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► **To cite this version:**

Caroline Strube, Florian Gackière, Layal Saliba, Fabien Tell, Jean-Pierre Kessler. Variability of quantal NMDA to AMPA current ratio in nucleus tractus solitarii neurons. 2017. hal-03501363

**HAL Id: hal-03501363**

**<https://hal.science/hal-03501363v1>**

Preprint submitted on 23 Dec 2021

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1 Title : Variability of quantal NMDA to AMPA current ratio in nucleus tractus solitarii neurons

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1

## 20 **Abstract**

21 The ratio between AMPA and NMDA receptors is a key factor governing integrative and plastic  
22 properties of excitatory glutamatergic synapses. To determine whether the respective proportions of  
23 AMPA and NMDA receptors are similar or vary across a neuron's synapse, we analyzed the variability  
24 of NMDA and AMPA currents in quantal responses recorded from neurons located in the nucleus  
25 tractus solitarii. We found that the average NMDA to AMPA current ratio strongly differed between  
26 recorded neurons and that most of the intra-neuronal current ratio variability was attributable to  
27 fluctuations in NMDA current. We next performed computer simulations with a Monte Carlo model of  
28 a glutamatergic synapse to estimate the part of AMPA and NMDA currents fluctuations induced by  
29 stochastic factors. We found that NMDA current variability mainly resulted from strong channel noise  
30 with few influence of release variations. On the contrary, partly because of the presence of  
31 subconductance states, AMPA receptor channel noise was low and AMPA current fluctuations tightly  
32 reflected changes in the amount of glutamate released. We next showed that these two factors, channel  
33 noise and fluctuations in glutamate release, were sufficient to explain the observed variability of the  
34 NMDA to AMPA current ratio in quantal events recorded from the same neuron. We therefore  
35 concluded that the proportion of AMPA and NMDA receptors was similar, or roughly similar, across  
36 synapses onto the same target cell.

37

## 38 **INTRODUCTION**

39 Excitatory glutamatergic synapses in the vertebrate central nervous system (CNS) transmit via two  
40 types of ligand gated ion channels, the AMPA and the NMDA receptors. These two types of receptors  
41 differ by their pharmacological and biophysical properties. AMPA receptors are low affinity ligand-  
42 gated channels with fast deactivation whereas NMDA receptors are high affinity receptors with  
43 prolonged activation (Traynelis et al., 2010). Consequently, they have different roles. AMPA receptors  
44 mainly detect fast glutamate transients whereas NMDA receptors also sense slowly changing and steady  
45 state glutamate levels (Yang and Xu-Friedman, 2015). In addition, being highly permeable to calcium  
46 ions, NMDA receptors play a key role in activity-induced long term changes in synaptic strength and  
47 neuronal excitability. Because of these differences in role and behavior between the two receptor types,  
48 the NMDA to AMPA receptor ratio is a key parameter that strongly influences the integrative properties  
49 of excitatory synapses. Expression levels of AMPA and NMDA receptor subunits in post synaptic  
50 membranes are highly variable and depend on the region investigated, the target neuron type and/or the  
51 origin of the fibers that give rise to the presynaptic boutons (Nusser et al., 1998; Nyiri et al., 2003;  
52 Shinohara et al., 2008; Tarusawa et al., 2009; Dong et al., 2010; Fukazawa and Shigemoto 2012; Rubio  
53 et al., 2014). Furthermore, several forms of synaptic plasticity rely on changes in postsynaptic receptor  
54 numbers, especially AMPA receptors numbers, indicating that receptor expression levels at synapses  
55 may vary with time and state (Turrigiano, 2000). The factors that determine the relative abundance of  
56 AMPA and NMDA receptors in a particular synapse remain largely unidentified. Several studies suggest  
57 that the ratio between the two receptors is for a large part a pathway-specific property. In CA1  
58 pyramidal cells for instance, responses from perforant path and Schaffer collateral synapses differ by  
59 their AMPA to NMDA charge ratio (Otmakhova et al., 2002). Likewise, cortico-striatal and thalamo-  
60 striatal pathways elicit responses with different NMDA/AMPA current ratios in striatal neurons (Smeal  
61 et al., 2008; Ellender et al., 2013). Thalamic reticular neurons also receives two types of inputs with  
62 different NMDA/AMPA current ratios (Deleuze and Huguenard, 2016). However, these data should be

63 interpreted with caution. As discussed in Myme et al (2003), synaptic responses evoked by electrical  
64 stimulation of afferent pathways may fail to provide a reliable view of receptor equipment at synapses.  
65 Other studies provide a different view. Recordings performed on hippocampal and neocortical neurons  
66 show that the amplitudes of AMPA and NMDA receptor currents are correlated across quantal events  
67 recorded from the same cell, suggesting that different synapses onto the same target neuron have a  
68 relatively constant ratio of each receptor type (Gompert et al., 1998 ; Umemiya et al., . 1999; Watt et al.,  
69 2000; Myme et al., 2003; Watt et al., 2004).

70 The aim of the present study was to determine whether AMPA to NMDA receptor ratio is similar or  
71 varies across synapses onto the same neuron. We investigated this question by analyzing the sources of  
72 current fluctuations across quantal synaptic responses recorded from a single neuron. Our main  
73 objective was to determine whether current ratio variability was high, suggesting heterogeneity of  
74 synapses as regards receptor ratio, or low enough to be fully explainable by stochastic factors known to  
75 induce current fluctuations at a single synapse (channel noise, variations in vesicular transmitter  
76 content). Recording of miniature excitatory post-synaptic currents (mEPSCs) were obtained from  
77 retrogradely-identified output neurons of the nucleus tractus solitarii (NTS), a brainstem sensory relay  
78 nucleus which receives glutamatergic inputs from visceral afferent fibers via the glossopharyngeal and  
79 the vagus nerves and in turn projects onto various brain regions (see Baude et al., 2009 for review). The  
80 contribution of stochastic factors to AMPA and NMDA current variability was estimated both by a  
81 theoretical approach based on the binomial law and by computer simulations performed using a  
82 stochastic synapse model.

## 83 **METHODS**

84 Experiments were performed on young (3-6 weeks old) male Wistar rats. All procedures were in  
85 agreement with the European Communities Council directive (86/609/EEC).

### 86 *Electrophysiological recordings*

87 Recordings were obtained from NTS projections neurons identified by retrograde tracing (Strube et al.,  
88 2015). Briefly, young adult rats were anesthetized by an intraperitoneal injection of a mixture of  
89 ketamine (50 mg/kg, Imalgène 1000, Centravet, Lapalisse, France) and xylazine (15 mg/kg, Rompun

