

# The sigma-1 receptor modulates NMDA receptor synaptic transmission and plasticity via SK channels in rat hippocampus

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The sigma receptor ( $\sigma$ R), once considered a subtype of the opioid receptor, is now described as a distinct pharmacological entity. Modulation of *N*-methyl-D-aspartate receptor (NMDAR) functions by  $\sigma$ R-1 ligands is well documented; however, its mechanism is not fully understood. Using patch-clamp whole-cell recordings in CA1 pyramidal cells of rat hippocampus and (+)pentazocine, a high-affinity  $\sigma$ R-1 agonist, we found that  $\sigma$ R-1 activation potentiates NMDAR responses and long-term potentiation (LTP) by preventing a small conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  current (SK channels), known to shunt NMDAR responses, to open. Therefore, the block of SK channels and the resulting increased  $\text{Ca}^{2+}$  influx through the NMDAR enhances NMDAR responses and LTP. These results emphasize the importance of the  $\sigma$ R-1 as postsynaptic regulator of synaptic transmission.

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The sigma receptor ( $\sigma$ R) was first described as a subtype of the opioid receptor (Martin *et al.* 1976). Further studies using ligands with high affinity and selectivity have demonstrated that it is a distinct pharmacological entity (Zukin & Zukin, 1979; Su, 1993). Two types of  $\sigma$ Rs have been described:  $\sigma$ R type 1 ( $\sigma$ R-1) and type 2 ( $\sigma$ R-2) (Bowen, 2000). Molecular characterization has shown that the  $\sigma$ R-1 is a novel protein with a molecular mass of 26 kDa (Hanner *et al.* 1996). This protein has a single putative membrane-spanning segment (Kekuda *et al.* 1996; Seth *et al.* 1997; Seth *et al.* 1998). The amino acid sequence of the  $\sigma$ R-1 has no homology with known mammalian proteins, but a weak homology with fungal sterol isomerase has led some investigators to speculate that  $\sigma$ R-1 may be involved in steroid hormone biosynthesis (Jbilo *et al.* 1997; Moebius *et al.* 1997).

$\sigma$ R-1 are widespread in the central nervous system and present in high levels in the prefrontal cortex, hippocampus and striatum (Hayashi & Su, 2004). Many studies have shown that  $\sigma$ R-1 can modulate several physiological and cellular events (Su & Hayashi, 2003). They have been implicated in the regulation of inositol 1,4,5-triphosphate ( $\text{IP}_3$ ) receptors and  $\text{Ca}^{2+}$  signalling at the endoplasmic reticulum (Hayashi *et al.* 2000), mobilization of cytoskeletal adaptor proteins, modulation of nerve growth factor-induced neurite sprouting and alteration of psychostimulant-induced gene expression

(Hayashi & Su, 2004).  $\sigma$ R-1 ligands have also been described to regulate ion channels such as  $\text{K}^{+}$  channels (Wilke *et al.* 1999) and voltage-dependent  $\text{Ca}^{2+}$  channels (Zhang & Cuevas, 2002). Recently, Aydar and his co-workers (Aydar *et al.* 2002) have shown that the  $\sigma$ R-1 modulate  $\text{K}^{+}$  channels as a regulatory subunit by a direct interaction. Importantly, the  $\sigma$ R-1 modulate *N*-methyl-D-aspartate receptor (NMDAR) functions *in vivo* and *in vitro* preparations (Hayashi & Su, 2004).

NMDARs are  $\text{Ca}^{2+}$ -permeable ligand-gated channels that contribute to synaptic transmission and long-term events such as dendritic growth, synaptic modification, and control of gene expression (Waxman & Lynch, 2005). The  $\text{Ca}^{2+}$  influx through NMDARs is responsible for several forms of synaptic plasticity such as long-term potentiation (LTP) and depression (LTD; Collingridge *et al.* 2004). It is well documented that in the hippocampus  $\sigma$ R-1 ligands increase the NMDAR response (Monnet *et al.* 1990; Ishihara & Sasa, 2002; Hayashi & Su, 2004); however, the mechanism through which the  $\sigma$ R-1 modulates the NMDAR response is still not fully understood. Using patch-clamp whole-cell recordings in CA1 pyramidal cells of rat hippocampus, we show that  $\sigma$ R-1 activation enhances NMDAR currents and LTP by preventing small conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels to open.

## Methods

### Preparation of hippocampal slices

Coronal brain slices containing the hippocampus were obtained from Sprague-Dawley rats (21–28 days old). Prior to decapitation, the animals were anaesthetized using an isoflurane vaporizer (Stoelting, Wood Dale, IL, USA) in agreement with the guidelines of the Canadian Council of Animal Care. The concentration of isoflurane was 2–5% and the O<sub>2</sub> flow rate was 1 l min<sup>-1</sup>. The brain was removed and placed in an oxygenated (95% O<sub>2</sub>–5% CO<sub>2</sub>) physiological solution, artificial cerebrospinal fluid (ACSF) at 4°C, containing (mM): 126 NaCl, 2.5 KCl, 1 MgCl<sub>2</sub>, 26 NaHCO<sub>3</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 2 CaCl<sub>2</sub> and 10 glucose. The osmolarity of the ACSF was adjusted to 300 mosmol l<sup>-1</sup> and the pH to 7.2. A block containing the region of interest was prepared, and sections (300 μm) were obtained with a vibrating microtome (Leica VT 1000S, Germany). The slices were stored for 1 h in an oxygenated chamber at room temperature before they were used for the experiments.

