# Involvement of NR2A- or NR2B-containing N-methyl-D-aspartate receptors in the potentiation of cortical layer 5 pyramidal neurone inputs depends on the developmental stage.

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# Involvement of NR2A or NR2B-containing NMDA receptors in the potentiation of cortical layer 5 pyramidal neurons inputs depends on the developmental stage

Running title: NR2A or NR2B-containing NMDA receptors in LTP

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## Abstract

In the cortex, NMDA receptors (NMDARs) play a critical role in the control of synaptic plasticity processes. We have previously shown in rat visual cortex that the application of a high frequency of stimulation (HFS) protocol used to induce long-term potentiation (LTP) in layer 2/3 leads to a parallel potentiation of excitatory and inhibitory inputs received by cortical layer 5 pyramidal neurons without changing the excitation/inhibition (E/I) balance of the pyramidal neuron, indicating a homeostatic control of this parameter.

We show here that the blockade of NMDARs of the neuronal network prevents the potentiation of excitatory and inhibitory inputs and this result opens to question the role of the NMDAR isoform involved in the induction of LTP, actually being strongly debated. In P18-P23 rat cortical slices, the blockade of synaptic NR2B-containing NMDARs prevents the induction of the potentiation induced by the HFS protocol, whereas the blockade of NR2A-containing NMDARs reduced the potentiation itself. In P29-P32 cortical slices, the specific activation of NR2A-containing receptors fully ensures the potentiation of excitatory and inhibitory inputs. These results constitute the first report of a functional shift in subunit composition of NMDARs during the critical period (P12-P36) which explains the relative contribution of both NR2B- and NR2A-containing NMDARs in synaptic plasticity processes. These effects of HFS protocol are mediated by the activation of synaptic NMDARs but our results also indicate that the homeostatic control of the E/I balance is independent of NMDARs activation and is due to specialized recurrent interactions between excitatory and inhibitory networks.

# Introduction

N-methyl D-aspartate receptors (NMDARs) are among the most important receptors in the central nervous system. Largely expressed in the brain, they play a major role in glutamatergic transmission due to their unique functional properties, which include a high $Ca^{2+}$ permeability, slow activation and deactivation kinetics, and a voltage-sensitive block by external $Mg^{2+}$ (Jahr & Stevens, 1990; Lester *et al.*, 1990). As a result of these properties, NMDARs play key roles in numerous physiological and pathological brain processes such as synaptic plasticity (Choi & Rothman, 1990), schizophrenia (Coyle *et al.*, 2002; Coyle *et al.*, 2003) or epilepsy (Dingledine *et al.*, 1990).

The role of NMDARs in the two major forms of synaptic plasticity that contribute to phenomena of learning and memory, long-term potentiation (LTP) and long-term depression (LTD) has been largely determined. Modest elevations of intracellular calcium due to NMDARs activation are thought to trigger depression, whereas large increases of intracellular calcium trigger potentiation (Bear & Malenka, 1994; Yang *et al.*, 1999).

NMDARs are heterotetramers that consist of the NMDA receptor 1 (NR1) subunit which binds glycine or D-serine and one or more of the NR2 subunits: NR2A-NR2D which bind glutamate (Forrest *et al.*, 1994; Hollmann & Heinemann, 1994). While, NR3A (Ciabarra *et al.*, 1995; Sucher *et al.*, 1995) and NR3B (Chatterton *et al.*, 2002; Matsuda *et al.*, 2002) have also been recently identified, it is reported that NR2A and NR2B subunits dominate in the forebrain (Monyer *et al.*, 1994).

The functional properties of the receptor strongly depend on the type of NR2 subunit incorporated (Cull-Candy *et al.*, 2001; Cull-Candy & Leszkiewicz, 2004) which changes with age. In the cortex, the NR2B subunit is predominantly expressed early in development (Babb *et al.*, 2005) whereas expression of the NR2A subunit increases later. According to the NR2 composition, NMDARs will have different roles in synaptic plasticity, and it has been proposed that potentiation and depression are differentially mediated by NR2A and NR2B, respectively (Liu *et al.*, 2004a; Massey *et al.*, 2004). This differential effect could be related to the specific localization of NMDARs subtypes; the NR1/NR2A complex, characterized by rapid offset kinetics, would be present at the synapse, while the NR1/NR2B complex, characterized by slow kinetics, would mainly lie in extrasynaptic sites (Rumbaugh & Vicini, 1999; Tovar & Westbrook, 1999). However, recent studies suggest that NR2A and NR2B

each contribute equally to the induction of potentiation and depression (Toyoda *et al.*, 2005; Weitlauf *et al.*, 2005; Zhao *et al.*, 2005).

In the rat visual cortex it has been shown that the NR1 subunit is co-expressed with both the NR2A and NR2B subunits (Hawkins *et al.*, 1999; Tongiorgi *et al.*, 2003; Babb *et al.*, 2005). The role of NMDARs in the induction of potentiation in the visual cortex is well established (Bear & Kirkwood, 1993; Bliss & Collingridge, 1993) but which NR2 subunit(s) might be involved in this plasticity process remains controversial. We have previously shown that application of a high frequency stimulation (HFS) protocol in layer 2/3 does not change the excitatory/inhibitory (E/I) ratio due to parallel potentiation of excitatory and inhibitory inputs received by pyramidal neurons (Le Roux *et al.*, 2006). Here, we investigate the role of NMDARs in the induction of the potentiation of excitatory and inhibitory inputs of layer 5 pyramidal neurons after application of an HFS protocol in layer 2/3 in rat visual cortex (between P18 and P23 or between P29 and P32). We find that for young rats (between P18 and P32) the potentiation of excitatory and inhibitory inputs by an HFS protocol mainly depends on the activation of NR2B-containing receptors, whereas for P29-P32 old rats this plasticity becomes dependent on NR2A-containing receptors. We show that the prevention of potentiating effects on excitation and inhibition by blockade of NMDARs did not change the E/I ratio. Our results bring direct evidence of a functional shift in subunit composition of NMDARs during the critical period (P12-P36) which would explain the relative contribution of both NR2B- and NR2A-containing NMDARs in synaptic plasticity processes.

## Materials and Methods

### Slice preparation

Parasagittal slices containing primary visual cortex were obtained from P18-P23 or P29-P32 day-old Wistar rats. Briefly, in accordance with the guidelines of the American Neuroscience Association, a rat was decapitated, its brain quickly removed and placed in chilled (5°C) artificial cerebrospinal extracellular solution. Slices of 250 µm thickness were made using a vibratome from the primary visual cortex and then incubated for at least 1 hr at 36°C in a solution containing (in mM) : 126 NaCl, 26 $NaHCO_3$, 10 Glucose, 2 $CaCl_2$, 1.5 KCl, 1.5 $MgSO_4$ and 1.25 $KH_2PO_4$ (pH 7.5, 310-330 mOsm). This solution was bubbled continuously with a mixture of 95 % $O_2$ and 5 % $CO_2$.

### Electrophysiological recordings and cell identification

Current-clamp and voltage-clamp recordings were performed using an Axopatch 1D (Axon Instruments, USA); filtered by a low-pass Bessel filter with a cut-off frequency set at 2 kHz, and digitally sampled at 4 kHz. Layer 5 pyramidal neurons, identified from the shape of their soma and primary dendrites and from their current-induced excitability pattern, were studied using the patch-clamp technique in whole-cell configuration. Somatic whole-cell recordings were performed at room temperature using borosilicate glass pipettes (of 3-5 MΩ resistance in the bath) filled with a solution containing (in mM) : 140 K-gluconate, 10 HEPES, 4 ATP, 2 $MgCl_2$, 0.4 GTP, 0.5 EGTA (pH 7.3 adjusted with KOH, 270-290 mOsm). The membrane potential was corrected off-line by –10 mV to account for the junction potential. Estimation of the access resistance (Rs) is critical to quantitatively evaluate the relative change of input conductance in response to synaptic activation. After capacitance neutralisation, bridge balancing was done on-line in current-clamp conditions, which provided us with an initial estimate of Rs. This value was checked and revised as necessary off-line by fitting the voltage response to a hyperpolarizing current pulse with the sum of two exponentials. Under voltage-clamp conditions, the holding potential was corrected off-line using this Rs value. Only cells with a resting membrane potential more negative than -55 mV and recordings with an access resistance lower than 25 MΩ were kept for further analysis. The firing behavior of neurons was determined in response to depolarizing current pulses ranging from –100 to 200 pA under current-clamp conditions.

The stimulating electrodes were positioned in layer 2-3. Electrical stimulations (1-10 μA, 0.2 ms duration) were delivered in these layers using 1 MΩ impedance bipolar tungsten electrodes (TST33A10KT, WPI). The intensity of the stimulation was adjusted in current-clamp conditions to induce a subthreshold postsynaptic response due to coactivation of excitatory and inhibitory circuits. Under voltage-clamp conditions, the frequency of stimulation was 0.05 Hz and five to eight trials were repeated for a given holding potential.

