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Human Neuroepithelial Cells Express NMDA Receptors

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Abstract

L-glutamate, an excitatory neurotransmitter, binds to both ionotropic and metabotropic glutamate receptors. In certain parts of the brain the BBB contains two normally impermeable barriers: 1) cerebral endothelial barrier and 2) cerebral epithelial barrier. Human cerebral endothelial cells express NMDA receptors; however, to date, human cerebral epithelial cells (neuroepithelial cells) have not been shown to express NMDA receptor message or protein. In this study, human hypothalamic sections were examined for NMDA receptors (NMDAR) expression via immunohistochemistry and murine neuroepithelial cell line (V1) were examined for NMDAR via RT-PCR and Western analysis. We found that human cerebral epithelium express protein and cultured mouse neuroepithelial cells express both mRNA and protein for the NMDA receptor. These findings may have important consequences for neuroepithelial responses during excitotoxicity and in disease.

Background

Glutamate receptor stimulation is an important physiological event, which helps regulate learning and memory development. [1]. In the mammalian nervous system, L-glutamate binds to several classes of 'glutamatergic' receptors, which are classified into two major groups, metabotropic and ionotropic. Within the ionotropic family of glutamate receptors there are three subtypes (based upon their binding and activation by AMPA, kainic acid, and NMDA): 1) α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), 2) kainic acid (KA) receptors, and 3) N-methyl-D-aspartate (NMDA) receptors [2].

Glutamate is present at concentrations ranging from 18–25 μM in plasma [3], 0.3 μM in the cerebral spinal fluid (CSF), and as high as 3 mM within the parenchymal cell stores [4-6]. However, under ischemic or traumatic conditions the glutamate concentration levels in the brain interstitial space can increase 55-fold [7] to levels that are toxic to neurons.

During stroke and trauma it has been suggested that majority of glutamate released (although not the only pathway) is due to neuronal injury within the cerebrum, which is provoked by cerebral oxygen and glucose deprivation, resulting in the excessive release of stored synaptic

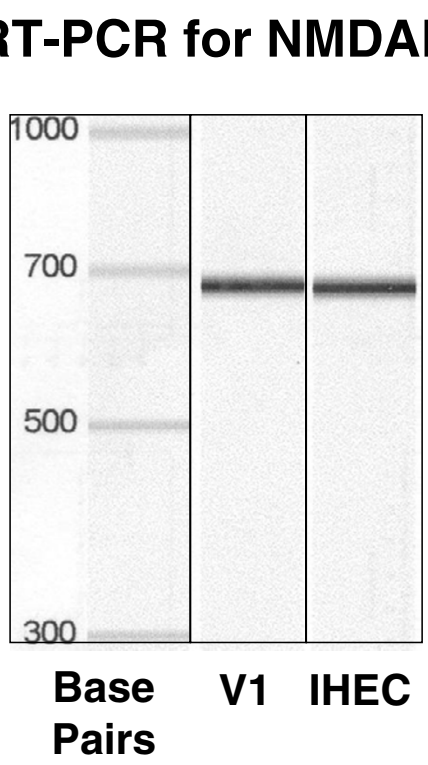

Figure 1
RT-PCR (30 cycles) was performed with primers specific for mouse and human $NMDAR_1$, which revealed that both V1 (mouse neuroepithelial cells) and IHEC (Human cerebral endothelial cells – positive control) express mRNA for $NMDAR_1$. The bands were determined to be ~680 bps and ~670 bps and match the anticipated size of mouse and human $NMDAR_1$ (respectively).

glutamate. Glutamate synaptic release is due to the loss of ATP stores and dissipation of membrane ion gradients leads to K^+ efflux, and membrane depolarization. Eventually anoxia triggers a massive depolarization and opening of voltage-dependent Na^+ channels. As a result, glutamate is released by synaptic exocytosis and trapped in the interstitium, due to the reversal of the glutamate transporters flooding the synaptic space with glutamate. This response in turn leads to the massive over-stimulation of NMDA receptors, referred to as '*glutamate excitotoxicity*' [8-11]. In addition to neuronal excitotoxicity, increased extracellular glutamate may also contribute to 'vasogenic edema' (characterized by an increase in microvascular solute permeability) [12-18].