90 2%, Centravet) and placed in a stereotaxic apparatus with the incisor bar 2 mm below horizontal.  
91 Tracing was performed using either red RetroBeads (undiluted Rhodamine-labeled latex microspheres,  
92 Lumafluor Inc., Naples, FL, USA) or Fluorogold (2% in 0.2% saline, Fluorochrome LLC., Denver, CO,  
93 USA). Tracer (100 nl) was pressure-delivered through a Hamilton syringe connected to a stainless  
94 needle (ID: 0.15 mm, OD: 0.25 mm) at a rate of 1 nl s<sup>-1</sup> in the parabrachial nucleus (PBN) or the  
95 caudal ventrolateral medulla (CVLM). After wound closure and recovery from anaesthesia, the animals  
96 were housed individually. Preparation of medullary slices was made as described before (Balland et al.,  
97 2006, 2008; Strube et al., 2015) four to seven days after retrograde tracer injection. For recordings,  
98 slices were perfused in a chamber at around 3 ml/min with oxygenated ACSF containing (in mM) 120  
99 NaCl, 3 KCl, 26 NaHCO<sub>3</sub>, 1.25 KH<sub>2</sub>PO<sub>4</sub>, 0.5 ascorbate, 2 pyruvate, 3 myoinositol, 10 glucose, 2.5  
100 CaCl<sub>2</sub>, 2.5 MgCl<sub>2</sub>, 0.02 D-serine and a mixture of GABA<sub>A</sub> receptors blockers (in  $\mu$ M: 20 bicuculline,  
101 100 picrotoxin) at 32°C. Labeled neurons were visualized using a upright microscope (BX51WI,  
102 Olympus Corp., Tokyo, Japan) equipped for fluorescence detection. Whole-cell patch-clamp of NTS  
103 neurons were made with an Axopatch 200B (Axon instruments, Foster city, CA, USA), filtered at 2  
104 kHz and digitized at 20 kHz. Series resistance was monitored throughout the experiment and neurons in  
105 which this parameter was > 20 M $\Omega$  or not stable were discarded. Patch electrodes (2-4 M $\Omega$ ) contained  
106 in mM: 120 cesium methane sulfonate, 10 NaCl, 1 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 10 EGTA, 2 ATP, 0.3 GTP, 10  
107 Glucose, 10 HEPES (pH 7.4). Recordings were performed at +40 mV in order to remove NMDA  
108 receptor magnesium block. To record mEPSCs, 1 $\mu$ M TTX was added to the external solution. A  
109 computer interfaced to a 12-bit A/D converter (Digidata 1200 using Clampex 9.x; Molecular Devices  
110 LLC, Sunnyvale, CA, USA) controlled the voltage clamp protocols and data acquisition.

### 111 ***Data analysis***

112 Detection of mEPSCs was carried out using the event detection module from the Clampfit software  
113 (pClamp, Molecular Device). To prevent any loss of data, detection was performed with two templates,  
114 corresponding to events with high or low NMDA/AMPA current ratio respectively, using a loose  
115 template match stringency (threshold set to 4). False positives were removed by visual examination of  
116 each putative event. A minimum of 30 mEPSCs were collected per neuron. AMPA current amplitude  
117 ( $I_{AMPA}$ ) was measured at the peak of the mEPSC (current averaged over 0.3 ms). NMDA current

118 amplitude ( $I_{\text{NMDA}}$ ) was obtained by averaging current within a time window starting 5 ms after onset.  
119 We used 5ms duration time windows (as in Watt et al 2000, Hanse and Gustafsson 2001, Myme et al.  
120 2003) since long averaging periods (50 ms) resulted in very high dispersion of data. Measures of  
121 variability for  $I_{\text{AMPA}}$ ,  $I_{\text{NMDA}}$  and  $I_{\text{NMDA}}/I_{\text{NMDA}}$  ratio were obtained by calculating variances ( $\sigma$ ) and/or  
122 coefficients of variation (CVs). CVs were used when dimensionless comparison was required.  
123 Statistical analysis were performed using the Graphpad Instat software.

#### 124 ***Computer simulation***

125 Simulation was performed using a Monte-Carlo model of a glutamatergic synapse (Kessler, 2013). The  
126 radii of the axon-dendrite apposition and of the active zone-PSD interface were 500 nm and 200 nm,  
127 respectively. No glial membrane or glutamate transporter was included in the model. Glutamate was  
128 released in front of PSD center. Depending on the experiment, the number of glutamate molecules  
129 released at each synaptic event was either set to 3000 or made variable around a 3000 average value  
130 using a Gaussian random number generator. Quantum size was limited by low and high cut-offs set at  
131 1000 and 9000 molecules respectively. Glutamate diffusion was calculated using the equation for  
132 Brownian displacement in a three dimensional space:

$$133 \quad \langle r^2 \rangle = 6Dt$$

134 The elementary time step  $t$  was set to 10 ns and the coefficient of diffusion for glutamate  $D$  was set to  
135  $0.4 \mu\text{m}^2.\text{ms}^{-1}$ . AMPA and NMDA receptors were randomly placed in the PSD. NMDA receptors were a  
136 mix of GluN2A- and GluN2B-containing receptors (2:8 ratio) to comply with the known presence of  
137 GluN2B subunits in NTS NMDA receptors (Zhao et al., 2015). AMPA receptor activation was  
138 calculated using the kinetic scheme and rate constants for GluA2-containing receptors from Robert et  
139 al. (2005). NMDA receptor activation was calculated using the kinetic scheme 4 from Erreger et al.  
140 (2005) and temperature-adjusted rate constants from Santucci and Raghavachari (2008). Temperature  
141 correction of NMDA receptor rates was necessary to get rise and decay phases matching those obtained  
142 in recording experiments in order to perform measurements in similar conditions. Binding probabilities  
143 ( $P_{on}$ ) were calculated from association rate constants ( $k_{on}$ ) using the following formula:

144 
$$P_{on} = \frac{k_{on} dt}{0.5 N_A A_T \sqrt{2 D dt}}$$

145 where  $N_A$  is the Avogadro number,  $A_T$  is the receptor surface area set to 100 nm<sup>2</sup> and  $D$  is the diffusion  
146 coefficient for glutamate in water (see Kessler 3013, for details). The receptor surface area  $A_T$  was used  
147 to calculate both collisions of glutamate molecules with receptors and binding probabilities. Thus, it  
148 exact value had no incidence on the output of the simulation provided that it was set below an upper  
149 limit given by the inverse of the receptor density. The accuracy of binding probability calculation was  
150 verified by comparing association curves (without dissociation) obtained by Monte-Carlo methods with  
151 those obtained by solving ordinary differential equations using a very simple model consisting in a  
152 finite disk (500 nm radius, 12 nm height) populated with 1000 binding sites and 8000 homogeneously  
153 dispersed glutamate molecules. Unbinding and transition rates were converted to probabilities using the  
154 following general formula :

155 
$$P_i = k_i dt$$

156 For receptor current calculation, transmembrane potential was set to +40 mV. AMPA receptor  
157 conductance was set to 7, 14 and 20 pS for the di-, tri- and quadri-liganded states, respectively. NMDA  
158 receptor conductance was set to 50 pS.  $I_{AMPA}$  was measured at the peak of the response. Depending on  
159 the experiment,  $I_{NMDA}$  was either measured 5 ms after glutamate release or obtained by averaging  
160 current within a 5 ms duration time window (from 5 to 10 ms after release) in order to match  
161 measurements performed on recorded mEPSCs.

## 162 **RESULTS**

### 163 *Variability in mean $I_{NMDA}/I_{AMPA}$ ratio across NTS neurons.*

164 Recordings were obtained from a total sample of 43 NTS output neurons (see example in Fig. 1A),  
165 among which 20 sent projections to PBN and 23 to the CVLM. Data from the two groups of neurons  
166 were pooled after checking that there was no significant difference in main mEPSC characteristics  
167 according to the projection site (PBN vs CVLM). The frequency of mEPSCs was highly variable  
168 ranging from 0.1 to 1 Hz, depending on the neuron (median : 0.22 Hz). At +40 mV, most individual



169 mEPSCs were composite mEPSCs with both a fast and a slow component attributable to AMPA and  
170 NMDA receptor activation ( $I_{AMPA}$  and  $I_{NMDA}$ ), respectively (Aylwin et al 1997; Balland et al, 2006, 2008,  
171 Zhao et al. 2015). We verified that the slow component was suppressed by APV and thus entirely due to  
172 NMDA receptor activation (Fig. 1A). Mean  $I_{AMPA}$  ( $\mu_{AMPA}$ ) exhibited little variability between cells.  
173 Depending on the neuron, it ranged from 14 to 27 pA. On the contrary, mean  $I_{NMDA}$  ( $\mu_{NMDA}$ ) exhibited  
174 five-fold variation across neurons, ranging from 2 to 10 pA. As a consequence, mean  $I_{NMDA}/I_{AMPA}$  ratio  
175 ( $\mu_{RATIO}$ ) was also highly variable across neurons, ranging from 0.12 to 0.49 (Fig 1B).

