B'SYS

B'SYS GmbH

# NMDA DUO Cell Lines

Specification Sheet

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# 1 BACKGROUND

## 1.1 NMDA Receptors

The NMDA receptor is a ionotropic glutamate receptor. Its activation results in a none selective cation current. The equilibrium potential is near 0 mV. In the presence of $Mg^{2+}$ the NMDA receptor current shows an outward rectifying voltage dependence.
NMDA receptors are involved in synaptical plasticity and can influence learning and memory.
The receptors consist of heterotetramers between two NR1 and NR2 subunits. Multiple receptor isoforms with distinct brain distributions and functional properties arise by selective splicing of the NR1 transcripts and differential expression of the NR2 subunits. At the extracellular domain of the NR1 subunit a glycine binding site is located, the NR2 subunit exhibits an extracellular glutamate / NMDA binding site.
The activity of the receptors can be modulated by several compounds that bind to several allosteric binding sites (e.g. a binding site for ifenprodil (only NR2B) or neurosteroids).

## 1.2 B'SYS' NR1/NR2A, NR1/NR2B and NR1/NR2D cell lines

The host of B'SYS' NR1/NR2X cell lines are HEK293 cells. Cells were stably transfected and selected for high expression rates of the human NMDA receptor isoforms. They are suitable for manual and automated patch-clamping.

# 2 VALIDATION OF NMDA DUO CELLS

## 2.1 Electrophysiology

All cell lines were validated using manual patch-clamping and automated patch-clamping (Q-Patch). During validation, the dose dependence and the $EC_{50}$ for NMDA and Glycine were determined. Further during constant concentrations of NMDA and Glycine, increasing concentrations of positive allosteric modulators and antagonists were applied and the $EC_{50}$ / $IC_{50}$ calculated.

The extracellular solution was composed of 137 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 10 mM HEPES, 10 mM Glucose, pH 7.4 (NaOH). The pipette solution contained: 130 mM KCl, 1 mM $MgCl_2$, 5 mM MgATP, 10 mM HEPES, 5 mM EGTA, pH 7.2 (KOH). Cells were clamped to -80 mV for the duration of the complete experiment. To accomplish fast solution exchanges, a 8 to 1 manifold was placed in close neighborhood of the tested cell.

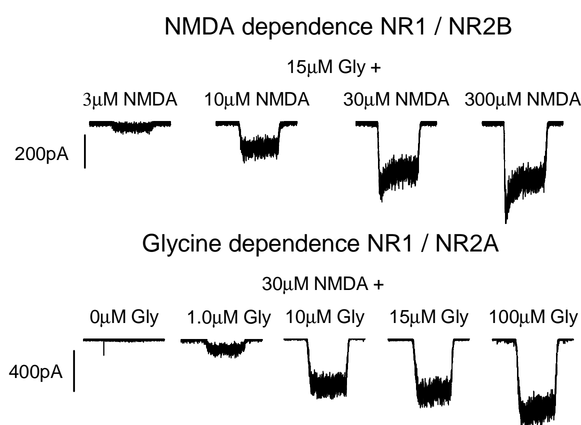
## 2.2 Agonists

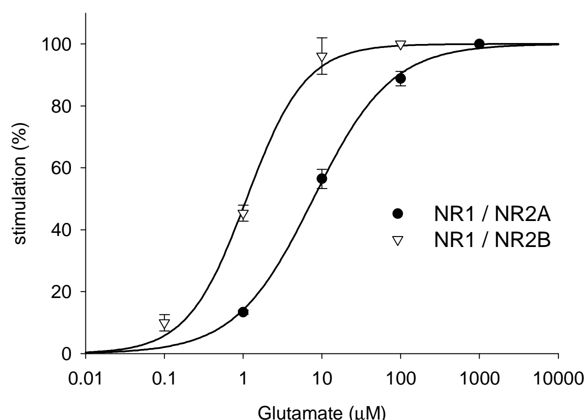
The following $EC_{50}$ for NMDA and Glycine were determined:

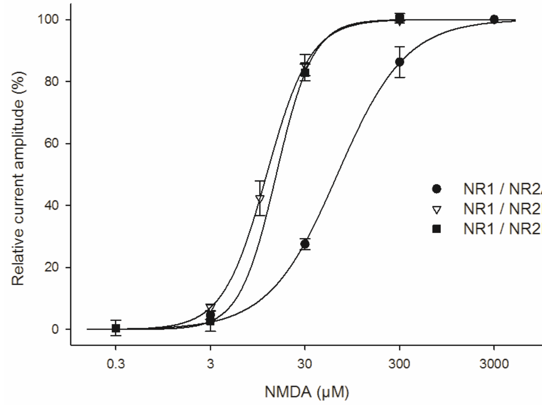
Table 1: Summary of stimulation by different agonists.

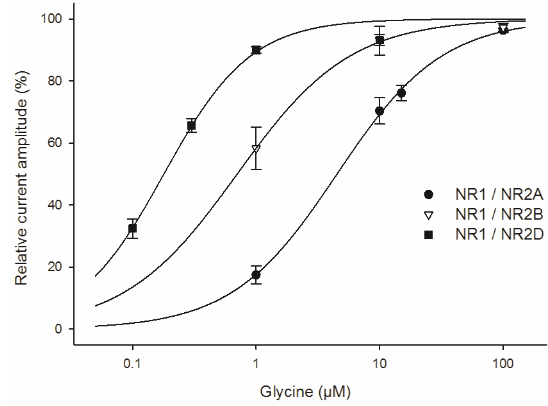
| Subtype | Agonist | $EC_{50}$ (manual / automated) | Hill Coefficient (manual / automated) |
|---|---|---|---|
| NR1/ NR2A | Glutamate | 7.76 µM / - | -0.88 / - |
| | NMDA | 65.80 µM / 55.51 µM | -1.20 / -1.49 |
| | Glycin | 4.53 µM / 4.74 µM | -1.02 / -1.79 |
| NR1/ NR2B | Glutamate | 1.10 µM / - | -1.16 / - |
| | NMDA | 11.78 µM / 25.56 µM | -1.88 / -2.03 |
| | Glycin | 0.71 µM / 0.23 µM | -1.14 / -1.30 |
| NR1/ NR2D | NMDA | 15.01 µM / - | -2.23 / - |
| | Glycin | 0.16 µM / - | -1.50 (set manually) / - |

Manual patch-clamping:



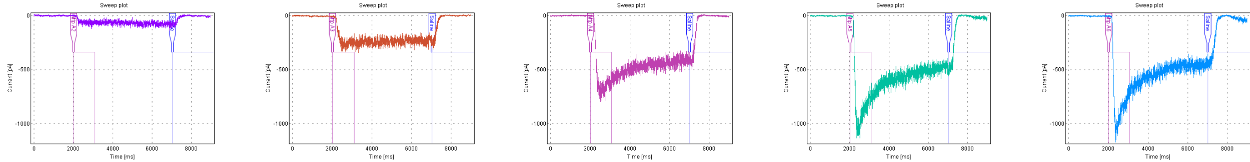





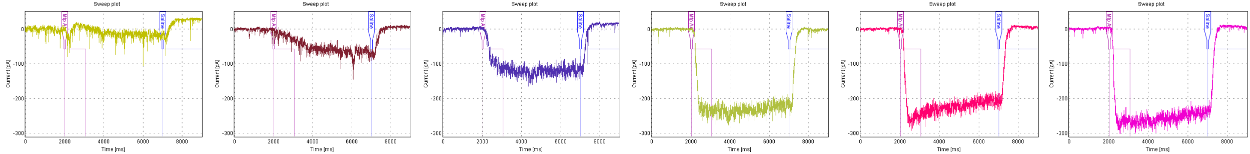




Automated patch-clamping (Q-Patch):

