Esmethadone (REL-1017) Blocks NMDA Receptors and Reduces Ca^{2+} Entry In Presence of Quinolinic Acid and Gentamicin

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INTRODUCTION

- Esmethadone (REL-1017; dextromethadone; DXT) is a novel NMDA receptor (NMDAR) channel blocker currently in clinical development for major depressive disorder (MDD).

OBJECTIVES

- To better understand esmethadone role as a channel blocker, we examined the functional interaction between esmethadone and quinolinic acid, a brain metabolite and endogenous neurotoxin acting as a partial agonist at the glutamate binding site of the NMDAR; and esmethadone and gentamicin, an antibiotic showing ototoxic and nephrotoxic effects mediated by NMDAR potentiation.

METHODS

- Intracellular calcium levels were measured by fluorometric imaging plate reader (FLIPR) in 384 well plate format, using Fluo-4 fluorescent free calcium indicator.
- Four CHO cell lines expressing human heterodimeric NMDA receptors were used: hGluN1/hGluN2A, hGluN1/hGluN2B, hGluN1/hGluN2C and hGluN1/hGluN2D.
- Test items were added together with 10 µM glycine and indicated concentration of L-glutamate in magnesium-free buffer.
- Area under the curve (AUC) values of fluorescence readings were measured for 5 minutes after L-glutamate addition
- Data were normalized to readings obtained in presence of 10 µM L-glutamate plus 10 µM glycine (100%) and buffer (0%).
- Four parameter logistic equation in GraphPad Prism was used to calculate test item EC_{50}.
- Statistical analysis was performed using one-way ANOVA, followed by Tukey's multiple comparisons test.

CONCLUSIONS

- Quinolinic acid and gentamicin are endogenous and exogenous substances, respectively, known to have neurotoxic effects, mediated by their action on NMDA receptors.
- Quinolinic acid acted as a partial agonist at NMDA receptors that contained hGluN2A, hGluN2B or hGluN2D subunits, with an EC_{50} of 850, 170, 520 µM, respectively, while it had no effect at NMDA receptors that included the hGluN2C subunit (Figure 1).
- 10 µM esmethadone significantly reduced calcium entry elicited by 1000 µM quinolinic acid in absence of L-glutamate (Figure 3), or in presence of low L-glutamate concentrations (Figure 4 and 5).
- Gentamicin did not elicited an agonist effect in the presence of 10 µM glycine at all tested NMDA receptors (Figure 2 panel A).
- Gentamicin acted as a positive modulator at all tested NMDA receptors in presence of 0.04 µM L-glutamate (Figure 6), while only at NMDA receptors that included the hGluN2B in presence of 10 µM L-glutamate, with an EC_{50} of 21 µg/ml and 128% effect at 100 µg/ml (Figure 2 panel B).
- 10 µM esmethadone significantly reduced calcium entry induced by 10 µg/ml gentamicin in presence 0.04 µM L-glutamate in all tested NMDAR expressing cell lines (Figure 6).
- Esmethadone inhibition of quinolinic acid or gentamicin evoked calcium influx through NMDA receptors might have neuroprotective potentials.

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

RESULTS

Figure 1 - Quinolinic acid CRC

Quinolinic acid is an endogenous neurotoxin, acting on NMDAR. Little was known about quinolinic acid effect on various NMDAR isoforms. Therefore, we studied quinolinic acid concentration response curve (CRC) on heterodimeric human NMDA receptor expressed in CHO cell lines, by FLIPR assay in presence of 10 µM glycine, but in absence of L-glutamate.
Quinolinic acid resulted as partial agonist at NMDAR containing hGluN2A, hGluN2B or hGluN2D subunit, while it was ineffective on NMDAR containing hGluN2C subunit. GraphPad Prism fitting values are reported in below table (N.A.: not available). Data are mean ± SEM, n = 6.

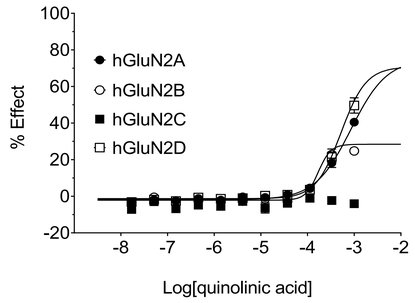


Table 1 - Quinolinic acid EC_{50}

Quinolinic acid EC_{50} values were calculated from fittings shown is Figure 1. Quinolinic acid resulted more potent on NMDAR containing hGluN2B subunit, while inactive on NMDAR containing hGluN2C subunit.

	hGluN-hGluN2A	hGluN1-hGluN2B	hGluN1-hGluN2C	hGluN1-hGluN2D
EC_{50} (µM)	850	170	> 1000	520
Min (%)	-1.9	-2.1	N.A.	-1.3
Max (%)	~ 75	28	N.A.	71
Slope	1.1	3.2	N.A.	1.6

Figure 3a - Gentamicin CRC

Gentamicin is an antibiotic which might result neurotoxic at high concentrations. Therefore, to investigated if gentamicin might activate NMDARs in absence of L-glutamate, gentamicin CRCs were run by FLIPR assay in presence of 10 µM glycine, but in absence of L-glutamate, using four different CHO cell lines expressing different heterodimeric human NMDA receptor. No effect was observed in any of the cell lines. Data are mean ± SEM, n = 6.