In the hypothalamus, neuroepithelial or ependymal cells create a second brain solute "barrier" [19], which separates the brain parenchyma from the CSF. If the neuroepithelium maintains the same tight junctional characteristics as non-cerebral epithelial cells, then they will create a significantly tighter barrier than those formed by most of the endothelium, including that of the blood brain barrier (BBB) [20-22]. During inflammation extracellular pathogens may penetrate the cerebrum through both the endothelial and epithelial barriers, demonstrating that infection of the cerebrum can occur from the direction of either the blood or the CSF [23]. These data suggest that the CSF barrier, like that of the endothelium, might also be compromised in disease.

In this study we demonstrated NMDA receptor expression on cerebral epithelia within human cerebral tissue and murine neuroepithelial cells. The presence of the NMDA receptor 1 ($NMDAR_1$) on human neuroepithelium is demonstrated via immunohistochemistry and mRNA and protein expression for the NMDA receptors in cultured murine neuroepithelial cells suggest that their function is conserved among species.

Results

V1 (neuroepithelial cells) and Normal Human Brain Tissue RT-PCR for $NMDAR_1$

Human cerebral endothelial cells (IHEC), which had previously been shown to express mRNA for $NMDAR_1$ [24], were used as a positive control for determining if the V1 cells contained message for the $NMDAR_1$. With the use of primer sets designed for $NMDAR_1$ we were able to demonstrate that the V1 cells contained message for $NMDAR_1$ (Figure 1). Figure 1 shows a ~680 bp band that was specifically amplified.

V1 and Normal Rat Cerebral Tissue Western Analysis for NMDAR 1 and 2A/B

Rat cerebral lysate (positive control supplied by Chemicon) and Neuroepithelial cell protein from V1 cells were separated on SDS-PAGE gels and western blots (using antibodies specific for $NMDAR^1$ and $NMDAR_{2A/B}$) showed a ~116 and ~160 KD bands, the anticipated sizes for NMDA receptor subunit 1 and 2A/B, respectively (Figure 2). This matches the two reported size bands by Sharp et al. 2002 [24].

Human Hypothalamic Immunohistochemistry for the $NMDAR_1$

Immunohistochemical staining of a human adult hypothalamic paraffin slide revealed that the cerebral epithelial cells (Choroid Plexus epithelial cells, Figure 3A, and ependymal cells, Figure 3B) express $NMDAR_1$, which appeared to be concentrated on the apical (ventricular/CSF) side. We found that staining appeared to be

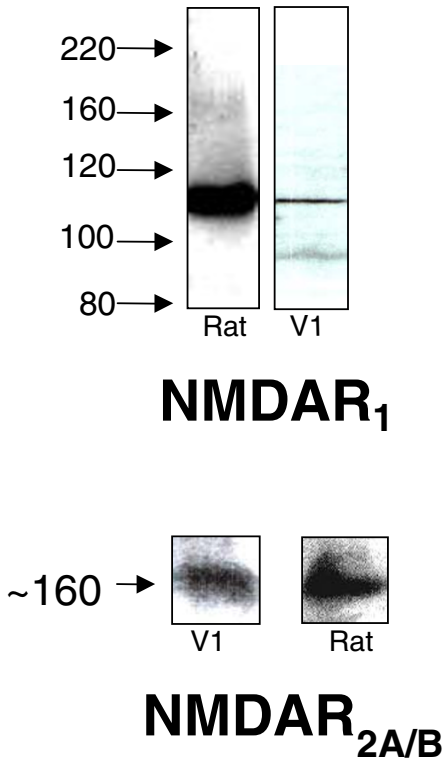

Figure 2
V1 cell protein and rat brain lysate positive control (supplied by Chemicon) were separated on 7.5% denaturing SDS-PAGE gels. Comparison of the control and V1 cell band (~116 kD) present after western analysis demonstrated that V1 cells do express protein for NMDA subunits 1 and 2A/B.