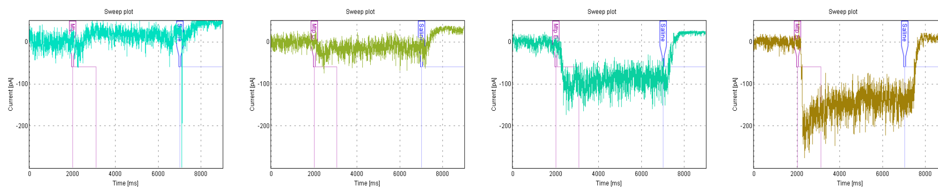
NR1/NR2A, 1, 10, 30, 100, 300 µM NMDA, 10 µM Glycine



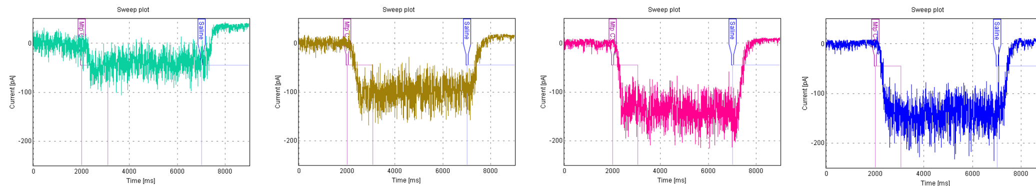
NR1/NR2A, 0.1, 1.0, 3.0, 10, 30, 100 µM Glycine, 30 µM NMDA

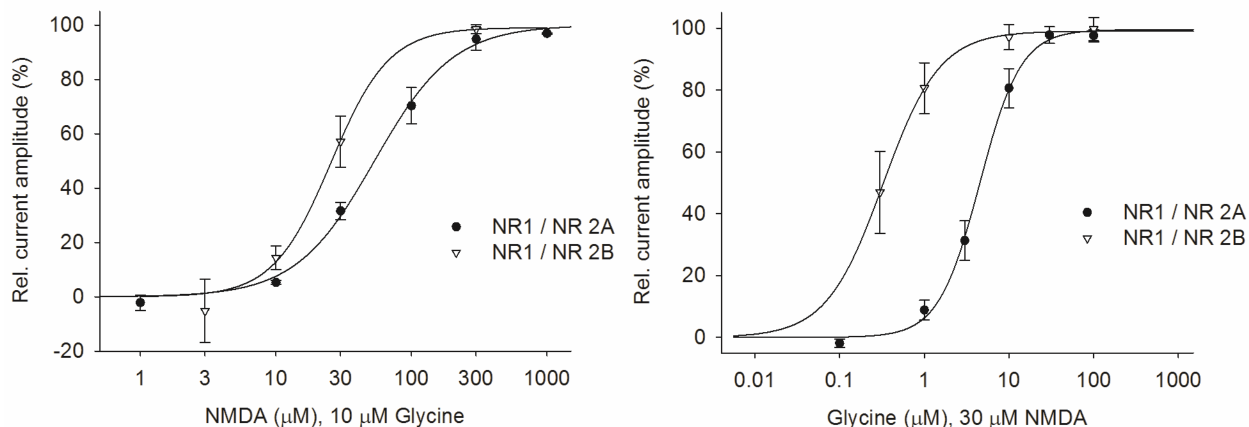


NR1/NR2B, 3, 10, 30, 300 µM NMDA, 10 µM Glycine



NR1/NR2B, 0.3, 1, 10, 100 µM Glycine, 30 µM NMDA

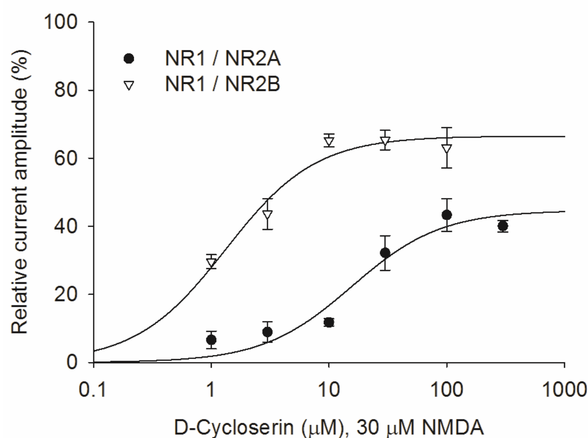





## 2.3 Partial Agonists

To validate the glycine binding site, the $EC_{50}$s for D-Cycloserin was measured in the subtypes NR1 / NR2A and NR1 / NR2B. D-Clycloserin is known to interact specifically with the glycine binding site in NMDA receptors.