A control recording was made after 15 min of patch-clamp equilibration at 0.05 Hz, and then drugs were continuously superfused during 20 min, a "drug recording" was made in the same conditions. We next applied an HFS (high frequency of stimulation) protocol in order to induce long term modifications of synaptic strength in the recruited circuits. The HFS protocol was elicited with theta burst stimulation known to induce long term potentiation (LTP) at the synaptic level. It consists of 3 trains of 13 bursts applied at a frequency of 5 Hz, each burst containing 4 pulses at 100 Hz (Abraham & Huggett, 1997). Recordings were made at 0.05 Hz after 15, 30, 45 and 60 min application of HFS protocol. A LFS (low frequency of stimulation) protocol was also used, it consists of a 1 Hz stimulation applied during 15 min.

**Drugs**

D-AP5, Ro-256981 were purchased from Sigma (St-Louis, Missouri), NVP-AAM077 was a gift from Dr. Y.P. Auberson (Novartis Institutes for BioMedical Research, Basel Switzerland) and CP 101,606 was a gift from Pfizer (Groton, Connecticut, USA). Drugs were dissolved in the perfusate for at least 15 min before recordings.

**Synaptic response analysis**

Data were analyzed off-line with specialized software (Acquis1$^{TM}$ and Elphy$^{TM}$: written by Gérard Sadoc, Biologic UNIC–CNRS, France). The method is based on the continuous measurement of conductance dynamics during stimulus-evoked synaptic responses, as primarily described *in vivo* on cat cortex (Borg-Graham *et al.*, 1998; Monier *et al.*, 2003) and recently validated on rat visual cortex (Le Roux *et al.*, 2006). This method has received further validation on other experimental models (Shu *et al.*, 2003; Wehr & Zador, 2003; Higley & Contreras, 2006; Le Roux *et al.*, 2006). Evoked synaptic currents were measured and averaged at a given holding potential. In I-V curves for each delay (t), the value of the holding potential (Vh) was corrected (Vhc) from the ohmic drop due to the leakage current

through Rs, by the equation: Vhc(t) = Vh(t) – I(t) x Rs. An average estimate of the input conductance waveform of the cell was calculated from the best linear fit (mean least square criterion) of the I-V curve for each delay (t) following the stimulation onset. Only cells showing a Pearson correlation coefficient for the I-V linear regression higher than 0.95 between –90 and –40 mV were considered in order to calculate the conductance change of the recorded pyramidal neuron from the slope of the linear regression.

The evoked synaptic conductance term (gT(t)) was derived from the input conductance by subtracting the resting conductance ($g_{rest}$) estimated 90 ms before the electrical stimulation. The apparent reversal potential of the synaptic current at the soma (Esyn(t)) was taken as the voltage abscissa of the intersection point between the I-V curve obtained at a given time (t) and that determined at rest. Assuming that the evoked somatic conductance change reflects the composite synaptic input reaching the soma, Esyn(t) characterizes the stimulation-locked dynamics of the balance between excitation and inhibition.

**Decomposition of the synaptic conductance**

To decompose the global evoked synaptic conductance (gT(t)) into excitatory and inhibitory components (gE(t) and gI(t)), we used the following simplifications: Isyn(t) = gE(t) (Esyn(t) – Eexc) + gI(t) (Esyn(t) – Einh) and gT(t) = gE(t) + gI(t) where Isyn(t) is the total synaptic current, Esyn(t) is the apparent reversal potential at the soma (see the previous paragraph), gE(t) and gI(t) are excitatory and inhibitory conductances respectively and Eexc and Einh are the reversal potentials for excitation and inhibition. Values of these reversal potentials were equal to 0 mV for excitation (Eexc) and to -80 mV for inhibition (Einh). The value of -80 mV used in the decomposition method is the reversal potential of $GABA_A$ (and not an intermediate value between $GABA_A$ and $GABA_B$) because in the presence of QX314 (which blocks $K^+$ efflux), no variation of the synaptic response was observed.

We showed that the I-V curve in the presence of excitatory synaptic transmission blockers (CNQX, D-AP5) is linear between -80 to +10 mV with a reversal potential equal to -80 mV (Le Roux *et al.*, 2006; supplementary material). In the presence of bicuculline, which blocks the inhibitory inputs on the layer 5 pyramidal neuron, the I/V curve for excitation is linear between -80 to +10 mV with a reversal potential equal to 0 mV (data not shown) as already shown by other studies (Wehr & Zador, 2003; Higley & Contreras, 2006). Under our experimental conditions of stimulation of cortical layers leading to subthreshold postsynaptic

responses, Esyn(t) which was extrapolated from I-V curves took any intermediate values between -80 mV and -40 mV (Le Roux *et al.*, 2006; supplementary material) *i.e.* within the limits of our voltage excursion (-90 to -40 mV) corresponding to the linearity of I-V curves and between the respective values of Einh and Eexc in such a way that the mathematical conditions of the oversimplification used to calculate gI(t) and gE(t) were fulfilled.

Like all somatic recordings, our recordings cannot make rigorous estimates of synaptic events in the distal dendrites and the conductance estimates are ratios of the overall excitatory and inhibitory drive contained in the local stimulated network (Haider *et al.*, 2006). However, our measurements give relative changes in conductance magnitude which reflect the cumulative contributions of excitation and inhibition arriving at proximal portions of the neuron. These relative conductance changes at the somatic level is precisely the result of synaptic dendritic integration that we wish to record at the somatic level because it determines the output signal from the neuron (Wehr & Zador, 2003; Higley & Contreras, 2006).

To quantify the synaptic conductance changes we calculate the integral (int) over a time window of 200 ms. The contribution of each component was expressed by the ratio of its integral value (intgE or intgI) to that of total conductance change (intgT).

**Statistical analysis**

Data are expressed as the mean ± the standard deviation of the mean (SD) of n cells. Paired samples for IntgT, IntgE, IntgI, %E or %I between the control condition (before HFS) and a given time after HFS (15, 30, 45 or 60 min) were analysed using the parametrical *t*-test. Data were considered statistically significant for $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

## Results

### *Involvement of NMDARs in the potentiation of excitatory and inhibitory inputs on cortical layer 5 pyramidal neurons induced by HFS protocol application in layer 2/3*

Figure 1A-C presents representative current recordings in a layer 5 voltage-clamped pyramidal neuron to electrical stimulation (0.05 Hz) in layer 2/3. Current responses show a strong inward current followed by an outward component (Fig. 1A). Decomposition of these current responses into distinct conductance changes showed that, as already reported (Le Roux *et al.*, 2006), inhibition (gI) represents roughly 80 % (light grey trace) and excitation (gE) 20 % (dark grey trace) of the global conductance change (black trace, Fig. 1A1). Application of 50 µM D-AP5, a specific antagonist of NMDA receptors (Yoshimura *et al.*, 2003; Huemmeke *et al.*, 2004), for 20 min did not induce significant differences in the global, excitatory and inhibitory conductance changes (Fig. 1A2). These results clearly show that the excitatory and inhibitory components recorded in a layer 5 pyramidal neuron in response to a low frequency of stimulation (0.05 Hz) of layer 2/3 does not depend on activation of NMDA receptors but corresponds to the activation of AMPA and $GABA_A$ receptors mainly located on the apical dendrite of the layer 5 pyramidal neuron.

In the brain, information is encoded as action potential trains of higher frequency than that used in our protocol (0.05 Hz). We applied an HFS protocol with a higher frequency (see methods), usually used to induce LTP in numerous preparations. Application of this HFS protocol in layer 2/3 induced a long term potentiation of both excitatory and inhibitory inputs on layer 5 pyramidal neurons. Figure 1B shows representative current recordings in a voltage clamp pyramidal neuron, under control condition (Fig. 1B1) and 15 minutes after application of an HFS protocol (Fig. 1B2). HFS application elicits enhanced current responses and increases conductance changes. The statistical analysis of the global population (n = 24) is presented on figure 1D and shows that 15, 30, 45 and 60 min after the HFS protocol, the total, excitatory and inhibitory conductance changes are potentiated by about 45, 35 and 48 %, respectively. However, no significant change in excitation (intgE) and inhibition (intgI) conductance integrals expressed as percent of intgT was measured (Fig 1D, right part) in such a way that the E/I balance was unchanged.

It is worth noting that the detected potentiation is likely related to HFS protocol since in absence of such protocol, control experiments show no significant change of IntgT, IntgE or IntgI for one hour (n = 17, *data not shown*).