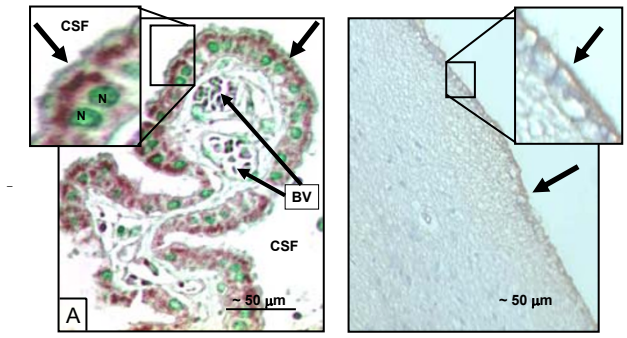

Figure 3
Human hypothalamus sections (A = choriod plexus and B = ependymal cell barrier) were stained for $NMDAR_1$ and demonstrated a uniform and consistent pattern of staining that is associated with the apical side (CSF) with very little staining basally. The magnified section of Figure 3, further illustrates this specific orientation (also pointed out by the arrows). The blood vessels are labeled 'BV' and nuclei are labeled "N".

segregated to the apical side (CSF compartment) of the nucleus. Other sections were used to examine control staining and confirmed the specificity of the antibody and procedure.

Discussion

Extracellular pathogens can penetrate the cerebrum through either the epithelial and endothelial barrier during events such as stroke and cerebral inflammation [23]. Many studies have considered possible mechanisms for the endothelial barrier disruption during trauma/excitotoxicity. We have recently reported that when the human cerebral endothelium was stimulated with either glutamate or NMDA there was a NMDA-type glutamate receptor dependent cerebral endothelial barrier dysfunction [24]); however, the mechanisms through which the neuroepithelial barrier might be disrupted have not been investigated. If neuroepithelial cells express $NMDAR_1$, then during excitotoxic events (like stroke), when the CSF glutamate concentration increases, there could be a massive efflux of Ca^{2+} into the neuroepithelial cells. Altered intracellular Ca^{2+} might then lead to an increase in solute permeability, as has been proposed within endothelial systems [25].

Neuroepithelial cells also express highly efficient glutamate transporters, which are at least partially responsible for maintaining the low concentrations of glutamate in the CSF by actively moving glutamate from the CSF to the parenchyma [26,27]. In fact these transporters create a 7-fold higher concentration of glutamate in the parenchyma (compared to the CSF) in under 5 min (with steady state reached within 15 min, as high as 30-fold) [26]. These cells also metabolize glutamine to glutamate in addition to transporting glutamate across the epithelia [26,27]. Therefore, NMDA receptors present on the "apical" surface might regulate the activity of these transporters to control glutamate homeostasis between CSF and parenchyma.

Immunohistochemical staining of the human ependymal sections demonstrated that the nuclei separated the neuroepithelial cells into apical (facing the CSF) and

basolateral (facing the parenchyma/vascular) surfaces. We saw a clear and distinct spatial association of $NMDAR_1$ staining with the apical side with a paucity of basal staining, suggesting that the neuroepithelial cells could possibly function as detectors of CSF glutamate concentration levels, which are normally 0.3 μM but have been shown to increase 55-fold under ischemic/traumatic conditions [7]. These data support possible roles for NMDA-receptors in regulating glutamate transporters, as previously described.

Conclusion

Therefore, the results of the present study, which evaluated neuroepithelial cell expression of NMDA-type glutamate receptors via RT-PCR, Western analysis, and immunohistochemistry supports the position that cerebral epithelia express the NMDA-receptor at the mRNA (Figure 1), protein (Figure 2), and tissue levels (Figure 3). As stated above, two potential roles for neuroepithelial NMDA receptors may include: 1) control of CSF homeostasis by controlling the activity of the glutamate transporters and 2) disruption of the neuroepithelial barrier during disease/excitotoxic processes; however, we can only currently speculate on the function of these receptors. Future studies will examine each of these possible functions of the neuroepithelial NMDA receptor.