For all measurements, 30 µM NMDA was used. The average of two applications with 30 µM NMDA and 100 µM Glycine of each cell were taken as 100%, followed by applications of increasing concentrations of D-Cycloserin.




The following $EC_{50}$ for D-Cycloserin were determined:

Table 2: Summary of stimulation by different subtype.

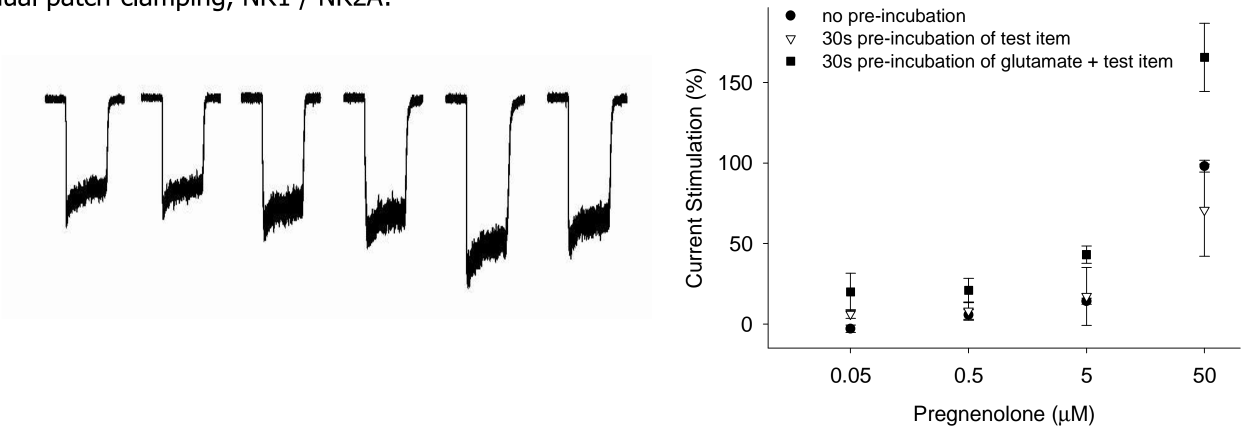
| Subtype | Agonist | $EC_{50}$ (manual) | Hill Coefficient (manual) |
|---|---|---|---|
| NR1/ NR2A | D-Cycloserin | 15.62 µM | -1.15 |
| NR1/ NR2B | D-Cycloserin | 1.33 µM | -1.14 |

## 2.4 Positive modulators

### 2.4.1 Pregnenolone sulphate

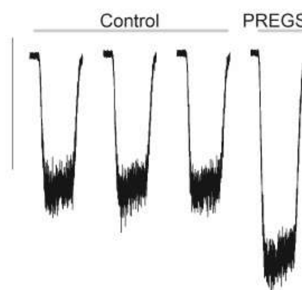
Pregnenolone sulphate is a neurosteroid, which can allosterically modulate NMDA currents. Four concentrations were tested in the manual patch-clamp using three different pre-applications before the receptor was stimulated with Glycine. Pre-incubation with test item together with glutamate resulted in the strongest stimulating effects of pregnenolone sulphate. Additionally the impact of pregnenolone sulphate on NR1 / NR2B currents was determined and the effect on both subtypes were also measured in automated patch-clamping.

