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Glioblastoma invasion and NMDA receptors: A novel prospect

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Purpose: Glioblastoma cells create glutamate-rich tumor microenvironment, which initiates activation of ion channels and modulates downstream intracellular signaling. N-methyl-D-aspartate receptors (NMDARs; a type of glutamate receptors) have a high affinity for glutamate. The role of NMDAR activation on invasion of glioblastoma cells and the crosstalk with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) is yet to be explored. *Main methods:* LN18, U251MG, and patient-derived glioblastoma cells were stimulated with NMDA to activate NMDAR glutamate receptors. The role of NMDAR activation on invasion and migration and its crosstalk with AMPAR were evaluated. Invasion and migration of glioblastoma cells were investigated by *in vitro* trans-well Matrigel invasion and trans-well migration assays, respectively. Expression of NMDARs and AMPARs at transcript level was evaluated by quantitative real-time polymerase chain reaction. *Results:* We determined that NMDA stimulation leads to enhanced invasion in LN18, U251MG, and patient-derived glioblastoma cells, whereas inhibition of NMDAR using MK-801, a non-competitive antagonist of the NMDAR, significantly decreased the invasive capacity. Concordant with these findings, migration was significantly augmented by NMDAR in both cell lines. Furthermore, NMDA stimulation upregulated the expression of GluN2 and GluA1 subunits at the transcript level. *Conclusions:* This study demonstrated the previously unexplored role of NMDAR in invasion of glioblastoma cells. Furthermore, the expression of the GluN2 subunit of NMDAR and the differential overexpression of the GluA1 subunit of AMPAR in both cell lines provide a plausible rationale of crosstalk between these calcium-permeable subunits in the glutamate-rich microenvironment of glioblastoma.

Keywords: calcium-permeable AMPAR, glutamate receptor, migration, NMDAR, signaling

Introduction

Glioblastoma (GBM) is the most common malignant primary brain tumor with an extremely invasive phenotype. Regardless of advances in current treatment regimens, prognosis remains poor with an overall survival rate of 18 months (30). Invasion is one of the most important hallmarks of GBM, which poses a serious clinical challenge and is a major factor playing a role in GBM recurrence after neurosurgical resection of tumor mass and chemoradiotherapy (28). GBM cell invasion is a multifaceted, dynamic process similar to metastasis (31). Understanding the mechanisms underlying the aggressive invasive behavior of GBM and its

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intracellular biology is of paramount importance. Although several studies have aimed at understanding the aggressive invasive behavior of GBM, it still remains to be deciphered.

Astrocyte-derived tumors co-opt neurotransmitters such as glutamate, and ion channels mediated signaling to support their unusual growth (18). The N-methyl-D-aspartate receptor (NMDAR) is one of the important subclasses of glutamate receptors. They are tetrameric complexes assembled from two NR1 (GluN1) and two NR2 (GluN2) subunits. The different GluN2 subunits (GluN2A–GluN2D) confer distinct gating and pharmacological properties on the heteromeric receptor channel (15). Even though NMDARs exist as multiple subtypes with distinct pharmacological and biophysical properties, major functions are largely determined by the GluN2 subunit (GluN2A–GluN2D) (8). NMDARs are shown to play a role in the regulation of calcium-dependent migration of neuronal progenitors during neural development (16). Earlier, we have shown that NMDARs play a role in GBM cell proliferation (22). However, the role of NMDARs in the aggressive migration and invasion of GBM pathobiology is still elusive.

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors assemble as homo- or heterotetramers of GluR1-4 (GluA1-4) subunits (25). Complexes composed of GluA1, GluA3, and GluA4 subunits of AMPA receptors (AMPARs) form calcium-permeable channels, whereas subunit GluA2-containing AMPAR form calcium-impermeable channels due to the presence of an arginine at the pore apex, which is introduced post-transcriptionally by RNA editing (32). Activation of AMPA-type glutamate receptors has been shown to play a crucial role in the growth and migration of GBM cells (11). Previous study has reported the role of calcium-permeable AMPA receptors in facilitating proliferation and mobility of GBM cells *via* Akt activation (12). However, in a glutamate-rich microenvironment, the crosstalk between NMDAR and AMPAR in the growth of GBM is yet to be explored.