176 ***Variability in quantal events recorded from the same NTS neuron. Fluctuations of  $I_{NMDA}/I_{AMPA}$  ratio***  
177 ***mainly result from variations of  $I_{NMDA}$***

178 To compare the variabilities of  $I_{NMDA}$ ,  $I_{AMPA}$  and  $I_{NMDA}/I_{AMPA}$  ratio across mEPSCs recorded from the same  
179 cell we calculated their respective CVs. Intra-neuronal  $I_{NMDA}/I_{AMPA}$  ratio variability was in some cases  
180 relatively high with  $CV_{RATIO}$  values up to 0.94 (range 0.22-0.94, depending on the neuron ; Fig 1C). We  
181 wondered whether this was due to fluctuations in  $I_{AMPA}$ , or  $I_{NMDA}$  or both. Whatever the neuron,  $CV_{I_{AMPA}}$   
182 was low ranging from 0.17 to 0.40, indicating little fluctuation from one quantal event to the other (Fig.  
183 1C).  $I_{NMDA}$  was far more variable with  $CV_{I_{NMDA}}$  being up to 0.93 and less than 0.4 for 1 neuron only (Fig.  
184 1C). We concluded that fluctuations in  $I_{NMDA}/I_{AMPA}$  ratio across mEPSCs recorded from the same cell  
185 originated from variations in  $I_{NMDA}$  rather than variations in  $I_{AMPA}$ . This finding was confirmed by  
186 regression analysis (coefficients of determination : 0.79 versus 0.02, respectively; see Fig. 1D,E). We  
187 wondered whether high intra-neuronal variability of  $I_{NMDA}$  as compared to  $I_{AMPA}$  resulted from  
188 differences in receptor channel properties or from stronger variations in NMDA than AMPA receptor  
189 content across synapses from the same target cell. To answer this question, we tried to estimate the  
190 contribution of stochastic factors to  $I_{NMDA}$  and  $I_{AMPA}$  variabilities.

191 ***Stochastic factors of  $I_{NMDA}$  variability***

192 Two main stochastic factors may contribute to receptor current variability across mEPSCs: random  
193 transitions between receptor channel closed and open states (channel noise) and fluctuations in quantal  
194 glutamate release. To estimate  $I_{NMDA}$  variability resulting from random receptor channel closing and  
195 opening, we first calculated channel noise variance according to the binomial distribution. Indeed, if  
196 variations of  $I_{NMDA}$  across mEPSCs were exclusively due to this factor (i.e. no variation in receptor

197 number, no variation in neurotransmitter quantum size, instantaneous equilibrium of glutamate  
198 concentrations within the cleft), then  $I_{\text{NMDA}}$  would follow a binomial distribution and the resulting  
199 variance  $\sigma^2_{\text{CN}}$  should be equal to (Sigworth, 1980 ; Robinson et al., 1991):

$$200 \quad \sigma^2_{\text{CN}} = i \cdot \mu_{\text{INMDA}} - \frac{\mu_{\text{INMDA}}^2}{N} \quad (1)$$

201 where  $N$  is the number of NMDA receptors and  $i$  the unitary receptor current. Since  $I_{\text{NMDA}}$  is the product  
202 of the unitary receptor current  $i$  by the number of open channels  $NP_{op}$  ( $P_{op}$  being the average open  
203 probability of NMDA receptors in the synapse), equation 1 may be linearized as follows:

$$204 \quad \sigma^2_{\text{CN}} = \mu_{\text{INMDA}} [i(1 - P_{op})] \quad (2)$$

205 In our experiments, the driving force was set + 40 mV. Thus,  $i$  was estimated to be about 2 pA (50 pS  
206 unitary conductance for GluN2B-containing NMDA receptors, see Traynelis et al., 2010). Assuming a  
207 realistic  $P_{op}$  value of 0.1 (Kessler, 2013), we compared  $I_{\text{NMDA}}$  variances ( $\sigma^2_{\text{INMDA}}$ ) obtained from recorded  
208 neurons with the  $\sigma^2_{\text{CN}}$  curve calculated from equation 2 (Fig. 2A). It should be kept in mind that  $\sigma^2_{\text{INMDA}}$   
209 values are likely to have been underestimated since averaging  $I_{\text{NMDA}}$  measurements over 5 ms duration  
210 time-window may have resulted in some smoothing of inter-event fluctuations (see methods).  
211 Nevertheless, this comparison suggests that a large part of  $I_{\text{NMDA}}$  variability across mEPSCS was  
212 accounted for by channel noise.

213 Equation 1 relies on the assumption that every NMDA receptor channel in a synapse has the same  $P_{op}$ .  
214 This may not be the case since glutamate concentrations decline with distance to the release site. We  
215 thus tried to obtain estimates of  $\sigma^2_{\text{CN}}$  that take into account possible differences in  $P_{op}$  between receptors  
216 according to their location relative to the release site. This was done by computer simulation using a  
217 Monte Carlo model of a glutamatergic synapse. Simulation was performed in 10 series of 50 runs each,  
218 each run representing a different quantal event. The amount of glutamate released was held constant  
219 (3000 molecules) throughout runs and series. The number of NMDA receptors in the synapse was  
220 adjusted between series (from 10 to 100) in order to span the entire range of mean  $I_{\text{NMDA}}$  values obtained  
221 from recorded neurons.  $I_{\text{NMDA}}$  values were measured 5 ms after onset. We compared  $\sigma^2_{\text{INMDA}}$  obtained by  
222 simulation with the  $\sigma^2_{\text{CN}}$  curve calculated from equation 2 using the average  $P_{op}$  value of NMDA

223 receptors in simulated data (0.07). The fit between the theoretical curve and the simulated data was  
224 nearly perfect (Fig. 2B) indicating that binomial distribution based on an averaged  $P_{op}$  provides an  
225 accurate description of the stochastic behavior of synaptic NMDA receptors.

226 We next used computer simulation to get estimate of  $I_{NMDA}$  variability resulting from fluctuations in  
227 glutamate release. Simulation was performed using a randomly determined amount of glutamate  
228 released for each run (see methods). The within-series average was close to 3000 glutamate molecules  
229 with either a low ( $CV_{Glu}$  ranging from 0.26 to 0.31, depending on the series) or a high ( $CV_{Glu}$  ranging  
230 from 0.50 to 0.62, depending on the series) variability. Surprisingly, we found little difference between  
231  $\sigma^2_{NMDA}$  values obtained using either a constant or a randomly varying amount of glutamate release (Fig.  
232 2C,D) suggesting that the part of  $I_{NMDA}$  variability resulting from release fluctuations is small as  
233 compared to that resulting from channel noise. This finding may seem at odds with the current view  
234 which states that channel noise minimally contribute to quantal current variability. This view was  
235 mainly based on studies dealing with  $I_{AMPA}$  variability (see for instance Franks et al., 2002 ; 2003). We  
236 therefore compared the stochastic behavior of AMPA and NMDA receptors placed in identical  
237 conditions.