Methods

Reagents for Tissue Culture

M-199, Insulin, Transferrin, Selenium, Heparin, and HEPES were purchased from Sigma (St. Louis, MO).

Cell culture

A mouse neuroepithelial cell line (termed "V1") was supplied by Dr. Harvey Ozer (University of Medicine and Dentistry of New Jersey, Newark). V1 cells were maintained in M-199 (with 10% FCS, 1% antibiotic/antimycotic, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml Selenium, and 600 USP units/l heparin) and grown to 100% confluency on T-75 flask. The neuroepithelial cultures were used at 1 week in culture (approximately 3 days after culture confluency was established).

Western Analysis

Protein samples (25 ug each) were separated on 7.5% SDS-PAGE gels, electro-transferred to nitrocellulose membranes and blocked overnight in 5% milk in PBS. Membranes were then incubated for 24 hours with anti-human $NMDAR_1$ and an anti-human $NMDAR_{2A/B}$ (Chemicon; Temecula, CA) polyclonal antibody at 1:250 dilution in 0.1% milk in PBS. The membranes were then washed 3 × 5' (each wash) and incubated overnight at 4°C in goat anti-mouse alkaline-phosphatase secondary antibody (Sigma; St. Louis, MO) at a 1:1000 dilution in 0.1% milk in PBS. The membranes were then reacted with NBT/BCIP chromogen to visualize the proteins.

RT-PCR

Message for $NMDAR_1$ was determined in both V1 cells and human brain tissue samples from total RNA (Qiagen kit number 52304; Qiagen; Seattle, WA). 1 ug of DNAse treated RNA was converted to cDNA using reverse transcriptase (Promega; Madison, WI) and amplified using the following primers (from ascension number BC039157; sense primer for base pairs 1192–1209 and antisense primer for base pairs 1854-1838): sense primer, 5'-GATGTCTTCCAAGTATGC-3' and antisense primer, 5'-ATCTCCTTCTTGACCAG-3'. The PCR mixture was amplified for 30 cycles using a three step protocol: denaturation at 94°C (1 min), annealing at 50°C for 1 min, and elongation at 72°C for 1 min. The corresponding 670 – 680 bp ($NMDAR_1$) product was separated in a 1.5% agarose gel stained with ethidium bromide and viewed and analyzed using Alpha Innotech gel documentation system (San Leandro, CA). RNA extracts from human cerebral endothelial cells (IHEC) were used as the positive control for all experiments [24]. Negative controls were performed in which the reverse transcriptase enzyme was omitted and replaced with RNAse free water.

Immunohistochemistry

Paraffin slides were deparaffinized with 2 changes of xylene (Sigma; St. Louis, MO) for 10 min each. The slides were transferred to 100% alcohol for 2 changes for 2 min each. They were incubated for 10 min in 3% H_2O_2 in methanol to block endogenous peroxidase activity. The slides were rinsed 1× in PBS and excess PBS removed. Slides were blocked with 5% normal serum for 1 hour and then the anti-human $NMDAR_1$ (Chemicon; Temecula, CA) polyclonal antibody at a 1:250 dilution in 0.1% milk (in PBS) was added and allowed to incubate at 4°C overnight. The next day the slides were rinsed 3× for 2 min each in PBS. HRP (Sigma; St. Louis, MO) at a 1:1000 dilutions in 0.1% milk was applied to the slides and incubated for 30 min at room temperature. The slides were washed 3 times in PBS and diaminobenzidine (DAB) solution (Pharmingen; San Diego, CA) was applied and allowed to incubate for 5 min. The DAB was drained and the slides were placed in water for 3 min. Slides were counterstained in hematoxylin (Pharmigen; San Diego, CA). The slides were then dehydrated with the use of 100% alcohol and then mounting medium and coverslips were applied.

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