GBM is characterized by tumor heterogeneity and accumulation of different genetic abnormalities. Tumor suppressor p53 is one of the most common genes mutated in GBM, and 40% of GBM tumors are reported to express mutant p53 (4). Patient-derived cells can contribute significantly to generating clinically relevant *in vitro* models of GBM invasion (19). Therefore, a greater understanding of GBM invasion through NMDA in a glutamate-rich microenvironment and the possible crosstalk among glutamate receptors is crucial. With this perspective, the present work was designed by incorporating p53 mutant GBM cell lines LN18 and U251MG along with patient-derived GBM cells to investigate migration, invasion, and crosstalk between NMDAR and AMPAR, upon stimulation by NMDA in glutamate-rich microenvironment.

Materials and Methods

Cell lines and primary cell culture

The LN18 cell line was procured from the National Centre for Cell Science, India, and the U251MG cell line was kindly provided by Prof. P. Kondaiah (Indian Institute of Sciences, Bengaluru, India). Based on clinical diagnosis and neuroimaging, the human high-grade glioma tumor samples were collected during surgeries performed at the Department of Neurosurgery, NIMHANS, Bengaluru, India. An informed consent was obtained from each patient before collecting the tumor samples, as per the protocol approved by the Institute Ethics Committee of NIMHANS. Both patient-derived glioma tumor samples obtained were

diagnosed as GBM (according to 2007 WHO classification of gliomas) by a neuropathologist and were code numbered as HGP-1 and HGP-3 (HGP-1 was 15% mutant for p53 and HGP-3 was 30% mutant for p53). Primary culture cells were obtained by processing these GBM tumor samples using Dulbecco's Modified Eagle's Medium-high glucose (DMEM; D5648-1L, Sigma-Aldrich, USA) with 20% fetal bovine serum (FBS; Gibco, USA) and 1% PenStrep (Gibco, USA) to obtain adherent cell cultures (23). These cells were made into 5–10 passages. The LN18 and U251MG cell lines were maintained in DMEM supplemented with 10% FBS and 1% PenStrep. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Materials

NMDA (product number: M3262-25MG, Sigma-Aldrich) was resuspended in sterile water as per manufacturer's instructions and working dilutions were made in serum-free DMEM. Glycine (product number: 50046-50MG, Sigma-Aldrich) was resuspended in sterile water as per manufacturer's instructions and working dilutions were made in serum-free DMEM. MK-801 hydrogen maleate (product number: M107-5MG, Sigma-Aldrich) was dissolved in dimethyl sulfoxide (cat. no. D2650, Sigma Chemicals, St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (cat. no.: M2128-1G, Sigma Aldrich, USA) was resuspended in DMEM.

NMDARs in GBM cells were activated by treating these cells with NMDA (100 μM) (Sigma-Aldrich) and the cofactor glycine (10 μM) (Sigma-Aldrich). Inhibition of NMDAR activation was done using a highly potent and selective non-competitive NMDA receptor antagonist, MK-801 (100 μM) (Sigma-Aldrich).

Invasion assay. Invasion of GBM cells was investigated by the *in vitro* trans-well Matrigel (cat. no. 354480, BD Biosciences, Franklin Lakes, NJ, USA) invasion assay (7). Cells were added to the upper chamber and incubated for 24 h with or without NMDA and the lower compartment contained standard culture medium with 10% FBS acting as a chemoattractant. Invaded cells were fixed and stained with crystal violet. Stained cells were counted in five random fields of the membrane using an inverted microscope. Effect of MK-801 on the invasion of GBM cells was analyzed by pretreating the GBM cells with MK-801 for 30 min and incubating them with or without NMDA for 24 h. Data shown are representative of three individual experiments.