### 238 ***Comparison between $I_{NMDA}$ and $I_{AMPA}$ stochastic behavior***

239 Simulation was performed with 100 NMDA receptors and 100 AMPA receptors in the PSD. A first  
240 series was obtained with a constant amount of glutamate release throughout runs. Subsequent series  
241 were obtained with randomly determined numbers of glutamate molecules released (series average  $\approx$   
242 3000), using parameters adjusted in order to obtain low, moderate or high release variability ( $CV_{Glu}$ : 0.3,  
243 0.54 and 0.62, respectively). To allow comparison between  $I_{NMDA}$  and  $I_{AMPA}$ , variances were converted  
244 into CVs. We found that contrary to  $CV_{NMDA}$ ,  $CV_{I_{AMPA}}$  was very low using constant release and steeply  
245 increased with  $CV_{Glu}$  (Fig. 3A,B). Plotting individual currents values within a series against the amount  
246 of glutamate released illustrated the different behaviors of the two receptors (Fig. 3C,D). While  $I_{AMPA}$   
247 amplitudes were strongly correlated with release (coefficient of determination : 0.77),  $I_{NMDA}$  amplitudes  
248 were only loosely correlated with glutamate molecules numbers (coefficient of determination : 0.21). A  
249 first factor that may explain this difference is the fact that AMPA receptors had an higher average open  
250 probability than NMDA receptors. In addition, AMPA receptors have subconductance states that depend

251 on the number of bound glutamate molecules (Traynelis et al. 2010). It should be kept in mind that  
 252 equation 2 derives from the binomial distribution and applies to channels that exist in conducting and  
 253 non-conducting states only, a more complex mathematical description being required for channels with  
 254 subconductance states, (see Neher and Stevens, 1977). Accordingly, we showed that removing the  
 255 partially-conducting states (i.e., the di and tri-liganded states) in the AMPA receptor scheme increased  
 256  $I_{AMPA}$  variability to levels expected from equation 2, indicating that the presence of subconductance  
 257 states decreases channel noise (Fig. 3E). Noise reduction by subconductance states was substantial as  
 258 shown by the two-third decrease in variance. Taken as a whole these data point out the fact that,  
 259 contrary to  $I_{NMDA}$  variability which mainly results from receptor noise,  $I_{AMPA}$  variability at a single  
 260 synapse tightly reflects fluctuations in glutamate release. These differences between AMPA and NMDA  
 261 receptor behaviors may have contributed to the variability of the  $I_{NMDA}/I_{AMPA}$  ratio across mEPSCs  
 262 recorded from the same cell.

### 263 *The origin of of $I_{NMDA}/I_{AMPA}$ ratio variability in quantal events recorded from NTS neuron*

264 We next wondered what would be  $I_{NMDA}/I_{AMPA}$  ratio variability if there were no difference in  
 265 NMDA/AMPA receptor proportions between synapses onto the same target cell. We estimated  $\sigma^2_{Ratio}$  by  
 266 using first the order Taylor expansion (van Kempen and van Vliet, 2000):

$$267 \quad \sigma^2_{Ratio} \approx \frac{\mu_{INMDA}^2}{\mu_{IAMPA}^2} \left[ \frac{\sigma_{INMDA}^2}{\mu_{INMDA}^2} + \frac{\sigma_{IAMPA}^2}{\mu_{IAMPA}^2} - \frac{2\rho_{(INMDA, IAMPA)}\sigma_{INMDA}\sigma_{IAMPA}}{\mu_{NMDA}\mu_{IAMPA}} \right] \quad (3)$$

268 where  $\rho_{(INMDA, IAMPA)}$  is the correlation coefficient between  $I_{NMDA}$  and  $I_{AMPA}$ . Since  $I_{NMDA}/I_{AMPA}$  ratio  
 269 variability was primarily due to variations in  $I_{NMDA}$  (see Fig. 1E), we reasoned that receptor ratio  
 270 heterogeneity across synapses, if present, would primarily result in increased  $I_{NMDA}$  fluctuation. Thus, to  
 271 eliminate potential effects of synapses heterogeneity, we replaced  $\sigma^2_{INMDA}$  and  $\sigma_{INMDA}$  in equation 3 by  $\sigma^2_{CN}$   
 272 and  $\sigma_{CN}$  values obtained from equation 2 :

$$273 \quad \sigma^2_{Ratio} \approx \frac{\mu_{INMDA}^2}{\mu_{IAMPA}^2} \left[ \frac{i(1-P_{op})}{\mu_{INMDA}} + \frac{\sigma_{IAMPA}^2}{\mu_{IAMPA}^2} - \frac{2\sqrt{i(1-P_{op})}\rho_{(INMDA, IAMPA)}\sigma_{IAMPA}}{\sqrt{\mu_{NMDA}\mu_{IAMPA}}} \right] \quad (4)$$

274 Calculation was performed for each neuron using the experimentally obtained values for  $\mu_{INMDA}$ ,  $\mu_{IAMPA}$ ,  
 275  $\sigma^2_{IAMPA}$  and  $\rho_{(INMDA, IAMPA)}$ . Comparing  $\sigma^2_{Ratio}$  values directly obtained from recorded data and those

275 recalculated from equation 4 showed that the observed  $I_{\text{NMDA}}/I_{\text{AMPA}}$  ratio variability was largely  
276 attributable to stochastic factors (Fig. 4A,B). Especially, the slope of the regression line of recorded  
277 values on calculated values was close to 1 (Fig. 4B) indicating that experimentally observed variability  
278 was on average close to that expected if  $I_{\text{NMDA}}$  fluctuations were entirely due to channel noise.

279 To confirm this finding, we performed simulation according to two scenarios, one assuming that the  
280 ratio of NMDA to AMPA receptors at synapses varies between neurons but is strictly identical across  
281 the different synapses onto the same neuron (scenario 1, Fig. 4C), the other assuming that the relative  
282 abundance of AMPA and NMDA receptors at synapses depends on the afferent pathway only and thus  
283 differs between synapses onto the same target cell (scenario 2, Fig 4E). Each neuron was simulated by a  
284 series of 50 runs, each run representing a quantal event occurring at a different synapse. The numbers of  
285 AMPA and NMDA receptors in the simulation were adjusted in order to fit the averaged quantal  
286 currents recorded in actual NTS neurons. We kept the AMPA receptor number constant (100 per  
287 synapse) across simulated neurons and we adjusted the overall number of NMDA receptors neuron by  
288 neuron in order to span the entire range of  $\mu_{\text{INMDA}}$  values found in recorded cells. Release variability was  
289 adjusted ( $CV_{\text{Glu}} \approx 0.3$ ) in order to obtain  $\sigma^2_{I_{\text{AMPA}}}$  close to those calculated for recorded neurons.  $I_{\text{NMDA}}$   
290 value was obtained by averaging current over a 5 ms time windows (see methods).

291 We then plotted  $\sigma^2_{\text{Ratio}}$  values obtained from either recorded or simulated neurons against  $\mu_{\text{INMDA}}$ .  
292 Variances measured from recorded mEPSCs were very close to values provided by simulation using  
293 scenario 1 (Fig. 4D) and far below those obtained using scenario 2 (Fig. 4F), confirming that the  
294 variability of the  $I_{\text{NMDA}}/I_{\text{AMPA}}$  ratio found in mEPSCs recorded from the same cell resulted from channel  
295 noise and fluctuations in glutamate release rather than from heterogeneity of receptor ratio across  
296 synapses.

### 297 ***Correlation between $I_{\text{NMDA}}$ and $I_{\text{AMPA}}$ across mEPSCs from the same neuron***

298 We wondered whether a similar NMDA to AMPA receptor ratio across a neuron's synapses would  
299 invariably result in a strong correlation between  $I_{\text{NMDA}}$  and  $I_{\text{AMPA}}$  in mEPSCs. Even with fully-identical  
300 receptor ratio, one may expect the correlation to vanish if NMDA channel noise is high enough. Signal  
301 to noise ratio (SNR) calculated from the binomial distribution is equal to:

$$SNR = \sqrt{\frac{N \cdot P_{op}}{[(1 - P_{op})]}}$$

302  
303 We reasoned that, since SNR increases with the square root of the receptor number  $N$ , the strength of  
304 the correlation between  $I_{NMDA}$  and  $I_{AMPA}$  should likewise increase with NMDA receptor number. We first  
305 look at quantal events simulated using scenario 1. We found a significant correlation between  $I_{NMDA}$  and  
306  $I_{AMPA}$  for most but not all simulated neurons. Furthermore, the strength of the correlation was highly  
307 variable (see examples in Fig. 5A). For the 27 neurons simulated using scenario 1, Pearson  $r$   
308 coefficients ranged from 0.14 to 0.65. As expected, correlation strength was found to linearly increase  
309 with receptor number in synapses (the only changing parameter between neurons in scenario 1) and  
310 hence with  $\mu_{INMDA}$  (Fig. 5B). We next examined recorded neurons. Most but not all (34 out of 43)  
311 exhibited significant correlation between  $I_{NMDA}$  and  $I_{AMPA}$  (see example in Fig 5C). Pearson  $r$  coefficients  
312 ranged from 0.27 to 0.89 and increased with  $\mu_{INMDA}$  (Fig 5D), consistent with the view that loose or  
313 lacking correlation resulted from high relative NMDA channel noise rather than synapses heterogeneity.

