Cl; this NH_4Cl concentration is the IC_{50} value for the inhibition of non-NMDA-type fast EPSPs (198 ± 125 μM, n = 8). The PPF of the evoked EPSPs in hippocampal neurons was inversely correlated with the release probability of Glu [19, 24, 25]. Therefore, the increase of the PPF ratio in the presence of 200 μM NH_4Cl may be due to a reduction in release probability of Glu at the first stimulus. Action potentials recorded from CA3 neurons were not affected by 200 μM NH_4Cl. These results suggest that the reduction in the evoked fast EPSPs by superfusion with low concentrations of NH_4Cl may be due to the inhibition of presynaptic Glu release. The rapid increase in blood NH_4Cl levels in acute hepatic encephalopathy may reduce Glu release from the presynaptic terminals without affecting the postsynaptic membrane responses.

4.1.3. Effect of high concentrations of NH_4Cl on evoked EPSPs

The application of a high concentration of NH_4Cl (5 mM) depolarized the membrane with an increase in the input resistance (8 ± 3 mV, n = 15; 65 ± 28 MΩ, n = 15, respectively) in 63% of all tested CA1 neurons (n = 24). This observation is similar to the result of a study in which the application of 1–4 mM NH_4^+ resulted in the depolarization of hippocampal CA1 pyramidal neurons by approximately 15 mV [14]. Substitution of external Na^+ for NH_4^+ induced depolarization and anode brakes in squid giant axon [26], suggesting that the addition of NH_4^+ to the external solution may shift the threshold to the depolarized membrane potential level. Once the external Na^+ has been completely exchanged for NH_4^+, the relative permeability of the NH_4^+ ion through the TTX-sensitive Na^+ channel (PNH_4/PNa) in the squid giant axon has been reported to be 0.27. The relative permeability of the NH_4^+ ion through the TEA-sensitive K^+ channel (PNH_4/PK) in the squid giant axon has similarly been reported to be 0.2 [26]. Therefore, the membranes of CA1 neurons may be depolarized by increasing the input resistance by superfusion with 5 mM NH_4Cl. The amplitudes of non-NMDA-type fast EPSPs decreased in the use of high concentrations of NH_4Cl (1–5 mM) in a concentration-dependent manner, and these potentials were almost suppressed by 3–5 mM NH_4Cl. The PPF ratio of the field EPSPs showed a reversible increase at 3 mM NH_4Cl, suggesting that a high concentration of NH_4Cl may cause presynaptic inhibition of Glu release. It has been reported that the acid-sensitive ion channel (ASIC) 1α which is known as brain Na^+ channel 2 (BNaC2), is expressed in rat hippocampal neurons from the CA1 region [27]. The ASIC was gated by bath application of 4 mM NH_4Cl (at pH 7.5) and produced consistently measurable inward currents in midbrain dopamine neurons, and similar observations were recorded in mice hippocampal interneurons [28]. Recently, Cho and Askwith reported that the paired-pulse ratios (PPRs) have been reduced, and the frequency of the miniature EPSC has been increased in cultured hippocampal neurons from ASIC1 knockout mice [29]. This observation indicates that ASIC1 affects presynaptic mechanisms of synaptic transmission in mice hippocampal neurons. The high concentrations of NH_4Cl used in this study (1–5 mM) were comparable to the NH_4^+ concentrations in the blood and brain of a rat fulminant liver failure model (1 mM and 1–5 mM, respectively) [3, 4]. Therefore, suppression of Glu release from excitatory nerve terminals in CA1 pyramidal neurons caused by high concentrations of NH_4Cl (hyperammonemia conditions) may be regulated by ASICs. High concentrations of NH_4Cl may open ASICs and generate inward currents that depolarize the presynaptic membrane potential. As a result, the frequency of Glu release from synaptic terminals may be suppressed. AMPA-induced depolarization in the postsynaptic membrane was increased by NH_4Cl (5 mM); however, Fan and Szerb reported that inward currents induced by quisqualate were suppressed by NH_4Cl (3 mM) [14]. The difference between these two results may be due to the difference in the action of the drug used in the experiment. AMPA acts only on the AMPAR, while quisqualate acts on the AMPA/Kainate (KA) receptor. The simultaneous activation of AMPA and KA receptors in the presence of NH_4Cl might suppress the AMPAR-mediated response. The action potentials reversibly disappeared at 10 min after superfusion with 5 mM NH_4Cl. The abolishment of action potentials by NH_4Cl application was also reported in guinea-pig cerebellar slices, rat brain cortex [7], and in rat hippocampal CA1 neurons [14]. Failure of the propagation of action potential through the excitatory nerve following superfusion with 5 mM NH_4Cl may involve a reduction in the evoked fast EPSPs at high concentrations of NH_4Cl. Therefore, the reduction in evoked fast EPSPs in the presence of high concentrations of NH_4Cl may be due to the reduced probability of Glu release in nerve terminals in the presence of NH_4^+, and the failure of propagation of action potentials through the excitatory nerve.