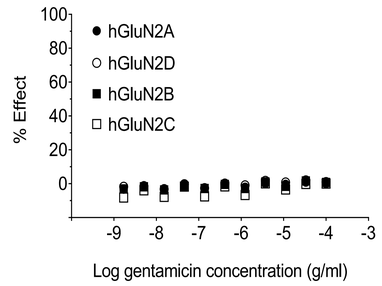


Figure 3b - Gentamicin CRC in 10 µM L-glutamate

To investigate if gentamicin neurotoxicity might be mediated by gentamicin potentiation of L-glutamate induced calcium entry through NMDAR, we first performed gentamicin concentration response curve by FLIPR assay in presence of 10 µM L-glutamate together with 10 µM glycine. Gentamicin resulted as positive modulator only at NMDA receptor containing hGluN2B subunit, with EC_{50} 21 µg/ml and 128% effect at 100 µg/ml. Data are mean ± SEM, n = 5.
Interestingly, additional studies (see Figure 4) showed that gentamicin was able to potentiate calcium entry through all NMDAR isoforms, in the presence of low (e.g. 0.04 µM) L-glutamate concentration.

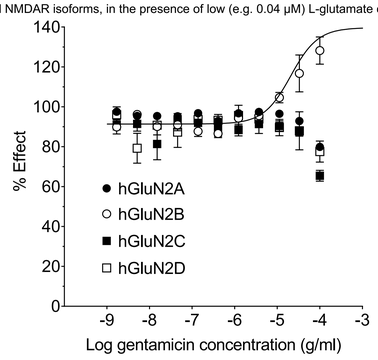


Figure 2a - Esmethadone reduced calcium entry induced by quinolinic acid alone

Quinolinic acid might be neurotoxic by activating NMDAR in absence of L-glutamate. Therefore, we decided to investigate if esmethadone might counteract such quinolinic acid effect. 10 µM esmethadone (DXT) ability to counteract 100-1000 µM quinolinic acid (QA) mediated increase in intracellular calcium levels through specific NMDARs was studied. FLIPR calcium assay was performed in presence of 10 µM glycine, using the 4 NMDAR cell lines. Data are shown as mean ± SEM, n=42 for each group.
Legend: * is P < 0.05; **** is P < 0.0001.

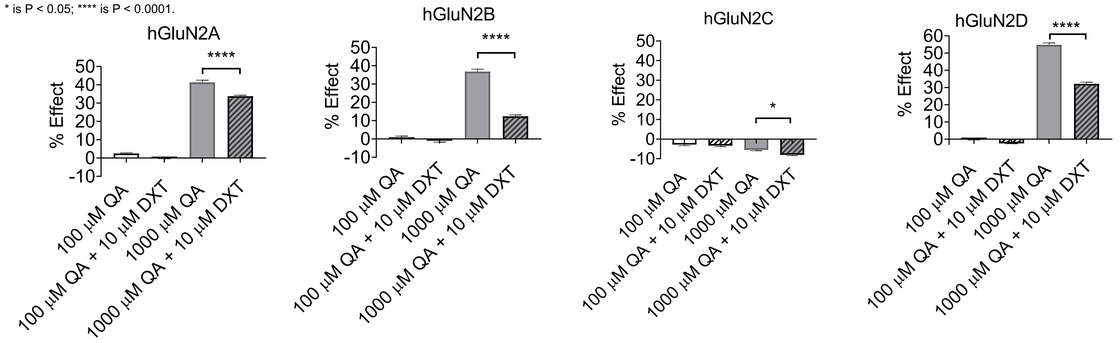


Figure 2b - Esmethadone reduced intracellular calcium entry induced by quinolinic acid in presence of 0.04 µM L-glutamate

Quinolinic acid might be neurotoxic by activating NMDAR in presence of low concentrations of ambient L-glutamate. Therefore 10 µM esmethadone effect on 100-1000 µM quinolinic acid in presence of 0.04 µM L-glutamate and 10 µM glycine, was studied in FLIPR calcium assay using the 4 NMDAR cell lines. Data are shown as % mean ± SEM, n=42 for each group. Statistical analysis was performed using one-way ANOVA, followed by Tukey's multiple comparisons test. Legend: * is P < 0.05; **** is P < 0.0001.