## 314 DISCUSSION

315 Here we found that quantal events recorded from the same NTS projection neuron exhibited substantial  
316 variations in  $I_{NMDA}/I_{AMPA}$  ratio. Using both a theoretical approach and numerical simulation, we showed  
317 that variability of  $I_{NMDA}/I_{AMPA}$  ratio was mostly if not entirely explainable by two factors: i) channel noise  
318 being especially strong at NMDA receptors, and ii) fluctuations in glutamate release having stronger  
319 effects on  $I_{AMPA}$  than on  $I_{NMDA}$ . These findings rule out any substantial contribution of synapse  
320 heterogeneity to the variability of  $I_{NMDA}/I_{AMPA}$  ratio. They therefore imply that the proportions of AMPA  
321 and NMDA receptors was similar, or roughly similar, across synapses onto the same target cell. In  
322 addition, we found strong differences in mean  $I_{NMDA}/I_{AMPA}$  ratio between neurons. Thus, our results  
323 support the idea that the receptor ratio in synapses is determined by the target cell rather than the  
324 afferent pathway. This conclusion is reminiscent of previous findings showing that different synapses  
325 onto the same neocortical neuron have very similar NMDA to AMPA receptor ratios (Umekiya et al.,  
326 1999; Watt et al., 2000; Myme et al., 2003; Watt et al., 2004) and raises the question of whether  
327 mechanisms exist that co-regulate AMPA and NMDA receptor expression in postsynaptic membranes.

328 As yet, AMPA and NMDA receptor trafficking are viewed as independent processes. To the best of our  
329 knowledge, there is no evidence for co-transport of AMPA and NMDA receptors through secretory or  
330 endosomal recycling pathways. Likewise, there is no data suggesting that AMPA and NMDA  
331 insertion/stabilization in postsynaptic membrane are tightly linked to each other. Alternatively, a  
332 conserved receptor ratio across synapses may be the passive consequence of structural constraints.  
333 Electron microscope studies performed in various CNS regions using either post-embedding  
334 immunogold labeling, freeze fracture replica immunolabeling or STEM tomography indicate that the  
335 number of AMPA receptors in synaptic clusters linearly scales with PSD size (Takumi et al., 1999;  
336 Racca et al., 2000; Tanaka et al., 2005; Masugi-Tokita et al., 2007; Antal et al., 2008; Shinohara et al.,  
337 2008; Dong et al., 2010; Rubio et al., 2014; Chen et al., 2015). NMDA receptor number in synaptic  
338 clusters also correlates with PSD size in several brain areas (Racca et al., 2000; Nyiri et al., 2003;  
339 Tarusawa et al., 2009; Rubio et al., 2014; but see Takumi et al., 1999; Shinohara et al., 2008; Chen et  
340 al., 2015). Thus, it may be hypothesized that the postsynaptic membrane includes finite numbers of  
341 specific potential slots for AMPA and NMDA receptors and that the number of slots for each receptor  
342 linearly scales with the PSD area. The slot hypothesis was originally proposed to explain how synapses  
343 acquire additional AMPA receptors during postsynaptic LTP (Shi et al., 2001; see also Lisman and  
344 Raghavachari, 2006; Opazo et al., 2012). It was also postulated that potential slots are not always fully  
345 filled with receptors. In this context, a possible interpretation for our data is that the degree of filling of  
346 potential NMDA slots is similar across a NTS neuron's synapses but differs between NTS neurons,  
347 presumably because of differences in readily available extrasynaptic receptors pools.

348 An unexpected finding from our simulation experiments was the fact that  $I_{\text{NMDA}}$  and  $I_{\text{AMPA}}$  fluctuations  
349 across mEPSCs originated from different sources.  $I_{\text{NMDA}}$  variability was mainly postsynaptic as it  
350 resulted from strong channel noise overwhelming the influence of release variations. On the contrary,  
351 AMPA receptor channel noise was low and  $I_{\text{AMPA}}$  variability was mainly presynaptic, tightly reflecting  
352 variations in the amount of glutamate released. The lower variability of  $I_{\text{AMPA}}$  as compared to  $I_{\text{NMDA}}$  as  
353 observed in the present study both *in vivo* and *in silico* is in line with previous results obtained by  
354 single synapse recording on hippocampal cell cultures (McAllister and Stevens, 2000). Data in Table 1  
355 from McAllister and Stevens (2000) indicate that the CV of the AMPA component across responses

356 from a single synapse ranged between 0.27 and 0.43, depending on the synapse, while the CV of the  
357 NMDA component across the same responses ranged between 0.56 and 0.82, depending on the synapse.  
358 It has been claimed previously that differences between the variability of AMPAR and NMDAR  
359 responses were due solely to unequal numbers of receptors at the synapse (Franck et al., 2002 ; 2003).  
360 This claim was based on simulations performed with simplified kinetic schemes including few receptor  
361 states (Lester and Jahr, 1992 for NMDA receptors and Jonas et al, 1993 for AMPA receptors). Here,  
362 using recently published more realistic Markov models (Roberts et al, 2005 for AMPA receptors and  
363 Erreger et al. 2005 for NMDA receptors), we unraveled an unexpected biophysical difference between  
364 the two receptors. We found that the intrinsic noise of AMPA channels is lower than that of NMDA  
365 channels partly as a consequence of AMPA receptors having subconductance states. In addition, the  
366 gradual opening of the AMPA receptor pore with the number of bound glutamate molecules provides a  
367 mechanism by which unitary receptor current increases with cleft glutamate concentration. In conclusion,  
368 our data show that AMPA receptors are endowed with specific features that reduce the variability of the  
369 early as compare to the late NMDA receptor-dependent phase of the postsynaptic response. From a  
370 functional point of view, these AMPA receptor specific features may fulfill an important role by  
371 increasing the temporal precision and the reliability of fast excitatory transmission.

372

373 Acknowledgments. We wish to thank Dr Lydia Kerkerian and Dr Francis Castets for their helpful  
374 comments on the manuscript. We also express our gratitude to Dr Boris Barbour for judicious advice in  
375 early steps of the study.