4.2. Effect of hyperosmolarity and a high concentration of NH_4Cl on evoked EPSPs

The effect of hyperosmolarity on evoked EPSPs was investigated. By superfusion with ACSF containing 10 mM sucrose (301 ± 1 mOsm/kgH_2O, n = 4), the amplitude of non-NMDA-type fast EPSPs was reduced by approximately 46% compared with the value in normal ACSF (290 ± 3 mOsm/kgH_2O, n = 5). However, this reduction was not significant. Increased osmotic pressure of 10 mOsm/kgH_2O may not affect the amplitude of non-NMDA-type fast EPSPs. Hypertonic stimulation (360 mOsm/kgH_2O) was not affected by the transient receptor potential vanilloid 4 (TRPV4) receptor (a cellular osmotic sensor) and the AMPA-induced current; however, the PPF ratio was increased, which could possibly be due to the reduction of presynaptic high voltage-gated calcium current (I_{HVA}) in CA3 neurons [17]. The reduction in the amplitude of the non-NMDA-type fast EPSPs may, in part, contribute to the inhibition of I_{HVA} by hyperosmolarity (10 mOsm/kgH_2O). To distinguish the effect of 5 mM NH_4Cl and hyperosmolarity on the evoked EPSPs, 5 mM NH_4Cl was applied continuously after superfusion with 10 mM sucrose containing ACSF. By superfusion with ACSF containing 5 mM NH_4Cl, the amplitude of the non-NMDA-type fast EPSPs was reduced by approximately 85% compared with the value in normal ACSF, and this reduction was statistically significant (Figure 2B, $P < 0.01$). The amplitude of the non-NMDA-type fast EPSPs in 5 mM NH_4Cl was reduced by approximately 71% compared with the value in ACSF containing 10 mM sucrose (Figure 2B, $P < 0.05$). The significant reduction of the evoked non-NMDA-type fast EPSPs in 5 mM NH_4Cl may be due to the high concentration of NH_4Cl, and may not due to hyperosmolality.

4.3. Physiological relevance

Under normal physiological conditions, the arterial NH_4^+ levels are kept low (50–100 μM range) via the removal of gut-derived NH_3 from the blood by the liver [1]. Under these conditions, NH_4Cl reduced the EPSP in a concentration-dependent manner without affecting the postsynaptic Glu responses. This reduction of EPSP by NH_4Cl via presynaptic mechanisms may not affect neural activity. In coma patients, the concentration of ammonia is 300–500 μM in arterial blood [2]. In a rat model of fulminant liver failure, the *in vivo* concentrations of ammonia in the blood and brain were 1 mM and 1–5 mM, respectively [3, 4]. In this study, 300 μM NH_4Cl significantly reduced EPSPs; therefore, NH_4Cl concentrations higher than 300 μM may affect neuronal activity. At high concentrations (1–5 mM), NH_4Cl significantly reduced EPSPs in a concentration-dependent manner. This significant reduction of excitatory synaptic transmission by NH_4Cl and the depolarization of the postsynaptic membrane may affect neural activity. Depolarization of the postsynaptic membrane may increase inactivation of Na^+ channels, and a high concentration of NH_4Cl may shift the threshold to the depolarized level of the membrane. This may reduce the excitability of the membrane. Presynaptic inhibition of EPSP by NH_4Cl and the depolarization of the postsynaptic membrane together may suppress neuronal activity.