In order to examine the involvement of NMDARs activation in these potentiating effects of HFS on excitatory and inhibitory inputs, HFS protocol was applied in the presence of 50 µM D-AP5. No significant increase of current responses and conductance changes was measured (Fig. 1C1 and 1C2). No variation of the total conductance change and of inhibitory or excitatory conductance changes was observed through the statistical analysis of the total neuronal population (n = 15; Fig. 1E). Under this condition, the HFS protocol failed to induce potentiation of excitatory and inhibitory inputs. No significant changes in excitation (intgE) and inhibition (intgI) conductance integrals expressed as percent of intgT was measured (Fig. 1E) and the E/I ratio did not change. These results indicate that NMDARs are recruited by high frequency of stimulation applied in layer 2/3.

***Involvement of NR2A and/or NR2B-containing receptors in the potentiation of excitatory and inhibitory inputs of cortical layer 5 pyramidal neurons induced by HFS protocol application in layer 2/3***

The involvement of NR2A and/or NR2B-containing receptors in synaptic plasticity is presently strongly debated (Liu *et al.*, 2004b; Massey *et al.*, 2004; Berberich *et al.*, 2005; Weitlauf *et al.*, 2005). To further characterise whether NMDA currents in layer 2/3 pyramidal neurons were due to the activation of NR2A- or NR2B-containing receptors, we then used antagonists for each of these receptors.

*Role of NR2B-containing receptors in P18-P23 old rats*

Ro 25-6981 (Yang *et al.*, 2006) or CP101,606 (Traxoprodil, (Chenard *et al.*, 1995; Menniti *et al.*, 1997) were used to specifically block NR2B-containing receptors. Representative recordings in the presence of 1 µM of Ro 25-6981 before and after application of HFS protocol showed that the HFS effect was blocked by the NR2B antagonist (Fig. 2A). The statistical analysis of the global population (n = 12; Fig.2B) confirms that no significant variations of the global, excitatory and inhibitory conductance changes were observed after application of HFS protocol in layer 2/3 in the presence of Ro 25-6981. Consequently, the E/I ratio was unchanged (Fig. 2C). Identical results were obtained after application of HFS

protocol in the presence of CP101,606 (n = 18; Fig. 2D, 2E, 2F). These results reproduced those observed in the presence of D-AP5 and provide evidence for an involvement of NR2B-containing receptors in the HFS-induced potentiation of both excitatory and inhibitory inputs on layer 5 pyramidal neurons.

*Role of NR2A-containing receptors*

NVP-AAM077 (Izumi *et al.*, 2006) or $Zn^{2+}$ (Paoletti & Neyton, 2006) were used to block NR2A-containing receptors. Representative recordings in the presence of 0.1 μM of NVP-AAM077, before and after application of HFS protocol showed an increase in the amplitude of current recordings (Fig. 3A, upper traces). Decomposition in conductance showed an increase of total, excitatory and inhibitory conductance changes (Fig. 3A, middle and lower traces). The statistical analysis of the global population (n = 13; Fig 3B) indicated that application of HFS protocol in the presence of NVP-AAM077 still induced a significant potentiation of the global and inhibitory conductance changes for 45 min. IntgT was enhanced by 26 % ($p < 0.01$), 21 % ($p < 0.05$) and 34 % ($p < 0.05$), 15, 30 and 45 min respectively after HFS protocol. IntgI was significantly enhanced in the same proportions but IntgE was not significantly increased ($p > 0.05$). In any case, no potentiating effect remains 60 min after HFS protocol. Consequently, the E/I ratio was decreased ($p < 0.01$, Fig. 3C) 15 min after HFS protocol but recovered its control value 30, 45 and 60 min after HFS protocol.

$ZnCl_2$ was also used to block selectively NR2A-containing receptors because low concentrations of $Zn^{2+}$ block NR2A receptors more than NR2B receptors (Amar *et al.*, 2001; Kohr, 2006; Neyton & Paoletti, 2006). As observed for NVP-AAM077, similar results were obtained in the presence of 200 nM of $ZnCl_2$, with excitatory and inhibitory conductance changes all increased (Fig. 3D). This result was confirmed by the statistical analysis of the global population (n = 13; Fig. 3E), which showed that HFS application in the presence of $Zn^{2+}$ leads to a mean increase by 26 %, 18 % and 30 % for the total, excitatory and inhibitory conductance changes, respectively. In this case, a slight but not significant ($p > 0.05$) decrease of the E/I ratio was observed (Fig. 3F). In the presence of NR2A-containing receptors blockers, the potentiating effects of the HFS protocol on layer 5 pyramidal neurons still occurred but were reduced.

***Involvement of extrasynaptic NR2B-containing receptors in potentiation of excitatory and inhibitory inputs on layer 5 pyramidal neurons***

NR2B receptors are known to be present in both synaptic and extrasynaptic domains at glutamatergic synapses (Cull-Candy *et al.*, 2001). To check the involvement of synaptic and/or extrasynaptic NR2B-containing receptors in HFS induced-potentiation of excitatory and inhibitory inputs on layer 5 pyramidal neurons, synaptic NMDARs were blocked according to the protocol described by Massey *et al.* (2004) using MK-801, an antagonist of NMDARs that acts only on open channels. MK-801 (10 μM) was bath-applied and a low frequency stimulation protocol (LFS) was delivered in order to activate synaptic NMDARs which can then be blocked whereas extrasynaptic receptors are not activated by this protocol of stimulation. MK-801 was then washed out for 30 min and HFS protocol of stimulation was applied in layer 2/3.

To start, a control condition (omitting MK-801) was used in order to check that application of LFS protocol does not prevent the potentiation induced by the HFS protocol. Significant potentiations were observed for IntgT (by 39 %, $p < 0.05$), for IntgI (by 42 %, $p < 0.05$) and for IntgE (by 22 %, $p < 0.05$) (Fig 4A for representative recordings and Fig. 4B for the statistical analysis of the global population, $n = 14$) and the E/I ratio slightly decreases (Fig. 4C). However, the increased percentage of intgE was lower than that obtained after a direct HFS protocol. This discrepancy might be due to the prolonged depressive effects (beyond 30 min) of LFS protocols (mainly on intgE) that we have shown (Le Roux *et al.*, 2006). Nevertheless, it was possible to obtain a potentiating effect with an HFS protocol, and we could thus test the HFS protocol in the presence of MK801. In the presence of MK-801, no potentiation of excitatory and inhibitory inputs was obtained, but a slight decrease of IntgT, IntgE and IntgI was observed (Fig. 4D for representative recordings and Fig. 4E for statistical analysis of the global population, $n = 13$) and the E/I ratio remains stable (Fig. 4F), indicating that synaptic NMDARs can account for the potentiation of excitatory and inhibitory inputs of layer 5 pyramidal neurons.

***Role of NR2A and/or NR2B-containing receptors in the potentiation of excitatory and inhibitory inputs in P29-P32 old rats***

Some studies suggest that the activation of NR2A but not of NR2B induces long-term potentiation (Liu *et al.*, 2004b; Massey *et al.*, 2004) while other studies proposed that both

types of NMDARs can contribute to the induction of LTP (Berberich *et al.*, 2005; Weitlauf *et al.*, 2005; Zhao *et al.*, 2005). These discrepancies may be related to temporal regulation of NR2 subunits (Monyer *et al.*, 1994; Liu *et al.*, 2004b). The NR2A/NR2B ratio increases with maturation (Yoshimura *et al.*, 2003; Liu *et al.*, 2004c). Our results indicate that for rats between P18 and P23, synaptic NR2B-containing receptors are functional while NR2A-containing receptors may not yet be fully functional. To test this hypothesis, a new series of experiments was done on P29-P32 old rats.

*Role of NR2B-containing receptors in P29-P32 rats*

Representative recordings in the presence of 1 μM Ro 25-6981, before and after application of HFS protocol showed an increase of the amplitude of current recordings (Fig. 5A, upper traces). Decomposition in conductance changes showed an increase of total, excitatory and inhibitory conductance changes (Fig. 5A, middle and lower traces). The statistical analysis of the global population (n = 12; Fig 5B) indicated that application of HFS protocol in the presence of Ro 25-6981 still induced a significant potentiation of the global and inhibitory conductance changes for 45 min. IntgT was enhanced by 42 %, intgE by 40 % and intgI by 51 % after HFS protocol application. Due to parallel potentiation of intgE and intgI, no significant change of the E/I ratio was observed (Fig. 5C).

*Role of NR2A-containing receptors in P29-P32 rats*

Representative recordings in the presence of 0.1 μM NVP-AAM077 before and after application of HFS protocol showed no significant variation (Fig. 5D). The statistical analysis of the global population (n = 13; Fig.5E) confirms the block of the effects of the HFS protocol in the presence of the NR2A-containing receptors blocker for P29-P32 old rats. Consequently, the E/I ratio was unchanged (Fig. 5F).