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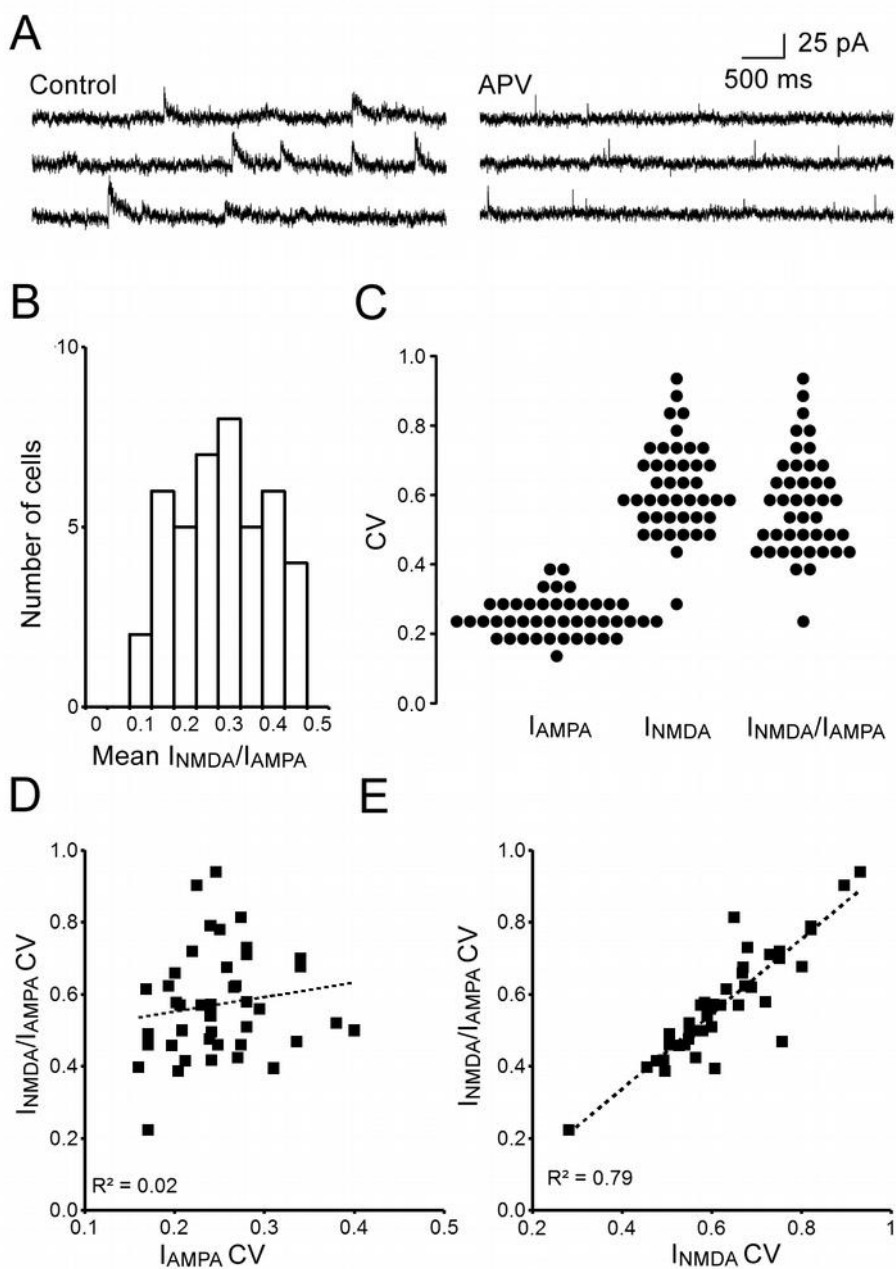
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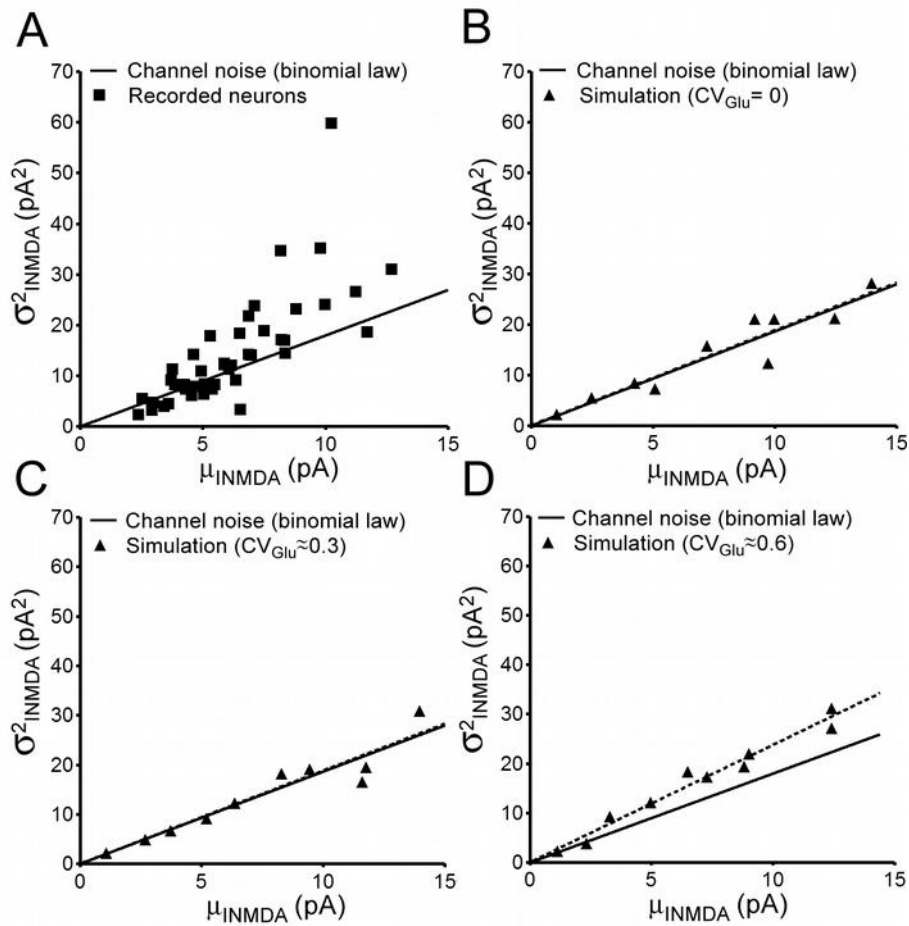
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488 **Figure Legends**



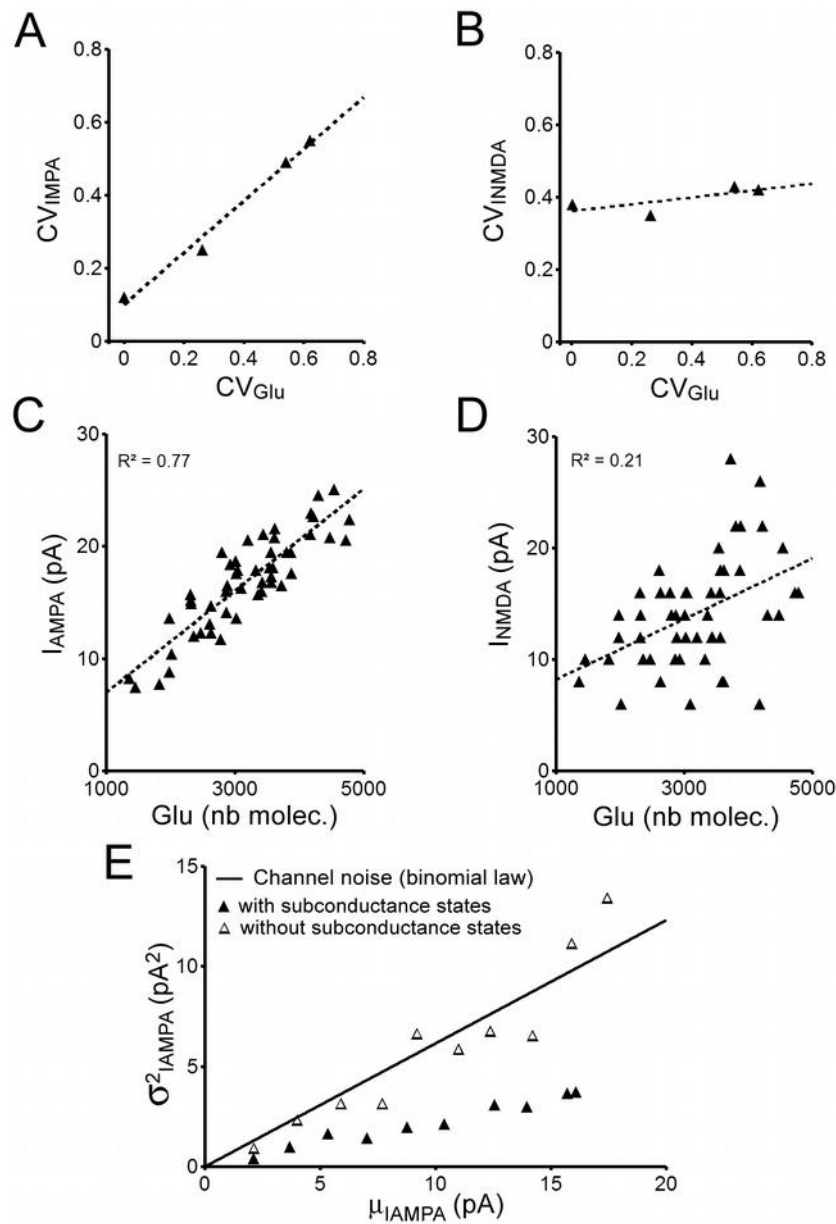
489 **Figure 1. A.** Example of recordings obtained from an NTS neuron (holding potential + 40mV). The  
490 slow component of composites mEPSC recorded in control conditions is no longer present when D,L-  
491 APV is added to perfusion medium (100  $\mu$ M) indicating that it is entirely due to NMDA receptors. **B.**  
492 Distribution histogram of mean  $I_{NMDA}/I_{AMPA}$  ratios ( $\mu_{RATIO}$ ) in mEPSCs from recorded neurons (n=43). **C.**