Migration assay. Migration of GBM cells was investigated by the trans-well migration method as described earlier with minor modification (13). Cells were added to the upper chamber and incubated for 24 h with or without NMDA and the lower compartment contained standard culture medium with 10% FBS acting as a chemoattractant. Migrated cells were fixed and stained with crystal violet. Stained cells were counted in five random fields of the membrane using an inverted microscope. Effect of MK-801 on the migration of GBM cells was analyzed by pretreating the GBM cells with MK-801 for 30 min and incubating them with or without NMDA for 24 h. Data shown are representative of three individual experiments.

RNA extraction and reverse transcription polymerase chain reaction (PCR). Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and cDNA synthesis was performed with EasyScript reverse transcriptase (Applied Biological Materials Inc., Richmond, British Columbia, Canada) following the manufacturer's instructions. PCR was performed in 2× Genei red dye master mix (Merck, Mumbai, India) using the primers listed below following manufacturer's instructions in a Nexus gradient mastercycler (Eppendorf AG, Hamburg, Germany) machine. The PCR machine was programmed for activation of the enzyme

at 95 °C for 5 min, followed by 30 cycles with each cycle containing 15 s for denaturation at 95 °C, 15 s for annealing at 53 °C, and 60 s extension at 72 °C.

Quantitative real-time PCR (RT-qPCR). RT-qPCR was conducted using 2× EvaGreen real-time PCR kit (Applied Biological Materials Inc.) following manufacturer's instructions in an ABI-7500 (Applied Biosystem Inc., Foster city, CA, USA) machine. The real-time qPCR machine was programmed for activation of the enzyme at 95 °C for 5 min, followed by 40 cycles each containing 15 s for denaturation at 95 °C, 15 s for annealing at 53 °C for all the genes except MMP-2 (61.9 °C), and 60 s extension at 60 °C or 72 °C. Melt curve analysis confirmed the specificity of the PCR products. Data were normalized to β-actin. Fold changes in gene expression were calculated using the delta–delta Ct method from the sample Ct values. A non-template control was included in each experiment. Primer sequences used for analysis of gene expression changes are displayed in Table I.

Statistical analysis

Results are presented as mean ± SD. Statistical analysis included Student's *t*-test method for two groups or one-way ANOVA followed by the suitable *post-hoc* test for multiple group comparisons. The values of $p < 0.05$ were considered as significant.

Table I. Primer sequences for gene expression analyses

Subunits	Primer	Sequence
GluN2A	Forward primer	5′-AACAATTCAACCAATGAGGG-3′
GluN2A	Reverse primer	5′-CAGATAGAGGTCGTAAGTAAAC-3′
GluN2B	Forward primer	5′-CAGAGTGAGAGATGGAATTG-3′
GluN2B	Reverse primer	5′-CCTATTTAGCATATTGGACTGG-3′
GluN2C	Forward primer	5′-CTTCTTTGCTGTCATCTTCC-3′
GluN2C	Reverse primer	5′-GAAACTTCTTGTCACTGAGG-3′
GluN2D	Forward primer	5′-TTTCATCTTCGAGTACCTCAG-3′
GluN2D	Reverse primer	5′-ACCGAATTATTGAACACCAG-3′
GluA1	Forward primer	5′-CTAGAA GATCCTTATGTGATGC-3′
GluA1	Reverse primer	5′-CTCCGTATTTTCCATCACTG-3′
GluA2	Forward primer	5′-GGAATCTCCGTATGTT ATGATG-3′
GluA2	Reverse primer	5′-TTGTACTTGAACCCACAATG-3′
GluA3	Forward primer	5′-TATTGTATCTGGGGCGTTAC-3′
GluA3	Reverse primer	5′-TTGAGAACTCA AGAAGGGAG-3′
GluA4	Forward primer	5′-GGTACGATAAAGG TGAATGTG-3′
GluA4	Reverse primer	5′-AAAAGGTCAGCTTCATTCTC-3′
β-actin	Forward primer	5′-CGTGCGTGACATTAAGGAGA-3′
β-actin	Reverse primer	5′-CACCTTCACCGTTCCAGTTT-3′

Results

NMDAR promotes invasion of GBM cells

To examine the effect of NMDAR on the invasion of GBM, LN18, U251MG, and patient-derived primary GBM cells (HGP-1 and HGP-3) were stimulated with NMDA (100 μM) and the cofactor glycine (10 μM). Invasion mediated by NMDAR was investigated by *in vitro* Matrigel invasion assay. NMDAR significantly increased the invasion of these GBM cells compared to vehicle-treated control. Furthermore, to confirm that invasion was mediated through NMDAR, we blocked NMDAR by MK-801, a specific non-competitive inhibitor. The results suggest that inhibition of NMDAR significantly reversed the invasion in both the cell lines and the patient-derived primary GBM cells (Fig. 1).