## Discussion

NMDAR activation is required for the potentiation of excitatory and inhibitory inputs on layer 5 pyramidal neurons after application of an HFS protocol in layer 2/3. Our results provide evidence for a major recruitment of synaptic NR2B-containing receptors in P18-P23 old rats, relative to the involvement of NR2A-receptors. As well as their possible differential role in physiological processes such as learning and memory (Collingridge *et al.*, 2004), NR2A and NR2B-containing receptors are thought to be differentially involved in certain central disorders such as chronic pain or dementia (Kemp & McKernan, 2002; Chazot, 2004; Paoletti & Neyton, 2006). It therefore becomes important to determine the contribution and the function of distinct NMDAR subtypes. However, this physiological classification is highly dependent on the selectivity of antagonists that can be used. Ro 25-6981 (ifenprodil) and CP,101-606 (traxoprodil) which bind the N-terminal lysine/isoleucine/valine-binding protein (LIVBP)-like domain of NR2B are now considered as selective antagonists of NR2B-containing receptors (Fischer *et al.*, 1997; Berberich *et al.*, 2005; Kohr, 2006). On the contrary, the specific blockade of NR2A-containing receptors is strongly debated (Feng & Macdonald, 2004; Paoletti & Neyton, 2006). First, $Zn^{2+}$ binds the (LIVBP)-like domain of NR2A and displays a better selectivity for NR2A than for NR2B with a maximal selectivity of 70 % (Amar *et al.*, 2001; Kohr, 2006; Neyton & Paoletti, 2006). Second, NVP-AAM077 was originally described as a highly selective antagonist of NR2A-containing receptors (Auberson *et al.*, 2002; Liu *et al.*, 2004b; Massey *et al.*, 2004) but later studies on rodents indicate that this compound lacks selectivity for NR2A *vs* NR2B receptors (Berberich *et al.*, 2005; Weitlauf *et al.*, 2005; Neyton & Paoletti, 2006). Other studies reported that NVP-AAM077 presents some selectivity at concentrations lower than 1 μM (Bartlett *et al.*, 2006; Izumi *et al.*, 2006) and concluded that it was not possible to find a concentration of NVP-AAM077 that fully block the NR2A receptors while sparing NR2B. According to the work by Bartlett *et al.* (2006), we used 0.1 μM NVP-AAM077 to check the specific blockade of NR2A receptors. Indeed, whatever the NR2A antagonist used, the potentiation of excitatory and inhibitory inputs of layer 5 pyramidal neurons occurred but was reduced by about 40 % in P18-P23 old rats. This result could be due to the blockade of NR2A, but also to an unspecific blockade of NR2B subunits.

Recent studies suggest that the activation of NR2A but not NR2B induces long-term potentiation (Liu *et al.*, 2004b; Massey *et al.*, 2004) although other studies proposed that both types of NMDARs can contribute to the induction of LTP (Berberich *et al.*, 2005; Weitlauf *et al.*, 2005; Zhao *et al.*, 2005). These discrepancies could be related to the differential distribution of NMDARs subtypes. For example, an important role for extrasynaptic receptors exclusively containing the NR2B subunit (Cull-Candy *et al.*, 2001) is reported in synaptic plasticity (Massey *et al.*, 2004) or in neuronal cell death (Hardingham *et al.*, 2002; Hardingham, 2006). However, both NR2A- or NR2B-NMDAR subtypes can be located either on synaptic or extrasynaptic sites (Thomas *et al.*, 2006). Here, we show that predominantly synaptic NMDARs are implicated in the observed potentiating effects.

Another possibility to explain these discrepancies is to consider changes in the spatial distribution of NMDA receptors during the developmental stages. While NR1 subunit is ubiquitously expressed in the CNS, NR2 subunit expression is temporally regulated (Monyer *et al.*, 1994; Liu *et al.*, 2004b) with the NR2A/NR2B ratio increasing during maturation (Yoshimura *et al.*, 2003; Liu *et al.*, 2004c). In the rat visual cortex, morphological studies showed that NR1 and NR2B subunits are already expressed at birth and reach their highest level of expression during the second and third postnatal weeks (Kew *et al.*, 1998; Tongiorgi *et al.*, 2003; Babb *et al.*, 2005). In contrast, NR2A subunits are poorly expressed at birth but progressively increase as development proceeds (Kew *et al.*, 1998) and the insertion of NR2A receptors displace NR2B receptors at extrasynaptic sites (Liu *et al.*, 2004c). Our results clearly show that the potentiation initially depends on NR2B-containing receptors in P18-P23 old rats, but becomes NR2A dependent for P29-P32 old rats. Thus in order to understand the particular role of NR2A or NR2B in the potentiation process, it is crucial to consider the temporal and spatial NMDARs expression. Hence, rather than a differential role for NR2A- or NR2B-containing receptors in the induction of LTP supposed by recent works (Berberich *et al.*, 2005, Weitlauf *et al.*, 2005, Zhao *et al.*, 2005), we proposed that both types can be involved in the potentiation but their respective involvement depends on the developmental stage of the cortex.

We show that without under estimating the problem of selectivity of the NR2A or NR2B-containing receptors antagonists, it is necessary to consider the developmental stages of the animal. In addition, we cannot exclude the possibility that, for P18-P23 old rats, NR2A-containing receptors were present but not yet fully functional (not antagonist sensitive), whereas for P29-P32 old rats specific blockers were rather efficient.

The blockade of NMDA receptors prevents the potentiation of excitatory and inhibitory inputs on layer 5 pyramidal neurons to HFS in layer 2/3 resulting in an unchanged E/I ratio. These results suggest that the NMDARs activation is not required for the induction of the homeostatic control of the E/I balance but is necessary for the homeostatic plasticity expression. Only a few studies report a similar observation in which the blockade of NMDARs prevents the potentiation of inhibitory synapses (IPSCs recorded in cerebellar stellate cells (Liu & Lachamp, 2006)). It is obvious that we cannot localize facilitated synapses in the stimulated networks by HFS protocol and we observed that the stimulation of inhibitory interneurons is mainly disynaptic, involving a synapse between a glutamatergic terminal and the interneuron (Le Roux *et al.*, 2006; Supplementary materials). Thus the potentiation of inhibitory inputs following HFS protocol in layer 2/3 is likely indirect, but in any case parallel increases of the strength of excitatory and inhibitory inputs on the layer 5 pyramidal neuron were observed resulting in a close control of the E/I ratio. Indeed, normal brain function requires a delicate balance between excitation and inhibition in neuronal networks, an imbalance of excitation and inhibition underlies various neurological diseases such as epilepsy (Cossart *et al.*, 2005), Parkinson's disease (Llinas *et al.*, 1999) or schizophrenia (Wassef *et al.*, 2003). A correlation between excitatory and inhibitory neuronal responses to sensory stimuli has been found in many cortical neurons in adult mammalian sensory cortices. For example, in cat primary visual cortex, excitation and inhibition exhibit similar tuning preference and tuning width in orientation-selective neurons (Anderson *et al.*, 2000; Martinez *et al.*, 2002; Monier *et al.*, 2003; Hirsch *et al.*, 2003; Marino *et al.*, 2005). In addition, a proportionality of recurrent excitation and inhibition in cortical networks has been demonstrated to be associated with the persistent activity and is thought to underlie the working memory (Shu *et al.*, 2003). So, neuronal networks adapt to environmental changes by learning and memory, in which the strength of excitatory or inhibitory synapses are changed. We show using a protocol of stimulation classically used in long-term plasticity, that the E/I ratio can remain unchanged while the strength of excitatory and inhibitory inputs altered. The present results provide evidence for the activation of the homeostatic control of the E/I balance previously reported by Le Roux *et al.* (2006). This control is independent of the activation of NMDARs but appears linked to recurrent interactions between excitatory and inhibitory circuits through layer 2/3 and layer 5 pyramidal neurons. This cortical organization can underlie the HFS-induced potentiation of some excitatory and inhibitory synapses (and

the imprinting of some information) and may control the main output pathway of the cortex, layer 5 pyramidal neurons, through a homeostatic mechanism avoiding hyper- or hypo-excitability.

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## Abbreviations

E, excitation; GABA, gamma amino butyric acid; HFS, high frequency of stimulation; I, inhibition; intgE, excitatory conductance integral; intgI, inhibitory conductance integral; intgT, total conductance integral; LTP, long-term potentiation; NMDAR, N-methyl D-aspartate receptor.

