Esmethadone (REL-1017) Restores NMDA Receptor 1 Subunit Expression in an In Vitro Model of Glutamatergic Excitotoxicity

Sara De Martin[1], Martina Colognesi[1], Charles E. Inturrisi[2], Andrea Mattarei[1], Ezio Bettini[3], Marco Pappagallo[2,4], Franco Folli[5], Stephen M. Stahl[6,7], Sergio Traversa[2], Paolo L. Manfredi[2]

[1]Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova, Italy, [2]Relmada Therapeutics, New York, NY, USA, [3]In Vitro Pharmacology Department, Aptuit, an Evotec Company, Verona, Italy, [4] Albert Einstein College of Medicine, Bronx, NY, USA, [5]Department of Health Sciences, University of Milano, Milano, Italy, [6]University of California, San Diego, CA, USA, [7]Neuroscience Education Institute, San Diego, CA, USA

INTRODUCTION

- The NMDAR channel blocker esmethadone (REL-1017; dextromethadone; DXT) reduces depressive-like behaviours in animal models via BDNF and mToR dependent mechanisms (Fogaca 2019).
- Esmethadone is currently in phase 3 clinical development for treatment of major depressive disorder (MDD). Hyperactive NMDARs and impaired neural plasticity have been implicated in the pathogenesis of MDD.
- NMDAR1 subunit is necessary for cell-membrane expression of NMDARs. In this study, we examined the effects of glutamate and esmethadone on the expression of the NMDAR1 subunit in ARPE-19 cells.

METHODS

- Retinal pigment epithelial ARPE-19 cells were plated in 25 cm^2 T-flasks at a seeding density of $2.5x10^5$, or onto sterilized coverslips placed into cell culture plates, for RNA extraction and IF assay, respectively.
- Cells were maintained in standard culture medium enriched with $MgCl_2$ (final concentration :1.2 mM Mg^{2+}), and $CaCl_2 \cdot 2H2O$ (final concentration : 1.4 mM Ca^{2+}).
- Cells were then passed in modified culture medium and treated with 10 μM L-glutamate, either alone or in combination with 1 μM or 10 μM esmethadone, for 24 hours.
- For RNA extraction, cells were detached, centrifuged, and the pellet was resuspended and subjected to RNA MiniPrep elution.
- RNA extracted from ARPE-19 cells was amplified and quantified by one-step qRT-PCR. The samples were diluted and loaded on a 48-well microplate for PCR together with a Mastermix, containing 2X One Step TB Green RT-PCR Buffer, PrimeScript RT enzyme Mix, and ROX Reference Dye. NMDAR1 mRNA was quantified using the $2^{-\Delta\Delta CT}$ method, as fold variation vs untreated control cells.
- For immunofluorescence analysis, cells were fixed for 20 min at room temperature in 4% paraformaldehyde and incubated overnight with a primary anti-NMDAR1 rabbit monoclonal antibody (ab68144; Abcam) in a humid chamber at 4°C. After extensive washing, cells were incubated with an Alexa Fluor 488-conjugated secondary anti-rabbit antibody (ab150077; Abcam) for 60 min at 37°C, and then with ribonuclease A for 5 min at room temperature. Cells were also incubated with DAPI Nucleic Acid Stain. Finally, the coverslips were mounted with Mowiol.
- Images were acquired with a confocal microscope Zeiss LSM 800 and the ImageJ was used to quantify the intensity of the fluorescent signal in the z-stack confocal images.
- Statistical analysis was performed using one-way ANOVA, followed by *post hoc* Tukey's multiple comparisons test.

RESULTS

- 10 μM L-glutamate significantly decreased mRNA ($p < 0.01$) and membrane protein ($p < 0.05$) expression.
- Esmethadone restored NMDAR1 mRNA and membrane protein expression in a dose-dependent manner.

Figure 1

mRNA expression of NMDAR1 in ARPE-19 cells. The results are obtained from 4 independent experiments, each performed in triplicate. Data are presented as mean ± SEM.

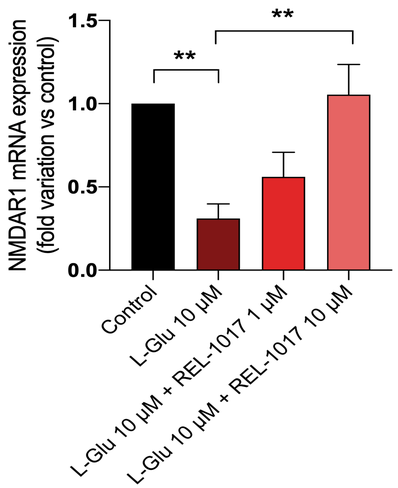


Figure 2

Membrane protein expression of NMDAR1 in ARPE-19 cells. The results are obtained from 3 independent experiments, each performed in duplicate. Data are presented as mean ± SEM.