NMDAR promotes migration of GBM cells

Migration is a prerequisite for invasion of GBM. To examine the effect of NMDAR on the migration of GBM, LN18 and U251MG cells were stimulated with NMDA (100 μM) and the cofactor glycine (10 μM). Migration mediated by NMDAR was investigated by trans-well migration assay. NMDAR significantly increased migration of these cells compared to vehicle-treated control, and the effect was reversed by inhibitor MK-801. The results suggest that NMDAR promotes the migration of GBM cells (Fig. 2).

Effect of NMDA stimulation on expression of GluN2 subunits in GBM

LN18 and U251MG cells were stimulated with NMDA (100 μM) and the cofactor glycine (10 μM) for 24 h. Gene expression was quantified by RT-qPCR. NMDAR significantly

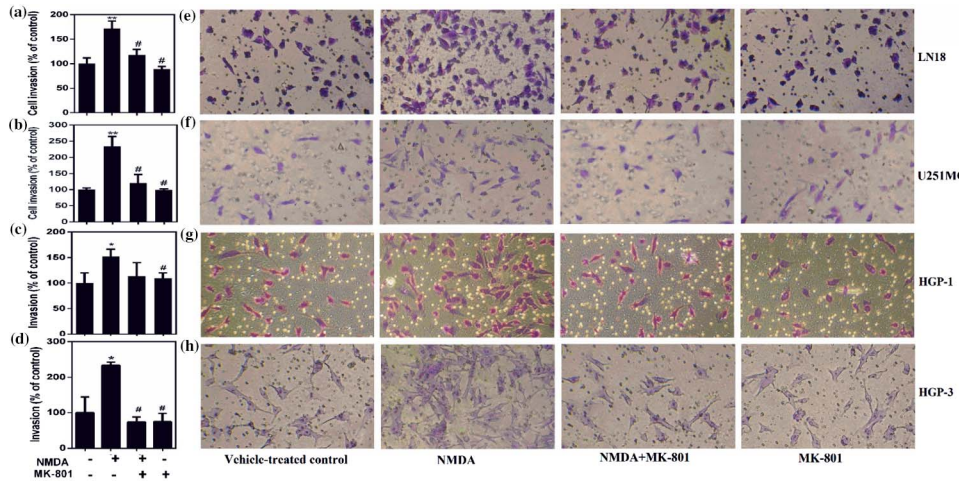

Fig. 1. Effect of NMDAR on GBM cell invasion: (a) LN18, (b) U251MG, (c) HGP-1, and (d) HGP-3 GBM cells were treated with NMDA (100 μM) and the cofactor glycine (10 μM) for 24 h with or without MK-801. (e), (f), (g), and (h) are the representative microscope pictures (magnification of these images is 10×) depicting cell invasion with the corresponding graphs (a), (b), (c), and (d), respectively. Cell invasion was examined by *in vitro* trans-well Matrigel invasion assay as described in "Materials and Methods." Shown are the averages of three independent experiments performed in duplicate. Data are presented as the mean ± SD. Statistics were performed using one-way ANOVA, followed by Tukey's test. *$p < 0.05$ and **$p < 0.01$ compared to respective vehicle-treated control. $^{\#}p < 0.05$ in comparison to NMDA-treated cells