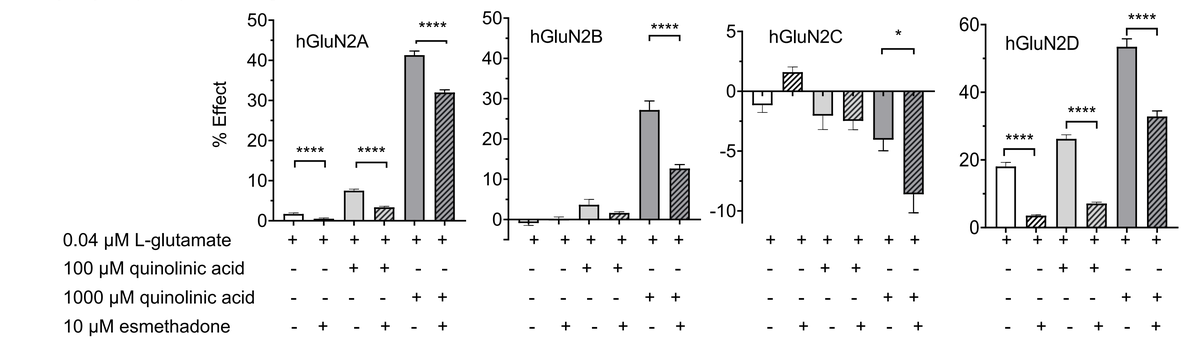


Figure 2c - Esmethadone reduced intracellular calcium entry induced by quinolinic acid in presence of 0.2 µM L-glutamate

Quinolinic acid might be neurotoxic by activating NMDAR in presence of low concentrations of ambient L-glutamate. Therefore, 10 µM esmethadone effect on 100-1000 µM quinolinic acid in presence of 0.2 µM L-glutamate and 10 µM glycine, was studied in FLIPR assay using the 4 NMDAR cell lines. Data are shown as % mean ± SEM, n=42 for each group. Statistical analysis was performed using one-way ANOVA, followed by Tukey's multiple comparisons test. Legend: **** is P < 0.0001.

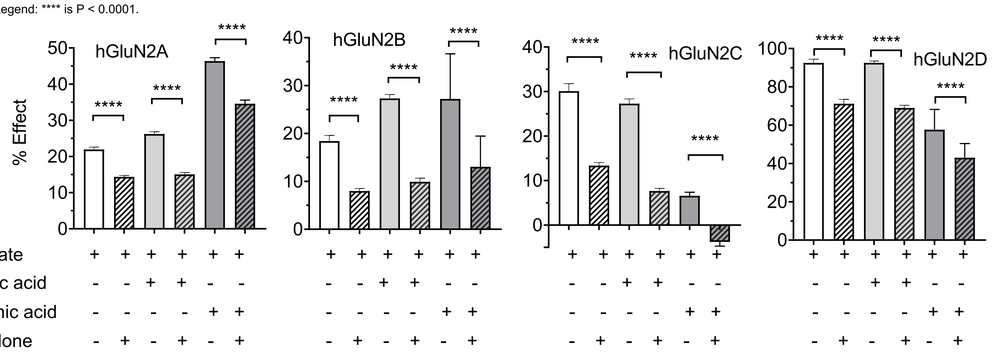
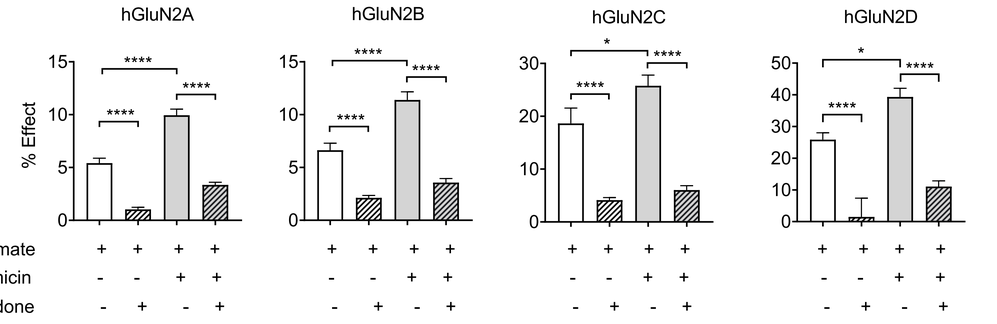


Figure 4 - Esmethadone reduced gentamicin induced calcium entry in presence of 0.04 µM L-glutamate

Gentamicin might be neurotoxic by activating NMDAR in presence of low concentrations of ambient L-glutamate. Therefore, we decided to investigate if esmethadone might counteract such gentamicin effect 10 µM esmethadone effect on calcium entry elicited by 10 µg/ml gentamicin in presence of 0.04 µM L-glutamate and 10 µM glycine was studied in FLIPR assay, using the 4 NMDAR cell lines. Data are shown as % mean ± SEM, n=30 for each group. Statistical analysis was performed using one-way ANOVA, followed by Tukey's multiple comparisons test. Legend: : * is P < 0.05; **** is P < 0.0001.



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