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**Figure 1. Effects of NMDA receptor blockade on the potentiation of layer 5 pyramidal neurons inputs.**

(**A-C**) **Upper traces**: current responses of a layer 5 pyramidal neuron to electrical stimulation (black arrow) applied in layer 2/3: (**A1**) under control conditions and (**A2**) 20 min after perfusion of 50 μM D-AP5; holding potentials scaled from – 75 (bottom trace) to – 55 mV (top trace, steps equal to 5 mV). (**B1**) Under control conditions and (**B2**) 15 min after HFS protocol; holding potentials scaled from – 70 (bottom trace) to – 50 mV (top trace, steps equal to 5 mV). (**C1**) 20 min after application of 50 μM D-AP5 and (**C2**) 15 min after HFS protocol in the presence of D-AP5; holding potentials scaled from – 75 (bottom trace) to – 55 mV (top trace, steps equal to 5 mV). **Medium traces**: decomposition of the responses in total conductance change (gT). **Lower traces**: decomposition of gT in excitatory (gE, dark grey) and inhibitory (gI, light grey) conductance changes. Note that, for stimulation in layer 2/3 in the presence of D-AP5, no significant change of the response was observed. HFS protocol induced an increase of total, excitatory and inhibitory conductance changes which was prevented in the presence of D-AP5.

(**D**) Left part: relative changes (compared to control) of intgT (black), intgE (dark grey) and intgI (light grey), after application of HFS protocol in layer 2/3 under control conditions (n = 26) (*** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$, t-test). Right part: relative contribution of excitatory and inhibitory conductances to the total conductance change, after HFS (c: control before HFS protocol).

(**E**) Left part: relative changes (compared to control) of intgT (black), intgE (dark grey) and intgI (light grey), after HFS in layer 2/3 (n = 15) in the presence of 50 μM D-AP5. Right part: relative contribution of excitatory and inhibitory conductances to the total conductance change, after HFS in the presence of 50 μM D-AP5 (c: control before HFS protocol).

**Figure 2. Effects of the blockade of NR2B-containing receptors on the potentiation of layer 5 pyramidal neurons inputs for P18-P23 old rats.**

(**A**) The left column shows representative recordings before HFS application and the right column 60 min after application of HFS in layer 2/3 in the presence of 1 μM Ro 25-6981. **Upper traces**: current responses of a layer 5 pyramidal neuron to electrical stimulation. Imposed membrane potential ranged for –55 to –75 mV. The amplitude of current responses was unchanged after HFS application in the presence of Ro 25-6981. **Medium traces**: decomposition of the responses in total conductance change (gT). **Lower traces**:

decomposition of gT in excitatory and inhibitory conductance changes (gE, dark grey and gI, light grey).

(**B**) Relative changes (compared to control) of intgT (black bars), intgE (dark grey bars) and intgI (light grey bars), after HFS in layer 2/3 (n = 12) in the presence of 1 μM Ro 25-6981.

(**C**) Relative contribution of excitation and inhibition conductance changes to the total conductance change, after HFS in the presence of 1 μM Ro 25-6981 (c:control before HFS protocol).

(**D-E-F**) Application of HFS protocol in the presence of 10 μM CP101,606 (n = 18). Legends are identical to those in **A-B-C**. No variations of total, excitatory and inhibitory conductance changes were observed after HFS (**D-E**) and the E/I ratio was unchanged (**F**).

**Figure 3. Effects of the blockade of NR2A-containing receptors on the potentiation of layer 5 pyramidal neurons inputs for P18-P23 old rats.**

(**A**) The left column shows representative recordings before HFS application and the right column 60 min after application of HFS in layer 2/3 in the presence of 0.1 μM NVP-AAM077. **Upper traces**: current responses of a layer 5 pyramidal neuron to electrical stimulation. Imposed membrane potential ranged for –55 to –75 mV. The amplitudes of current responses were slightly increased after HFS application. **Medium traces**: decomposition of the responses in total conductance change (gT). **Lower traces**: decomposition of gT in excitatory and inhibitory conductance changes (gE, dark grey and gI, light grey). Total, excitatory and inhibitory conductance changes were all increased.

(**B**) Relative changes (compared to control) of intgT (black bars), intgE (dark grey bars) and intgI (light grey bars), after HFS in layer 2/3 (n = 13) in the presence of 0.1 μM NVP-AAM077. (*** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$, t-test).

(**C**) Relative contribution of excitation and inhibition conductance changes to the total conductance change, after HFS in the presence of 0.1 μM NVP-AAM077 (c:control before HFS protocol).

(**D-E-F**) Application of HFS protocol in the presence of 200 nM $ZnCl_2$ (n = 13). Legends are identical to those in **A-B-C**. (**D**) Imposed membrane potential ranged for –60 to –80 mV. (**E-F**) Total, excitatory and inhibitory conductance changes were increased after HFS.

**Figure 4. Effect of synaptic NMDARs blockade on the potentiation of layer 5 pyramidal neurons inputs.**

(**A**) Left column: representative recordings after application of LFS protocol in layer 2/3. Right column: the LFS protocol was followed by an HFS protocol and recordings were done 60 min after the HFS protocol. **Upper traces**: current responses of a layer 5 pyramidal neuron to electrical stimulation. Imposed membrane potential ranged for –60 to –80 mV. The amplitudes of current responses were increased after HFS application. **Medium traces**: decomposition of the responses in total conductance change (gT). **Lower traces**: decomposition of gT in excitatory and inhibitory conductance changes (gE, dark grey and gI, light grey). Total, excitatory and inhibitory conductance changes were all increased.

(**B**) Relative changes (compared to control) of intgT (black bars), intgE (dark grey bars) and intgI (light grey bars), after HFS in layer 2/3 (n = 14) after application of LFS protocol. (*** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$, t-test).

(**C**) Relative contribution of excitation and inhibition conductance changes to the total conductance change, after application of LFS protocol, without MK 801. (c:control before HFS protocol).

(**D-E-F**) Application of HFS after application of a LFS protocol in the presence of 10 μM MK-801 (n = 13). Legends are identical to those in **A-B-C**. No significant variations of total, excitatory and inhibitory conductance changes were observed after HFS.

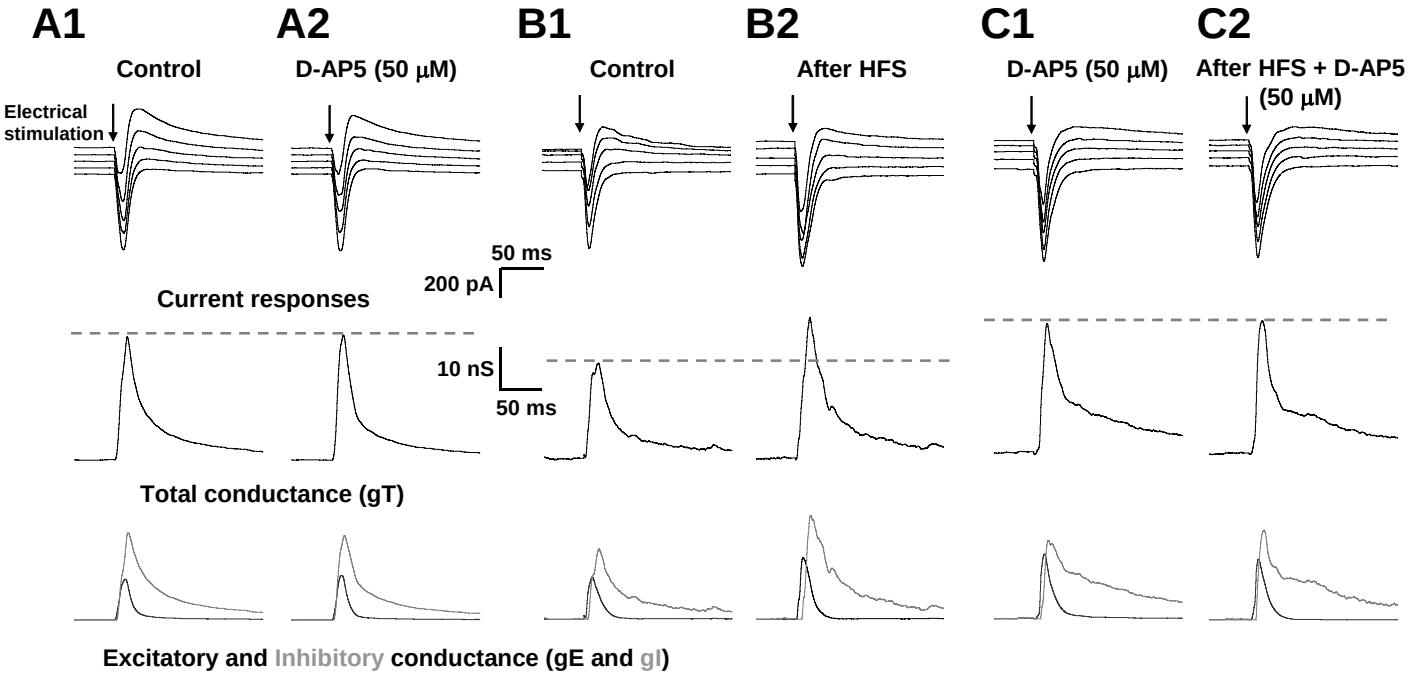
**Figure 5. Effects of the blockade of NR2B or NR2A-containing receptors on the potentiation of layer 5 pyramidal neurons inputs for P29-P32 old rats.**