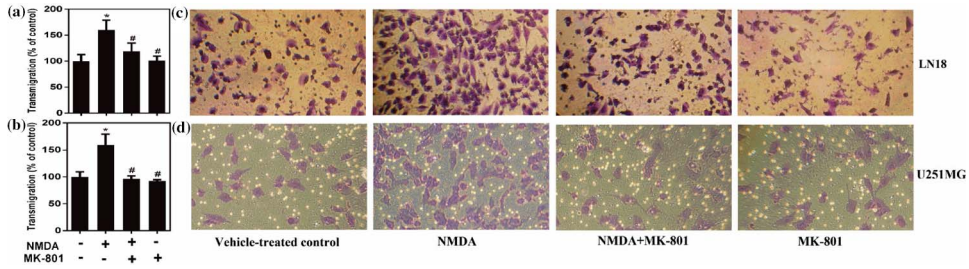

Fig. 2. Effect of NMDAR on migration of GBM cells: (a) LN18 (b) U251MG cells were treated with NMDA (100 μM) and the cofactor glycine (10 μM) for 24 h with or without MK-801. (c) and (d) are the representative microscope pictures (magnification of these images is 10X) depicting cell migration with the corresponding graphs (a) and (b), respectively. The trans-well migration assay was evaluated as described in "Materials and Methods. Shown are the averages of minimum three independent experiments performed in duplicate. Statistics were performed using one-way ANOVA, followed by Tukey's test. Data presented as the mean ± SD. $*p < 0.05$ in comparison to respective vehicle-treated control. $^{\#}p < 0.05$ in comparison to NMDA-treated cells

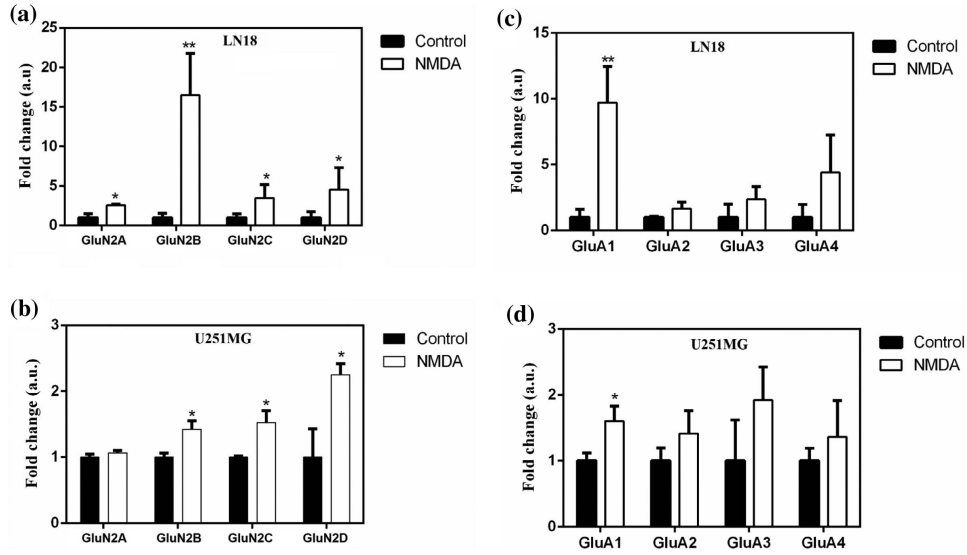

Fig. 3. Effect of stimulation of NMDA on expression of NMDAR and AMPAR subunits in GBM: (a, c) LN18, (b, d) U251MG cells were treated with NMDA (100 μM) and the cofactor glycine (10 μM) for 24 h. Expression of GluN2 and GluA subunits was quantified by RT-qPCR as described in "Materials and Methods" section. Shown are the averages of minimum three independent experiments. Statistics were performed using Student's *t*-test. Data are presented as the mean ± SD. $*p < 0.05$ and $**p < 0.01$ in comparison to control

increased the mRNA expression of all the subunits of GluN2 in LN18, whereas in U251MG cells, expression of GluN2B, GluN2C, and GluN2D subunits was enhanced (Fig. 3a, b).

Effect of NMDA stimulation on expression of GluA subunits in GBM

LN18 and U251MG cells were stimulated with NMDA (100 μM) and the cofactor glycine (10 μM) for 24 h. Gene expression was quantified by RT-qPCR. NMDAR significantly

increased the mRNA expression of only the GluA1 subunit of AMPAR in both cell lines, whereas there was no change in the expression of GluA2–A4 (Fig. 3c, d).