Manual patch-clamping, NR1 / NR2A:



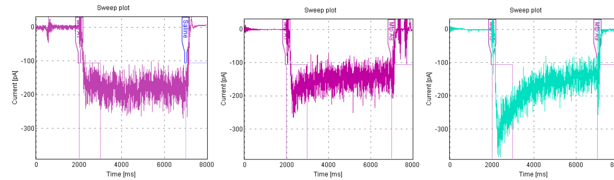

Manual patch-clamping, NR1 / NR2B:

50 µM pregnenolone sulphate:



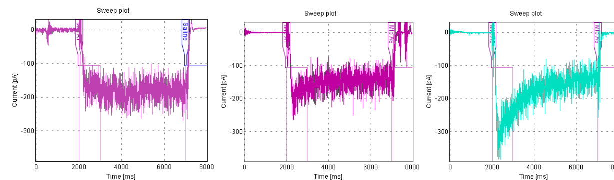
Automated patch-clamping, NR1 / NR2A:

0, 0, 50 µM Pregnenolone sulphate



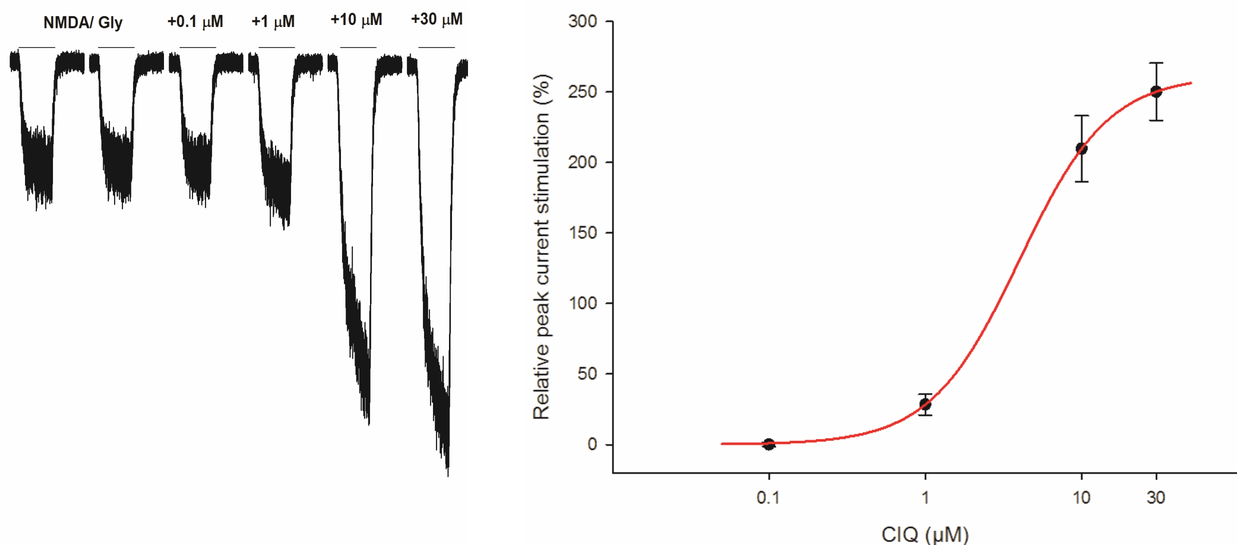
Automated patch-clamping, NR1 / NR2B:

0, 0, 100 µM Pregnenolone sulphate



### 2.4.2 CIQ

The substituted tetrahydoisoquinoline CIQ is a subunit-selective potentiator of NR2C- and NR2D- containing NMDA receptors. Four different concentrations were tested in the manual patch-clamp on the NR1 / NR2D cell line. The $EC_{50}$ was determined to 4.00 µM (Hill: 1.52).