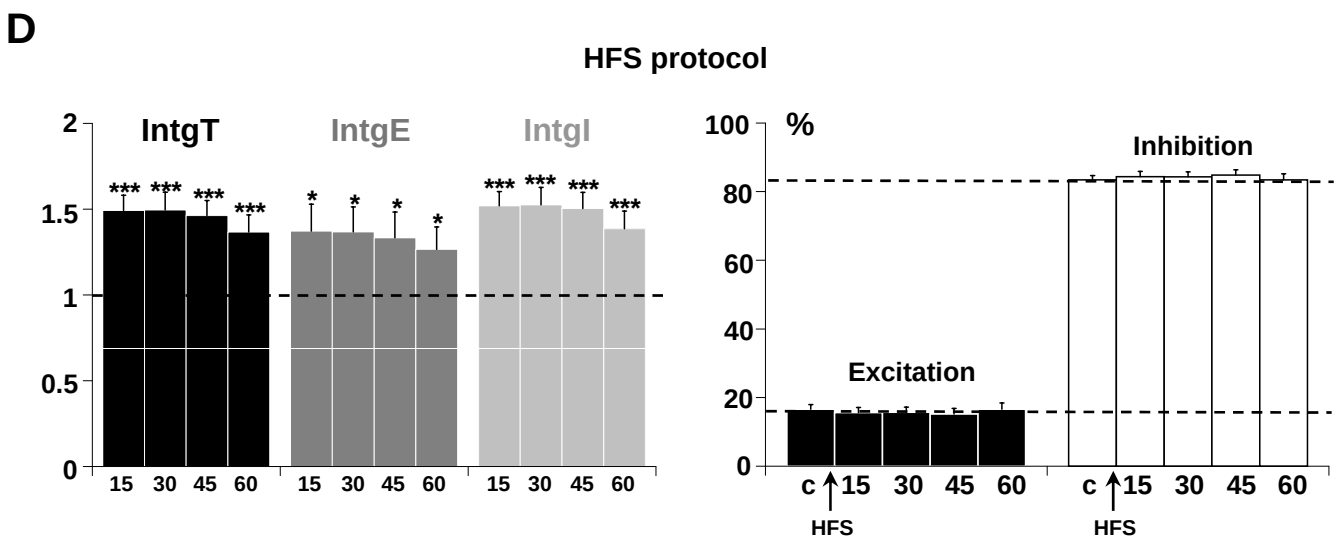
(**A**) The left column shows representative recordings from the statistical analysis of n = 12 experiments before HFS application and the right column 60 min after application of HFS in layer 2/3 in the presence of 1 μM Ro 25-6981. **Upper traces**: current responses of a layer 5 pyramidal neuron to electrical stimulation. Imposed membrane potential ranged for –55 to –75 mV. The amplitude of current responses was increased after HFS application. **Medium traces**: decomposition of the responses in total conductance change (gT). **Lower traces**: decomposition of gT in excitatory and inhibitory conductance changes (gE, dark grey and gI, light grey). Total, excitatory and inhibitory conductance changes were all increased.

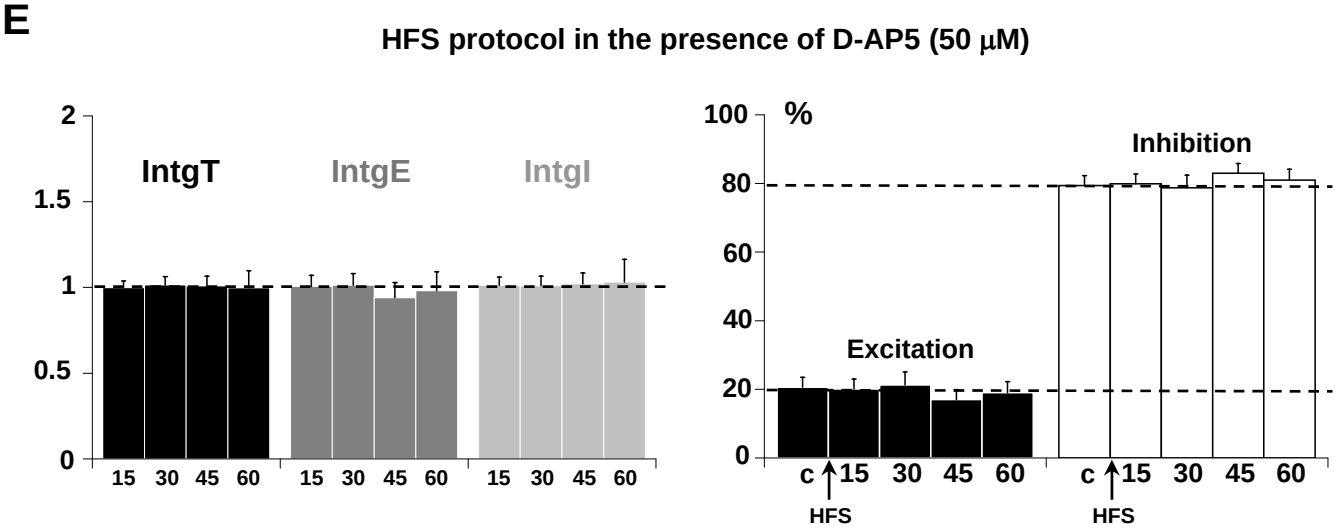
(**B**) Relative changes (compared to control) of intgT (black bars), intgE (dark grey bars) and intgI (light grey bars), after HFS in layer 2/3 (n = 12) in the presence of 1 μM Ro 25-6981.

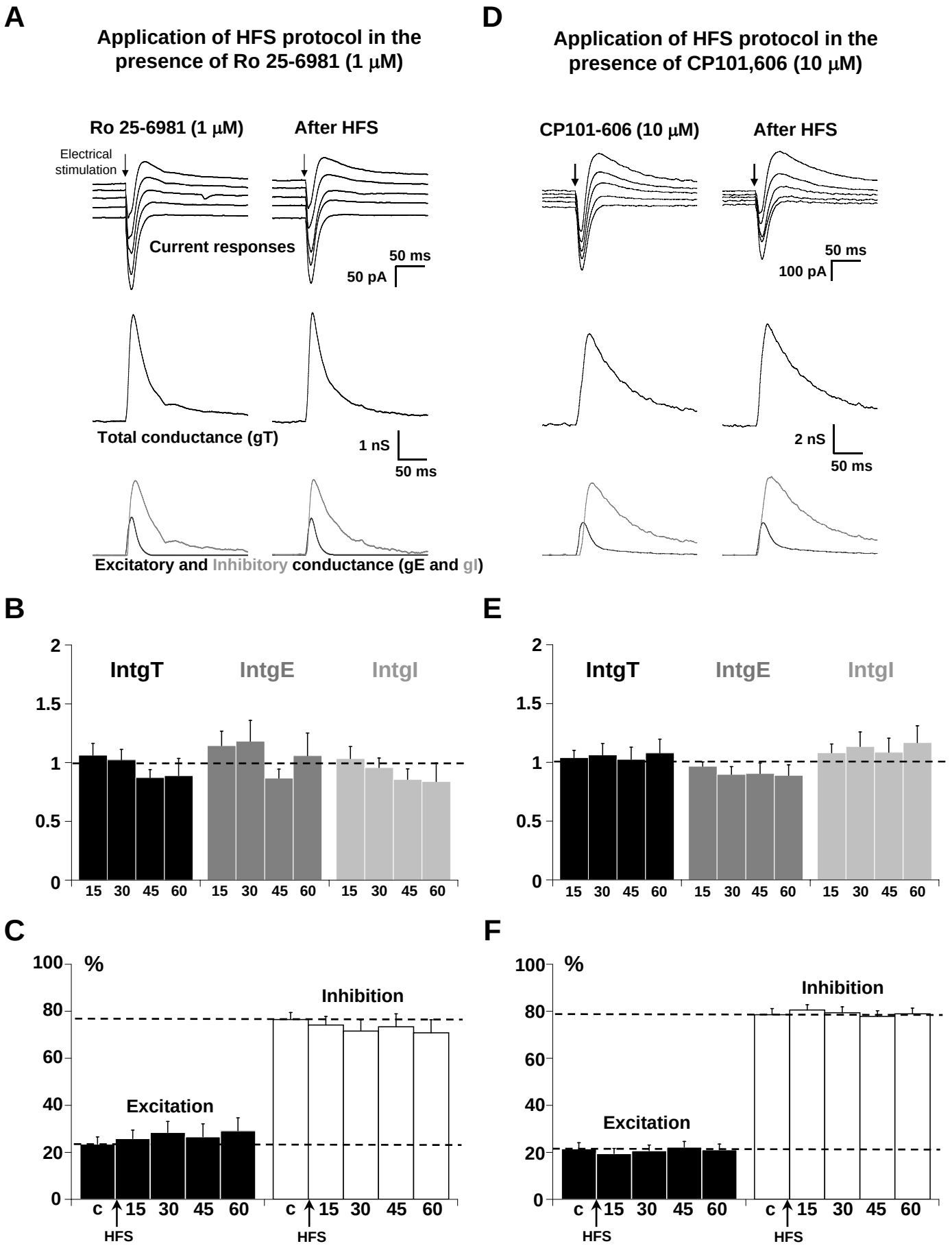
(**C**) Relative contribution of excitation and inhibition conductance changes to the total conductance change, after HFS in the presence of 1 μM Ro 25-6981 (c:control before HFS protocol).