Discussion

GBM cells secrete significant amounts of glutamate into the tumor microenvironment, thereby initiating the intracellular signaling by glutamate-induced modulation of their ion channels. NMDAR-type glutamate receptors have a high affinity for glutamate. Our earlier study has demonstrated the inhibition of GBM cell proliferation through NMDAR blockage. A recent study has highlighted memantine-derived compounds to have antiproliferative effect on GBM (5). In this study, we evaluated the effect of NMDA stimulation on NMDAR and AMPAR, and its role in invasion and migration of GBM cells.

NMDA receptors are highly permeable to calcium and do not readily desensitize. GluN2 (GluN2A–GluN2D) subunits determine the functional properties of NMDARs, although they are heteromultimeric protein complexes composed of GluN1 and GluN2 subunits (2). Hence, to decipher the status of GluN2 receptors in a glutamate-rich microenvironment, we examined the levels of mRNA expression of GluN2 subunits upon stimulation by NMDA in U251MG and LN18 cells. We observed that the mRNA expression of all the subunits of GluN2 was significantly increased in LN18 cells, whereas in U251MG cells, expression of all GluN2 subunits except GluN2A was significantly enhanced. As per the evidences, there is a difference in the various intrinsic factors between LN18 and U251MG cells. U251MG cells are phosphatase and tensin homolog (PTEN) deficient, whereas LN18 cells are PTEN proficient (17). In the similar way, U251MG cells have methylated O6-methylguanine-DNA methyltransferase (MGMT) promoter, whereas LN18 cells have non-methylated MGMT promoter (33). Calcium influx following NMDAR stimulation is biphasic in nature. GluN2A-containing NMDARs result in rapid and low levels of calcium influx, which is followed by a GluN2B-dependent, delayed, and more copious calcium inflow (21). We observed that on stimulation with NMDA, the expression of GluN2B in LN18 cells was increased many fold as compared to vehicle-treated control cells. It has been reported earlier that increased expression of GluN2B positively correlates with surface expression of GluA1 subunit in cortical neurons (14). Similarly, we evaluated the mRNA expression of AMPAR subunits in GBM cells on stimulation with NMDA. We observed significantly increased levels of mRNA expression of the GluA1 subunit of AMPAR in both GBM cells in comparison to vehicle-treated control cells. Overexpression of GluA1 in GBM cells favors calcium-permeable AMPA-type glutamate receptors assembled from GluA1 and/or GluA4 subunits, as opposed to GluA2 subunits that render these receptors calcium-impermeable (11, 20). Our experimental result demonstrated that NMDA-induced selective upregulation and activation of GluA1 leads to expression of calcium-permeable AMPA receptors. Prior study has highlighted the role of calcium-permeable AMPARs in the induction of GBM growth and invasion (11). Furthermore, evidences suggest that the intracellular calcium in U87MG cells activates mitogen-like sphingosine-1-phosphate (3) which through its downstream kinase activity stimulates migration and invasion (27). Accordingly, calcium-binding protein like S100A4 has been implicated in tumor invasion (26). Therefore, as evidenced from the effective increase in the expression of GluN2 and GluA1 subunits leading to calcium-permeable NMDAR and AMPAR, implication of NMDAR signal-mediated migration and invasion of GBM cells can be surmised. Calcium influx through

calcium-permeable glutamate receptors in turn contributes to the aggressive growth and invasive behavior of GBM (12).