5. Conclusion

Various concentrations of NH_4Cl induced depression of the evoked fast EPEPs elicited by the brief electrical stimulation of Schaffer

collaterals in rat CA1 neurons. The half-maximal inhibition of the evoked fast EPSPs was observed at approximately 200 μM of NH_4Cl. At a high concentration of NH_4Cl (5 mM) but not at a low concentration of NH_4Cl (200 μM), the threshold of action potential may shift to the depolarized membrane potential level. Depression of the evoked fast EPSPs at low concentrations of NH_4Cl may be due to the presynaptic mechanism. On the other hand, at high NH_4Cl concentrations, it may be due to both the presynaptic mechanism and the failure of propagation of the action potential through the excitatory nerve.

Declarations

Author contribution statement

Naomitsu Kameyama: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Yoshinaka Murai & Eiichiro Tanaka: Conceived and designed the experiments; Wrote the paper.

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Data will be made available on request.

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The authors declare no conflict of interest.

Additional information

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References

[1] V. Felipo, R.F. Butterworth, Neurobiology of ammonia, Prog. Neurobiol. 67 (2002) 259–279.
[2] J.O. Clemmesen, F.S. Larsen, J. Kondrup, B.A. Hansen, P. Ott, Cerebral herniation in patients with acute liver failure is correlated with arterial ammonia concentration, Hepatology 29 (1999) 648–653.
[3] M. Swain, R.F. Butterworth, A.T. Blei, Ammonia and related amino acids in the pathogenesis of brain edema in acute ischemic liver failure in rats, Hepatology (Baltimore, Md.) 15 (1992) 449–453.
[4] M.S. Swain, M. Bergeron, R. Audet, A.T. Blei, R.F. Butterworth, Monitoring of neurotransmitter amino acids by means of an indwelling cisterna magna catheter: a comparison of two rodent models of fulminant liver failure, Hepatology (Baltimore, Md.) 16 (1992) 1028–1035.
[5] M.E. Raichle, K.B. Larson, The significance of the NH_3- NH_4^+ equilibrium on the passage of ^{13}N-ammonia from blood to brain. A new regional residue detection model, Circ. Res. 48 (1981) 913–937.
[6] W. Raabe, S. Lin, Pathophysiology of ammonia intoxication, Exp. Neurol. 87 (1985) 519–532.
[7] A.M. Benjamin, K. Okamoto, J.H. Quastel, Effects of ammonium ions on spontaneous action potentials and on contents of sodium, potassium, ammonium and chloride ions in brain in vitro, J. Neurochem. 30 (1978) 131–143.
[8] A. Hamberger, B. Hedquist, B. Nystrom, Ammonium ion inhibition of evoked release of endogenous glutamate from hippocampal slices, J. Neurochem. 33 (1979) 1295–1302.
[9] B.E. Alger, R.A. Nicoll, Ammonia does not selectively block IPSPs in rat hippocampal pyramidal cells, J. Neurophysiol. 49 (1983) 1381–1391.
[10] Y. Theoret, J.L. Bossu, Effects of ammonium salts on synaptic transmission to hippocampal CA1 and CA3 pyramidal cells in vivo, Neuroscience 14 (1985) 807–821.
[11] Y. Theoret, M.F. Davies, B. Esplin, R. Capek, Effects of ammonium chloride on synaptic transmission in the rat hippocampal slice, Neuroscience 14 (1985) 798–806.