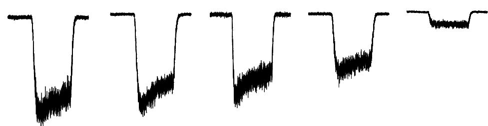
## 2.5 Antagonists

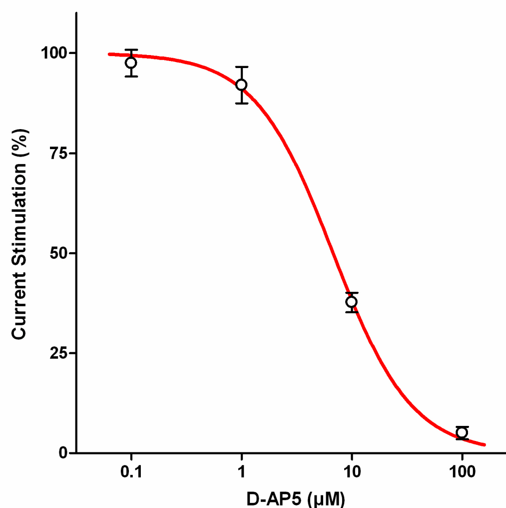
### 2.5.1 AP5

AP-5 (2R-amino-5-phosphonovaleric acid), is a competitive antagonist for the Glutamate binding site of NMDA receptors. The $IC_{50}$ value for NR1/NR2A was determined as 6.75 µM (Hill coefficient: 1.22) in manual patch clamping, and 0.39 µM (Hill coefficient: 0.99) in automated patch-clamping in the presence of 30 µM NMDA and 5.0 µM Glycine.

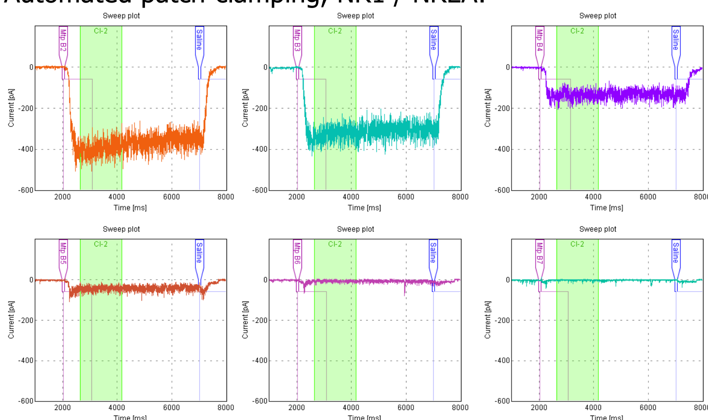
For Ketamine a $IC_{50}$ of 0.18 µM (Hill coefficient: 0.83) was determined under the same experimental conditions (automated patch-clamping).

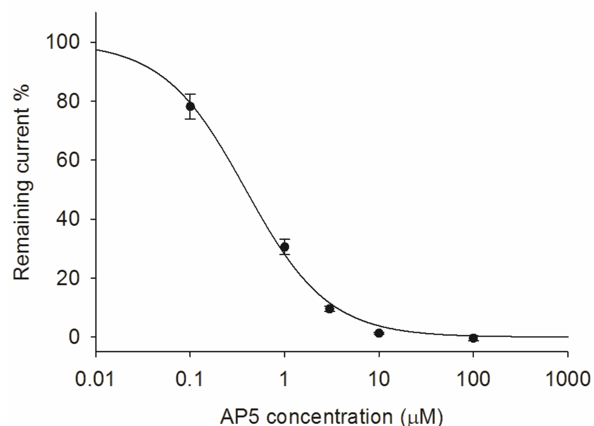
Manual patch-clamping, NR1 / NR2A:






Automated patch-clamping, NR1 / NR2A:

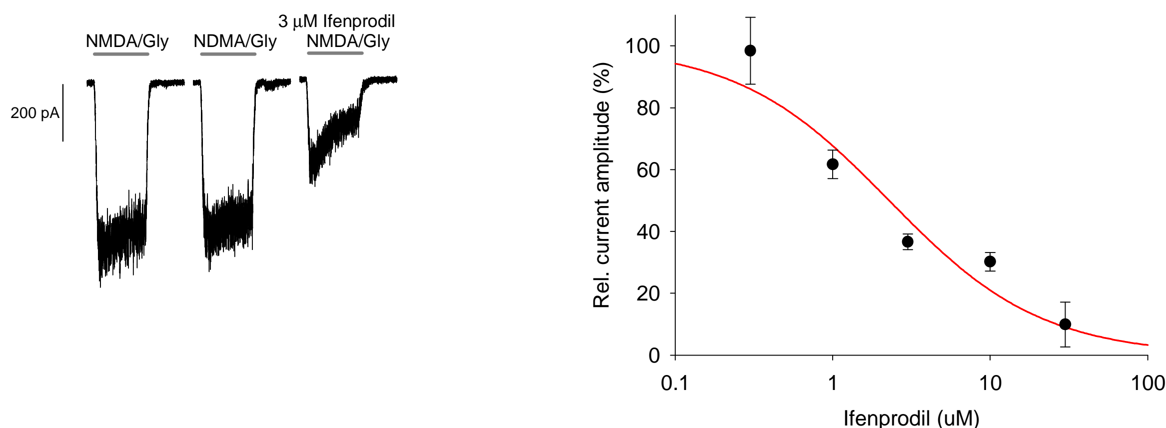


### 2.5.2 Ifenprodil

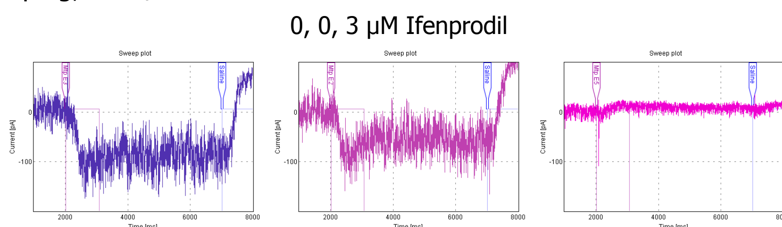
Ifenprodil, is a NR2B selective, atypical non-competitive antagonist. The $IC_{50}$ value for NR1/NR2B was determined as 2.28 µM (Hill coefficient: 0.90) in manual patch clamping in the presence of 20 µM NMDA and 1.0 µM Glycine with a sweep length of 5 s. Ifenprodil had no effect on NR1 / NR2A or NR1 / NR2D receptor currents (data not shown).

Manual patch-clamping, NR1 / NR2B:



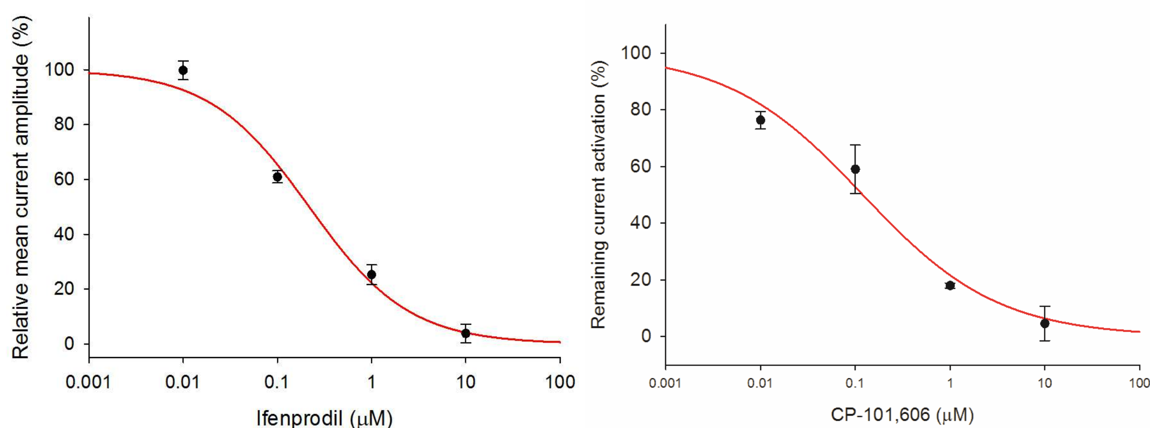

Automated patch-clamping, NR1 / NR2B:

0, 0, 3 µM Ifenprodil



A prolonged sweep length of the Ifenprodil application results in an $IC_{50}$ value of 0.22 µM with a Hill coefficient of 0.82. For these experiments concentrations of 30 µM NMDA and 5.0 µM Glycine were used. After five seconds of NMDA/Gly alone, the tested concentration of Ifenprodil in the same NMDA/Gly solution was applied for two minutes.

With the same application protocol and NMDA/Gly concentrations, an $IC_{50}$ of 120.10 nM with a hill coefficient of 0.61 was reached for CP-101,606 (Traxoprodil).