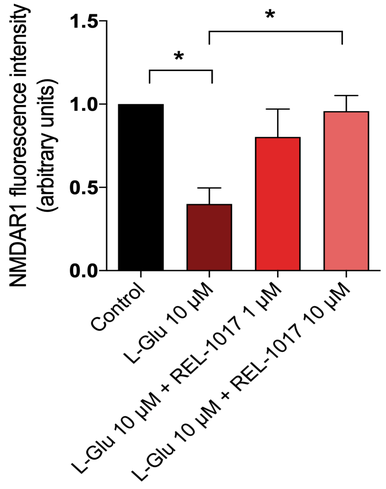
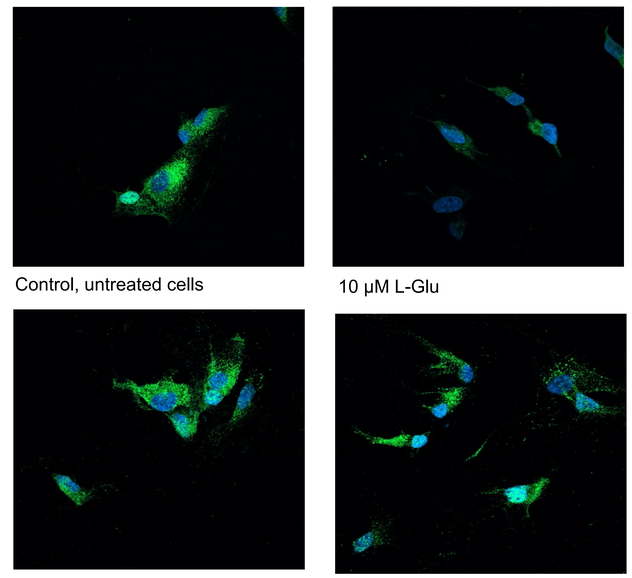


Figure 3

Representative images of of NMDAR1 in ARPE-19 cells. The quantification have been performed by analysing at least 10 different fields.



GENE	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')
GAPDH	ACATCAAGAAGGTGGTGAAGCA	GTCAAAGGTGGAGGAGTGGGT
NMDAR1	AAGCCAGAGTCATTCAGAGCAA	GGTCCTTAGCCACTCCTTCT

DISCUSSION

- NMDARs play a critical role in excitatory neurotransmission, brain development, and synaptic plasticity. High concentrations of L-glutamate induce excitotoxicity in neuronal cells expressing synaptic and extra-synaptic NMDARs. (Choi, 1992; Miladinovic et al., 2015).
- NMDA receptors are heteromeric complexes very permeable to Ca^{2+} and blocked by Mg^{2+}. Mg^{2+} and Ca^{2+} ion concentrations were adjusted in the medium to simulate physiological conditions.
- NMDAR1 was evaluated in the experiments as an indicator of NMDAR expression because it is a necessary subunit for the NMDAR tetrameric structure expression on cell membrane. (Lee et al., 2014).
- ARPE-19 cells were selected for their similarities with neuronal cells, and because they express NMDARs (Sharma et al., 2005; Shen et al., 2006).

CONCLUSIONS

- NMDARs tonically exposed to relatively low glutamate concentrations downregulate NMDAR1 transcription and expression. Esmethadone restores NMDAR1 transcription and expression to basal values in a dose-dependent manner. These results suggest that NMDARs autoregulate their own transcription and expression via Ca^{2+} signaling and confirm that esmethadone may have neural plasticity modulating effects via block of tonically and pathologically hyperactive NMDARs.
- The central role of NMDARs in neural plasticity is well established. The possibility of modulating NMDAR activity with esmethadone, a safe and well-tolerated drug, adds promise for new levels of understanding of the functional roles of NMDA receptors in physiology and disease.

DISCLOSURES

This research was sponsored by Relmada Therapeutics, Inc. Drs. Inturrisi, Stahl, Pappagallo, and Manfredi are paid consultants for Relmada Therapeutics. Drs. Inturrisi and Manfredi are inventors on esmethadone patents and other patents and patent applications.

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Corresponding Author: Paolo L. Manfredi, M.D. Email: pmanfredi@relmada.com