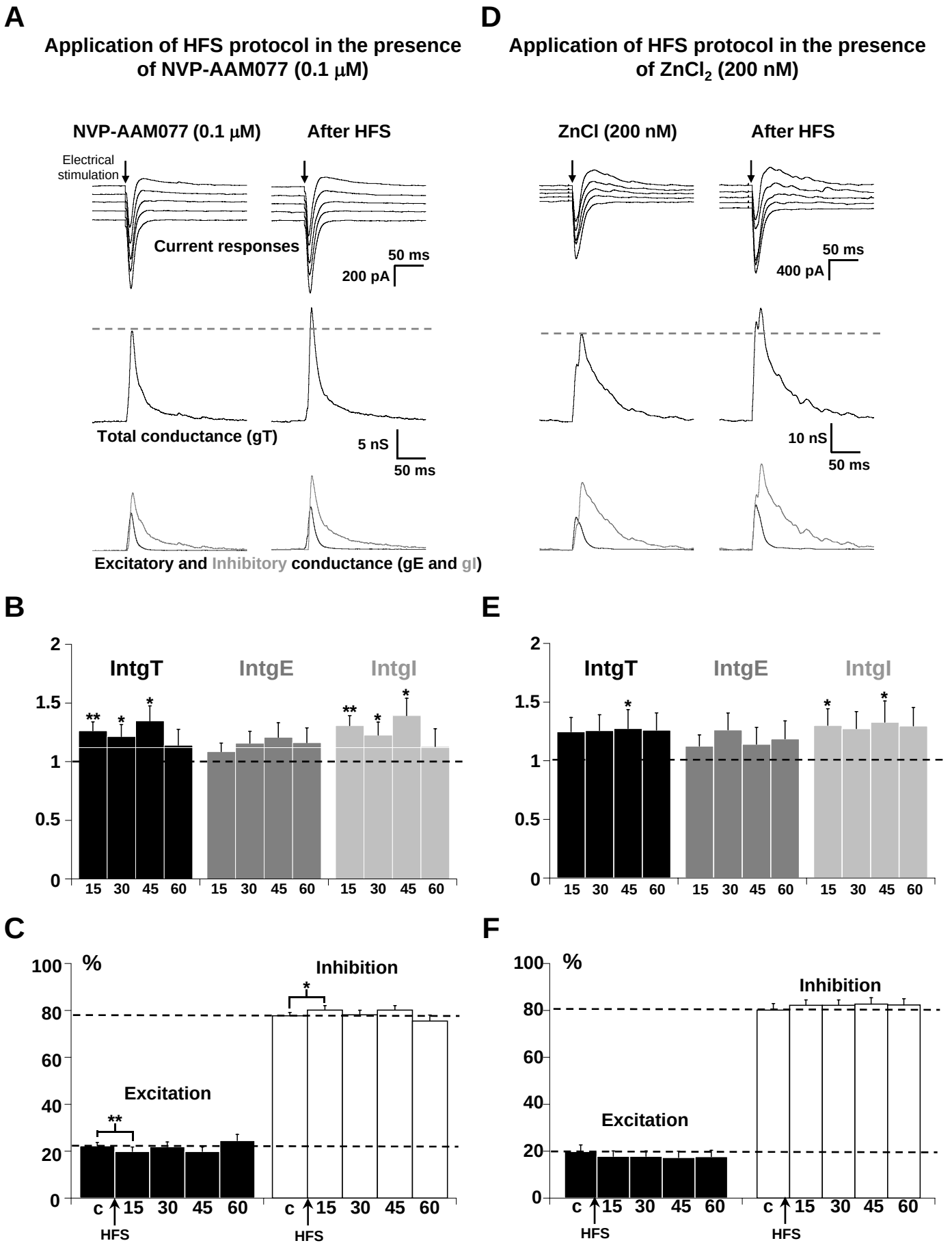
(**D-E-F**) Application of HFS protocol in the presence of 0.1 μM NVP-AAM077 (n = 13). Legends are identical to those in **A-B-C**. No variations of total, excitatory and inhibitory conductance changes were observed after HFS (**D-E**) and the E/I ratio was unchanged (**F**).

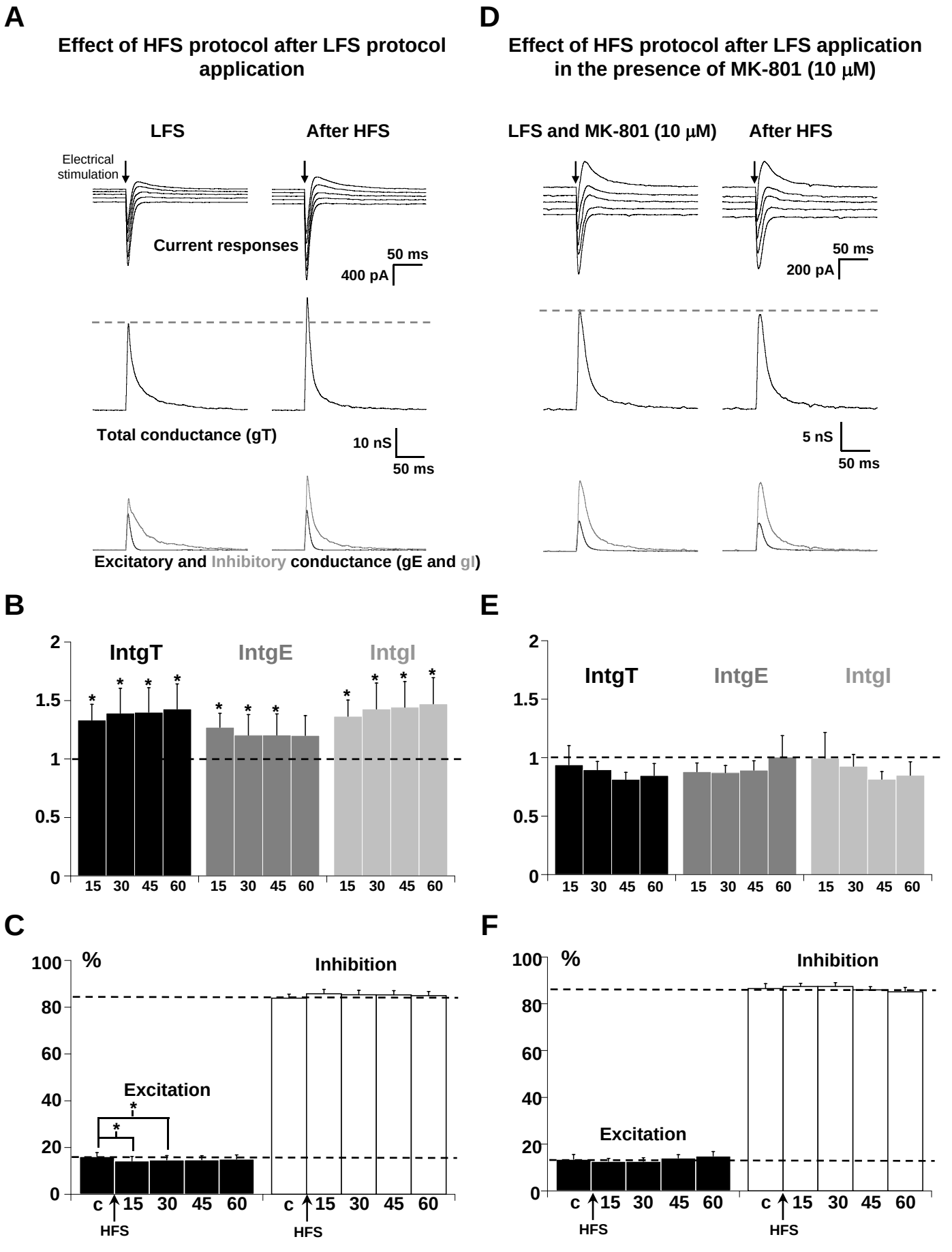

A1
A2
B1
B2
C1
C2
Control
D-AP5 (50 μM)
Control
After HFS
D-AP5 (50 μM)
After HFS + D-AP5 (50 μM)
Electrical stimulation
50 ms
200 pA
Current responses
10 nS
50 ms
Total conductance (gT)
Excitatory and Inhibitory conductance (gE and gI)