# 3 CELL CULTURE CONDITIONS

## 3.1 General

HEK NMDA DUO cells are incubated at 37°C in a humidified atmosphere with 5% $CO_2$ (rel. humidity > 95%). The cells are continuously maintained and passaged in sterile culture flasks containing medium supplemented with the NMDA receptor blocker AP5 and selection antibiotics (see table below). The HEK NMDA DUO cells are passaged at a confluence of about 50-80%. For electrophysiological measurements the cells are seeded onto e.g. 35 mm sterile culture dishes containing complete medium.

For the induction of NMDA receptor expression 2.5 µg/mL Tetracycline has to added 24-48 h before experimentation.

- All solutions and equipment coming in contact with the cells must be sterile.
- Use proper sterile technique and work in a laminar flow hood.
- Be sure to have frozen cell stocks at hand before starting experiments.
- Cells should be splitted every 4-5 days at 50% - 80% confluency at least 1:5 ratio (1:3 preferred).

## 3.2 Recommended Complete Medium

- DMEM/F12 with GlutaMAX I or L-Glutamine
- 9% FBS
- 0.9% Penicillin/Streptomycin
- 100 µM AP5

## 3.3 Antibiotics

HEK NR1 /NR2A: 100 µg/mL Hygromycine, 15 µg/mL Blasticidine, 1 µg/mL Puromycine

HEK NR1 /NR2B: 100 µg/mL Hygromycine, 15 µg/mL Blasticidine, 500 µg/mL G-418

HEK NR1 /NR2D: 100 µg/mL Hygromycine, 15 µg/mL Blasticidine, 1 µg/mL Puromycine

## 3.4 Thawing Cells

- Remove vial of cells from liquid nitrogen and thaw quickly at 37°C.
- Decontaminate outside of vial with 70% ethanol.
- Transfer cells to a T-25 culture flask containing 5 mL complete medium & AP5.
- Incubate cells at 37°C for 1-3 days to allow the cells to attach to the bottom of flask.
- Aspirate off the medium and replace with 5 mL complete medium after one day.
- Add antibiotics & AP5 as soon as the are attached and look healthy.
- Incubate cells and check them daily until 70% - 90% confluency is reached.

## 3.5 Splitting Cells

- When cells are 70% - 90% confluent remove complete medium.
- Wash cells once with 1x Detachin.
- Remove Detachin quickly and incubate cells for 3-5 min at room temperature.
- Detach cells, add complete medium and pipet up and down to break clumps of cells.
- Passage cells into new flask with complete medium and antibiotics & AP5 at **1:3** to **1:5** ratio.
- Use remaining suspension for counting the cells.

### 3.6 Freezing Medium

- Mix 0.9 mL fresh complete medium and 0.1 mL DMSO for every 1 mL freezing medium.
- Sterilize freezing medium by means of appropriate micro filter (0.1 µm – 0.2 µm).

### 3.7 Freezing Cells

- Prepare fresh freezing medium and keep it on ice.
- Cells should have 80% - 90% confluency prior to freezing.
- Remove the complete medium.
- Wash cells once with 1x Detachin.
- Remove Detachin quickly and incubate cells for 3-5 min at room temperature.
- Detach cells, add complete medium and pipet up and down to break clumps of cells.
- Pellet cells with centrifuge and carefully aspirate off medium.
- Resuspend cells at a density of approximately $3.0 \times 10^6$ cells per mL with fresh freezing medium (containing AP5).
- Aliquot 0.5 mL of cell suspension into each cryovial.
- Overnight incubate cells in a styropor box at –80°C.
- The next morning transfer cryovial in liquid nitrogen tank for long-term storage.

# 4 SEQUENCE INFORMATION

Codon optimized cDNA sequences of the human NMDA receptor subunits were stably expressed in HEK293 cells. The transfected subunits encode for the following proteins:

NR1: NP_015566.1

NR2A: AAB49993.1

NR2B: NP_000824.1

NR2D: NP_000827.2

# 5 CONTACT INFORMATION

### 5.1 Contact Address for Technical Support & Ordering Information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