[12] W. Raabe, S. Lin, Ammonia, postsynaptic inhibition and CNS-energy state, Brain Res. 303 (1984) 67–76.
[13] P. Fan, J. Lavoie, N.L. Le, J.C. Szerb, R.F. Butterworth, Neurochemical and electrophysiological studies on the inhibitory effect of ammonium ions on synaptic transmission in slices of rat hippocampus: evidence for a postsynaptic action, Neuroscience 37 (1990) 327–334.
[14] P. Fan, J.C. Szerb, Effects of ammonium ions on synaptic transmission and on responses to quisqualate and *N*-methyl-D-aspartate in hippocampal CA1 pyramidal neurons in vitro, Brain Res. 632 (1993) 225–231.
[15] W. Raabe, Ammonium decreases excitatory synaptic transmission in cat spinal cord in vivo, J. Neurophysiol. 62 (1989) 1461–1473.
[16] P.A. Schwartzkroin, Characteristics of CA1 neurons recorded intracellularly in the hippocampal in vitro slice preparation, Brain Res. 85 (1975) 423–436.
[17] L. Li, J. Yin, C. Liu, L. Chen, L. Chen, Hypertonic stimulation inhibits synaptic transmission in hippocampal slices through decreasing pre-synaptic voltage-gated calcium current, Neurosci. Lett. 507 (2012) 106–111.
[18] R. Creager, T. Dunwiddie, G. Lynch, Paired-pulse and frequency facilitation in the CA1 region of the in vitro rat hippocampus, J. Physiol. 299 (1980) 409–424.
[19] L.E. Dobrunz, C.F. Stevens, Heterogeneity of release probability, facilitation, and depletion at central synapses, Neuron 18 (1997) 995–1008.
[20] A. Baude, Z. Nusser, E. Molnar, R.A. McIlhinney, P. Somogyi, High-resolution immunogold localization of AMPA type glutamate receptor subunits at synaptic and non-synaptic sites in rat hippocampus, Neuroscience 69 (1995) 1031–1055.
[21] Z. Nusser, R. Lujan, G. Laube, J.D. Roberts, E. Molnar, P. Somogyi, Cell type and pathway dependence of synaptic AMPA receptor number and variability in the hippocampus, Neuron 21 (1998) 545–559.
[22] J.M. Bekkers, C.F. Stevens, NMDA and non-NMDA receptors are co-localized at individual excitatory synapses in cultured rat hippocampus, Nature 341 (1989) 230–233.
[23] Y. He, W.G. Janssen, J.H. Morrison, Synaptic coexistence of AMPA and NMDA receptors in the rat hippocampus: a postembedding immunogold study, J. Neurosci. Res. 54 (1998) 444–449.
[24] T. Manabe, D.J. Wyllie, D.J. Perkel, R.A. Nicoll, Modulation of synaptic transmission and long-term potentiation: effects on paired pulse facilitation and EPSC variance in the CA1 region of the hippocampus, J. Neurophysiol. 70 (1993) 1451–1459.
[25] R.S. Zucker, Short-term synaptic plasticity, Annu. Rev. Neurosci. 12 (1989) 13–31.
[26] L. Binstock, H. Lecar, Ammonium ion currents in the squid giant axon, J. Gen. Physiol. 53 (1969) 342–361.
[27] D. Alvarez de la Rosa, S.R. Krueger, A. Kolar, D. Shao, R.M. Fitzsimonds, C.M. Canessa, Distribution, subcellular localization and ontogeny of ASIC1 in the mammalian central nervous system, J. Physiol. 546 (2003) 77–87.
[28] V.L. Pidoplichko, J.A. Dani, Acid-sensitive ionic channels in midbraine dopamine neurons are sensitive to ammoniumu, which may contribute to hyperammonemia damage, Proc. Natl. Acad. Sci. 103 (2006) 11376–11380.
[29] J.H. Cho, C.C. Askwith, Presynaptic release probability is increased in hippocampal neurons from ASIC1 knockout mice, J. Neurophysiol. 99 (2008) 426–441.