493 Distribution histograms of  $I_{\text{AMPA}}$ ,  $I_{\text{NMDA}}$  and  $I_{\text{NMDA}}/I_{\text{AMPA}}$  ratio coefficients of variation (CV) in mEPSCs  
494 from recorded neurons. **D.** Lack of correlation between  $I_{\text{AMPA}}$  and  $I_{\text{NMDA}}/I_{\text{AMPA}}$  ratio CVs across recorded  
495 neurons. **E.**  $I_{\text{NMDA}}/I_{\text{AMPA}}$  ratio CV in mEPSCs from recorded neurons linearly increases with  $I_{\text{NMDA}}$  CV  
496 ( $R^2$  : coefficient of determination).



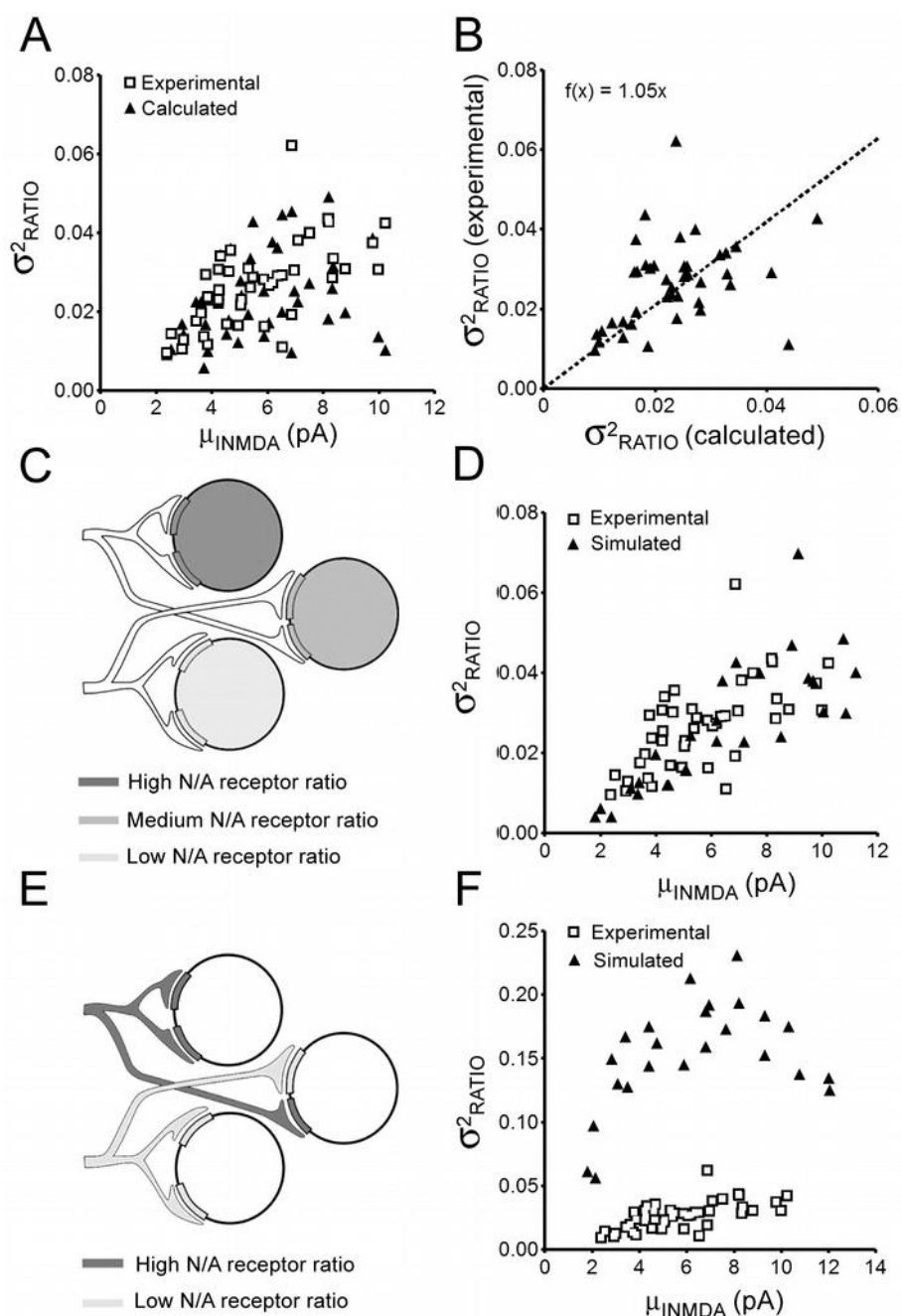
497 **Figure 2.A.**  $I_{\text{NMDA}}$  variance ( $\sigma^2_{\text{INMDA}}$ ) across mEPSCs of recorded neurons as a function of mean  $I_{\text{NMDA}}$ .  
 498 The solid line corresponds to NMDA receptor channel noise values predicted by equation 2 using a 50  
 499 pS conductance and a 0.1  $P_{\text{op}}$  value. **B.**  $I_{\text{NMDA}}$  variance in simulated mEPSCs series as a function of  
 500 mean  $I_{\text{NMDA}}$ . Each data point represents a different mEPSCs series. Data were obtained using a constant  
 501 amount of glutamate released (3000 molecules per quantal event). Note that the regression line of  
 502 simulation values (dashed line) perfectly fits with NMDA receptor channel noise values predicted by  
 503 equation 2 (solid line). **C and D.** As in B, except that the amount of glutamate released (3000  
 504 molecules per quantal event on average for each series ) was made variable from one quantal event to  
 505 the other within each simulated mEPSCs series. The coefficient of variation of glutamate released was  
 506 comprised between 0.26 and 0.31 in C and between 0.50 and 0.62 in D.