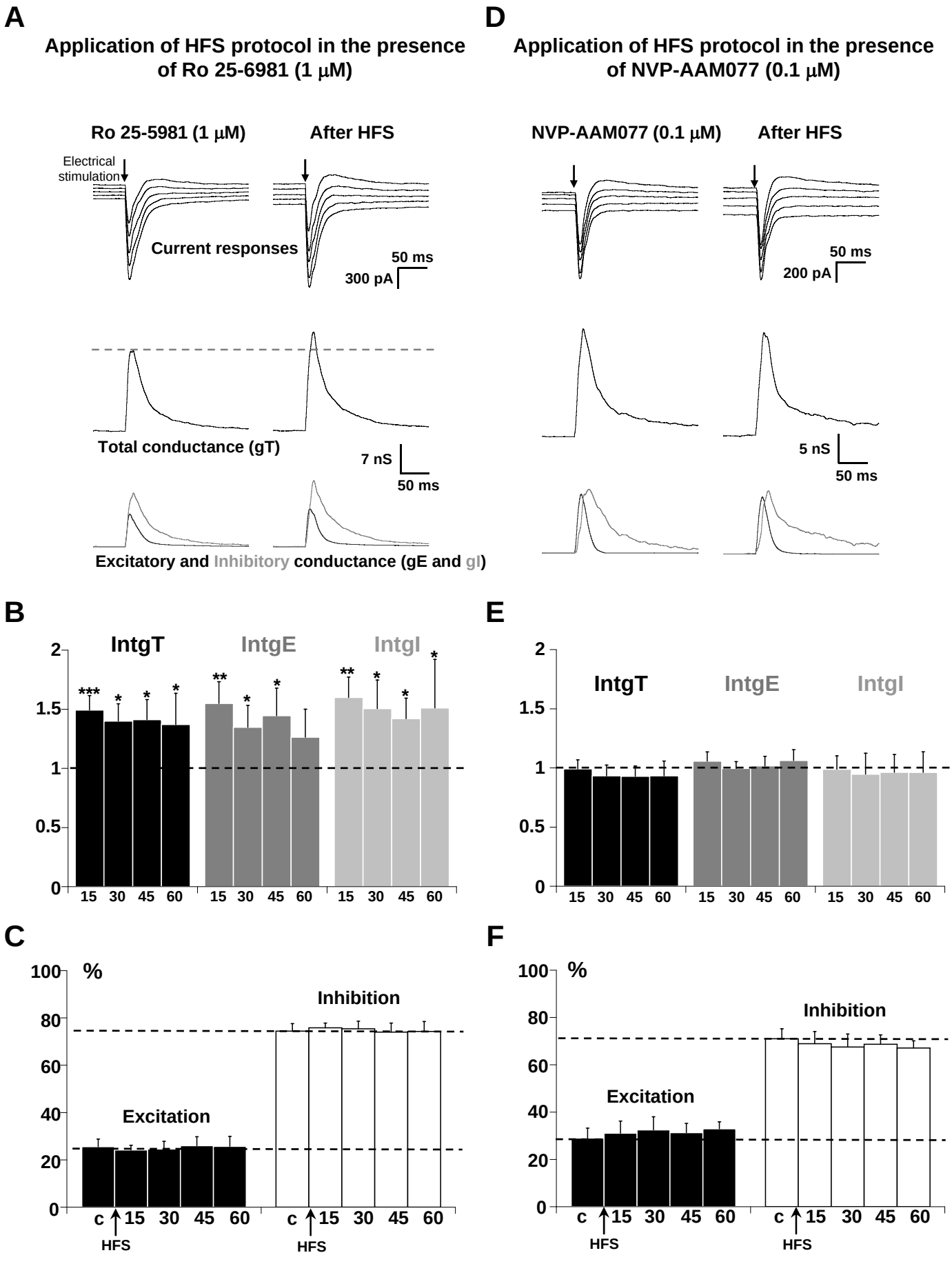

D
HFS protocol
IntgT
IntgE
IntgI
2
1.5
1
0.5
0
15 30 45 60 15 30 45 60 15 30 45 60
100
%
80
60
40
20
0
Inhibition
Excitation
c 15 30 45 60 c 15 30 45 60
HFS
HFS


E
HFS protocol in the presence of D-AP5 (50 μM)
IntgT
IntgE
IntgI
2
1.5
1
0.5
0
15 30 45 60 15 30 45 60 15 30 45 60
100
%
80
60
40
20
0
Inhibition
Excitation
c 15 30 45 60 c 15 30 45 60
HFS
HFS


A
Application of HFS protocol in the presence of Ro 25-6981 (1 μM)
Ro 25-6981 (1 μM)
After HFS
Electrical stimulation
Current responses
50 ms
50 pA
Total conductance (gT)
1 nS
50 ms
Excitatory and Inhibitory conductance (gE and gI)
D
Application of HFS protocol in the presence of CP101,606 (10 μM)
CP101-606 (10 μM)
After HFS
50 ms
100 pA
2 nS
50 ms
B
IntgT
IntgE
IntgI
2
1.5
1
0.5
0
15 30 45 60 15 30 45 60 15 30 45 60
E
IntgT
IntgE
IntgI
2
1.5
1
0.5
0
15 30 45 60 15 30 45 60 15 30 45 60
C
100
%
80
60
40
20
0
Inhibition
Excitation
c 15 30 45 60 c 15 30 45 60
HFS
HFS
F
100
%
80
60
40
20
0
Inhibition
Excitation
c 15 30 45 60 c 15 30 45 60
HFS
HFS


A
Application of HFS protocol in the presence of NVP-AAM077 (0.1 μM)
NVP-AAM077 (0.1 μM)
After HFS
Electrical stimulation
Current responses
50 ms
200 pA
Total conductance (gT)
5 nS
50 ms
Excitatory and Inhibitory conductance (gE and gI)
D
Application of HFS protocol in the presence of ZnCl2 (200 nM)
ZnCl (200 nM)
After HFS
50 ms
400 pA
10 nS
50 ms
B
IntgT
IntgE
IntgI
2
1.5
1
0.5
0
15 30 45 60 15 30 45 60 15 30 45 60
E
IntgT
IntgE
IntgI
2
1.5
1
0.5
0
15 30 45 60 15 30 45 60 15 30 45 60
C
100
%
Inhibition
Excitation
80
60
40
20
0
c 15 30 45 60 c 15 30 45 60
HFS
HFS
F
100
%
Inhibition
Excitation
80
60
40
20
0
c 15 30 45 60 c 15 30 45 60
HFS
HFS


A
Effect of HFS protocol after LFS protocol application
LFS
After HFS
Electrical stimulation
Current responses
50 ms
400 pA
Total conductance (gT)
10 nS
50 ms
Excitatory and Inhibitory conductance (gE and gI)
D
Effect of HFS protocol after LFS application in the presence of MK-801 (10 μM)
LFS and MK-801 (10 μM)
After HFS
50 ms
200 pA
5 nS
50 ms
B
IntgT
IntgE
IntgI
2
1.5
1
0.5
0
15 30 45 60 15 30 45 60 15 30 45 60
E
IntgT
IntgE
IntgI
2
1.5
1
0.5
0
15 30 45 60 15 30 45 60 15 30 45 60
C
%
Inhibition
Excitation
100
80
60
40
20
0
c 15 30 45 60 c 15 30 45 60
HFS
HFS
F
%
Inhibition
Excitation
100
80
60
40
20
0
c 15 30 45 60 c 15 30 45 60
HFS
HFS


A
Application of HFS protocol in the presence of Ro 25-6981 (1 μM)
Ro 25-5981 (1 μM)
After HFS
Electrical stimulation
Current responses
50 ms
300 pA
Total conductance (gT)
7 nS
50 ms
Excitatory and Inhibitory conductance (gE and gI)
D
Application of HFS protocol in the presence of NVP-AAM077 (0.1 μM)
NVP-AAM077 (0.1 μM)
After HFS
50 ms
200 pA
5 nS
50 ms
B
IntgT
IntgE
IntgI
2
1.5
1
0.5
0
15 30 45 60 15 30 45 60 15 30 45 60
E
IntgT
IntgE
IntgI
2
1.5
1
0.5
0
15 30 45 60 15 30 45 60 15 30 45 60
C
100
%
80
60
40
20
0
Inhibition
Excitation
c 15 30 45 60 c 15 30 45 60
HFS
HFS
F
100
%
80
60
40
20
0
Inhibition
Excitation
c 15 30 45 60 c 15 30 45 60
HFS
HFS