Invasion is a defining hallmark of GBM, which is characterized by an aggressive local growth (10) and is a product of continuous interaction between cancer cells with their surrounding microenvironment (9). In glutamate-rich microenvironment, significantly enhanced invasion in both LN18 and U251MG cells was observed on activation of NMDAR-type glutamate receptors by its agonist NMDA (100 μM) along with glycine (10 μM). This effect was significantly attenuated by the selective non-competitive NMDAR antagonist MK-801. A similar response was observed in patient-derived primary glioma cells. This novel finding illustrates the role of NMDAR in GBM cell invasion. In addition, this study demonstrated that NMDAR mediated increased migration in GBM cell lines, which was inhibited by an NMDAR antagonist. As observed in this study, the difference in the levels of expression of GluN2A in LN18 and U251MG cells on activation of NMDAR possibly reflects the same theme of phenotypic difference. However, in terms of recorded invasion and migration mediated through NMDAR, the observed difference was not found to be statistically significant. Although it is apparent that there is a certain difference in the receptor subunit expression pattern through NMDAR activation, it cannot be considered to have any functional significance with respect to invasion and migration in these cell lines. Another observation reported by Shipton and Paulsen (24) regarding short-term memory impairment indicated the decrease in NMDAR-mediated currents as the primary cause without any selective contribution of the GluN2A, GluN2B subunits of NMDAR. This evidence might have similar implications to our experimental results. More in-depth research to further study phenotypic/functional effects of NMDAR activation in GBM cells may provide new information.

To the best of our knowledge, this is the first study to evaluate the role of NMDAR in migration and invasion of GBM along with NMDAR and AMPAR interaction. The glutamate secreted from GBM cells functions in an autocrine and paracrine manner (6). In addition, autocrine and paracrine interactions implicated in the invasion of GBM have been reported to constitute recognized signaling systems in stages of neural development (10). NMDARs play a critical role in neural differentiation and calcium-dependent migration of

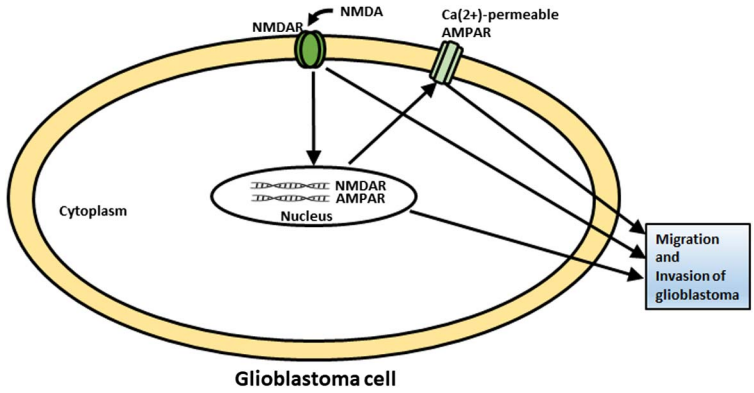

Fig. 4. Schematic diagram showing a potential role of NMDAR in migration and invasion of GBM cells: NMDA induces overexpression of NMDAR and calcium-permeable AMPAR subunits, and their crosstalk mediates migration and invasion of GBM

neurons (16). This study juxtaposes NMDAR-mediated normal neural developmental to NMDAR-induced migration and invasion of GBM cells. Interestingly, decane-1,2-diol ditosylated derivative interacts with NMDA receptor causing GBM cytotoxicity (29). A recent study has shown that nitromemantines mediate tumoricidal effect by inhibition of NMDAR signaling in high-grade gliomas (1). Current literature and our findings highlight the significance of NMDAR in GBM biology. High-glutamate microenvironment in GBM, crosstalk between NMDAR and AMPAR leading to downstream intracellular calcium signaling with multiple intermediaries, is a highly orchestrated process, which requires further exploration.

In summary, this study highlights the novel role of NMDAR in enhancing GBM cell migration and invasion. In the glutamate-rich microenvironment, NMDA induces over-expression of calcium-permeable NMDAR and AMPAR subunits, and their crosstalk provides a plausible rationale for increased GBM invasiveness (represented in schematic diagram of Fig. 4). Detailed mechanisms underlying effects mediated by NMDAR signaling in GBM await further exploration. Our observations emphasize the compelling need for in-depth analysis of glutamate receptor subunit-related pathways and the downstream signaling in GBM.

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Conflict of interest

The authors declare no competing interest.

Abbreviations

AMPA : α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
DMEM : Dulbecco's Modified Eagle's Medium
DMSO : dimethyl sulfoxide
GBM : glioblastoma
MMPs : matrix metalloproteinases
MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMDAR : N-methyl-D-aspartate receptor

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