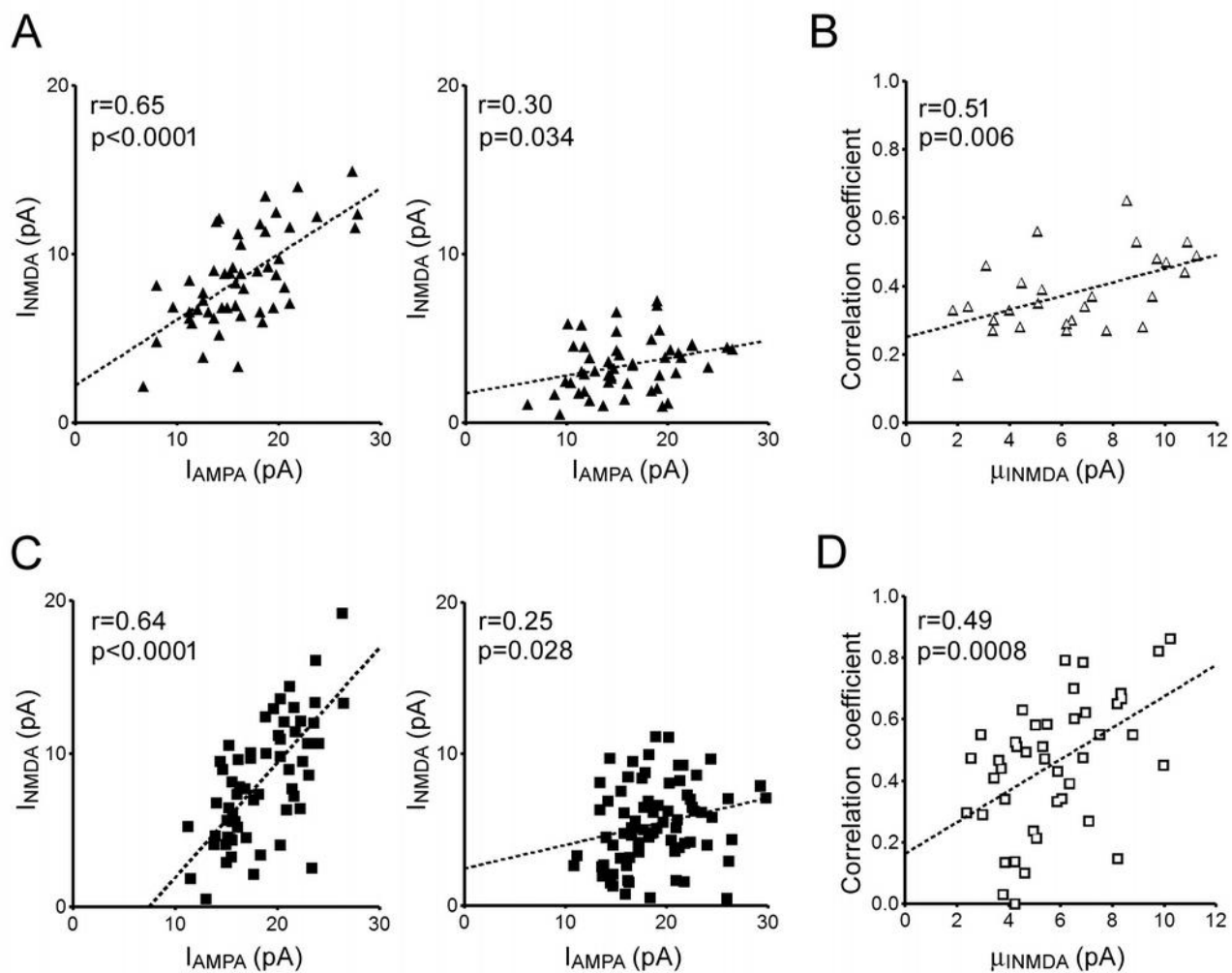
507 **Figure 3. A and B.** The influence of fluctuations in glutamate release on  $I_{AMPA}$  and  $I_{NMDA}$  variability.  
508 Each data point represents the CV of  $I_{AMPA}$  (A) or  $I_{NMDA}$  (B) within a series of simulated mEPSCs  
509 obtained with 100 AMPA receptors and 100 NMDA receptors and a fixed level of fluctuations in  
510 glutamate release. Note the strong correlation between  $CV_{I_{AMPA}}$  and  $CV_{Glu}$  and the lack of influence of  
511  $CV_{Glu}$  on  $CV_{I_{NMDA}}$ . **C and D.**  $I_{AMPA}$  (C) an  $I_{NMDA}$  (D) values in individual simulated mEPSCs plotted  
512 against the amount of glutamate release. Note that the relationship with the number of glutamate  
513 molecules released is strong for  $I_{AMPA}$  and considerably weaker for  $I_{NMDA}$ . **E.**  $I_{AMPA}$  variance in simulated

514 mEPSCs series obtained using a constant amount of glutamate released (3000 molecules per quantal  
515 event). Each data point represents a different mEPSCs series obtained using either the kinetic scheme  
516 from Robert et al. (2005) which includes a 20 pS conductance state and 7 and 14 pS subconductance  
517 states (solid triangles) or a simplified kinetic scheme including a single 20 pS conductance state (empty  
518 triangles). The solid line corresponds to AMPA receptor channel noise values predicted by equation 2  
519 using a 20 pS conductance and a 0.23  $P_{op}$  value. Note that subconductance states result in decreased  
520  $I_{AMPA}$  variance as compared to both expected channel noise and simulation values obtained using the  
521 simplified kinetic scheme.



522 **Figure 4.A**. Variance of  $I_{\text{NMDA}}/I_{\text{AMPA}}$  ratio across mEPSCs of recorded neurons as a function of mean  
 523  $I_{\text{NMDA}}$ . Note the overlap between experimental data (empty squares) and variances values recalculated  
 524 for the same neurons using equation 4 (solid triangles). **B**. Regression of experimental ratio variance  
 525 values on values recalculated using equation 4. The slope of the regression line (origin forced to 0,0) is

526 close to one indicating that  $I_{\text{NMDA}}$  contribution to ratio variability was mostly due to NMDA receptor  
527 channel noise. **C.** Schematic representation of scenario 1 assuming identical NMDA to AMPA receptor  
528 ratio across synapses onto the same target cell. Simulation was performed in 27 series (each  
529 representing a different neuron) of 50 runs (each representing a different quantal event). The number of  
530 AMPA receptor was set to 100 throughout runs and series. The number of NMDA receptors was  
531 identical across runs within a series but increased from 10 to 100 across series. **D.** Comparison between  
532 ratio variances obtained from recorded neurons (empty squares) and neurons simulated using scenario 1  
533 (solid triangles). Note the strong overlap between the two sets of data. **E.** Schematic representation of  
534 scenario 2 assuming different NMDA to AMPA receptor ratio across synapses onto the same target cell.  
535 Simulation was performed in 27 series (each representing a different neuron) of 50 runs (each  
536 representing a different quantal event). The number of AMPA receptor was set to 100 throughout runs  
537 and series. The number of NMDA receptor was either 5 or 120 depending on the run. The proportion of  
538 runs with 120 NMDA receptors increased (from 5:50 to 45:50) across series. **F.** Comparison between  
539 ratio variances obtained from recorded neurons (empty squares) and neurons simulated using scenario 2  
540 (solid triangles). Note that ratio variances obtained by simulation using different NMDA to AMPA  
541 receptor ratio across synapses onto the same target cell were much higher than those obtained  
542 experimentally.



543 **Fig. 5A.** Example of correlation between NMDA current amplitudes ( $I_{\text{NMDA}}$ ) and AMPA current  
544 amplitudes ( $I_{\text{AMPA}}$ ) across quantal events from two neurons simulated using scenario1. Note the  
545 difference in correlation strength between the two neurons. **B.** Relationship between  $\mu_{\text{INMDA}}$  and  $I_{\text{NMDA}}$ -  
546  $I_{\text{AMPA}}$  correlation strength across neurons simulated using scenario 1. **C.** Correlation between NMDA  
547 current amplitudes ( $I_{\text{NMDA}}$ ) and AMPA current amplitudes ( $I_{\text{AMPA}}$ ) across mEPSCs recorded from two  
548 NTS projection neurons. **D.** Relationship between  $\mu_{\text{INMDA}}$  and  $I_{\text{NMDA}}$ - $I_{\text{AMPA}}$  correlation strength across  
549 recorded neurons.