### Data recording and analysis

Voltage-clamp experiments were performed with borosilicate pipettes filled with a solution containing (mM): 130 potassium gluconate, 10 Hepes, 10 KCl, 2 MgCl<sub>2</sub>, 5 lidocaine *N*-ethyl bromide (QX-314), 2 ATP-Mg and 0.2 GTP-tris(hydroxy-methyl) aminomethane (pH 7.2 with KOH). When indicated in the text 0.2 mM ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetracetic acid (EGTA) or 10 mM caesium-BAPTA were added to this solution or a caesium-based solution was used. The caesium-based solution contained (mM): 130 caesium methanesulphonate, 10 Hepes, 10 CsCl, 2 MgCl<sub>2</sub>, 5 QX-314, 2 ATP-Mg and 0.2 GTP (pH 7.2 with CsOH). The osmolarity of both the solutions was adjusted to 280–290 mosmol l<sup>-1</sup>. With these solutions, the liquid junction potential was measured (~10 mV) and the membrane potential (*V*<sub>m</sub>) was corrected accordingly. The pipettes had a resistance of 3–6 MΩ. Recordings with series resistance higher than 20 MΩ were discarded. Bridge balance was monitored regularly during the recordings. Cells with a resting membrane potential > -60 mV were also discarded. To allow the drugs added in the pipette to induce their pharmacological action, a delay of 10–15 min was systematically observed prior to recording.

Whole-cell patch-clamp recordings were obtained with a Multiclamp 700A amplifier (Axon Instruments) under visual control using differential interference contrast and infrared video microscopy (IR-DIC; Leica DMLFSA). The recordings were performed at room temperature from individual pyramidal cells of the CA1 region of the hippocampus voltage-clamped at -65 mV.

Post-synaptic currents were evoked by electrical stimulation of the Schaffer collaterals with a bipolar microelectrode positioned in the stratum radiatum. The stimulation intensity consisted of 100 μs current pulses (10–200 μA) and was adjusted to evoke an EPSC amplitude in the range of 40–80 pA at *V*<sub>m</sub> = -65 mV. Stimuli were delivered every 10 s.

To isolate the NMDAR-mediated component of evoked responses, we used ACSF containing a low concentration of MgCl<sub>2</sub> (0.1 mM) with osmolarity maintained by CaCl<sub>2</sub>, and the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPA) antagonist 1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzof[*f*]quinoxaline-7-sulphonamide (NBQX, 20 μM), the GABA<sub>A</sub> receptor antagonist picrotoxin (50 μM), the GABA<sub>B</sub> receptor antagonist 3-[[[(3,4-dichlorophenyl)methyl]amino]propyl]diethoxymethylphosphinic acid (CGP 52432, 10 μM) and the glycine receptor antagonist strychnine (0.5 μM). Application of the NMDAR antagonist DL-2-amino-5-phosphonovaleric acid (AP-5, 50 μM) completely abolished the responses (*n* = 4; Supplementary Fig. 1).

Local drug injections were performed applying air pressure pulses (3–10 ms) with a picospritzer (Parker Hannifan Instrumentation, Fairfield, NJ, USA) to a patch pipette containing 100 μM NMDA. NMDA was dissolved in ACSF and applied every 30 s. The ejection pipette was positioned directly above the proximal dendrites. NMDA was then applied in the presence of TTX (0.5 μM) to avoid polysynaptic phenomena.

Kinetic analysis was performed on averaged EPSCs (usually 20–25 consecutive traces). The rise times of NMDAR currents were measured at peak to the end. Their decays were fitted with the exponential functions:  $y = A_f e^{-t/\tau_f} + A_s e^{-t/\tau_s}$  for double and  $y = A_1 \exp^{-t/\tau}$  for single exponential decay, where *A* is the amplitude, *t* is the time,  $\tau$  is the decay time constant, and the subscript *f* and *s* denote fast and slow components, respectively. Weighted time constants ( $\tau_{\text{mean}}$ ) were calculated using the equation:

$$\tau_{\text{mean}} = [A_f/(A_f + A_s)]\tau_f + [A_s/(A_s + A_f)]\tau_s$$

(Stocca & Vicini, 1998).

Currents underlain by small-conductance voltage-insensitive Ca<sup>2+</sup>-activated K<sup>+</sup> channels (SK channels; Shah & Haylett, 2002) sensitive to apamin (Sah, 1996) were evoked in voltage clamp by giving a 100 ms, 50 mV step from a holding potential of -50 mV in low Mg<sup>2+</sup> ACSF. This procedure evoked unclamped Ca<sup>2+</sup> spikes (Pedarzani & Storm, 1993) followed by outward tail currents. It has been shown that apamin abolished the early part of the outward tail currents (Stocker *et al.* 1999; Sailer *et al.* 2002; Gu *et al.* 2005). The current blocked using apamin, was examined by subtracting the current recorded in the

presence of apamin from that recorded in absence (control) of apamin.

In the slices used for LTP experiments, the CA3 region of the hippocampus was removed by a surgical cut. Post-synaptic currents were evoked by electrical stimulation of the Schaffer collaterals with a bipolar micro-electrode positioned in the stratum radiatum. Stimuli were delivered every 10 s. The recordings for the experiments using the pairing protocol to induce LTP were obtained in ACSF in the presence of picrotoxin ( $50 \mu\text{M}$ ) at  $V_m = -65 \text{ mV}$ . The pairing protocol used to induce LTP was composed of three brief high-frequency tetani (50 pulses at 100 Hz, 4 s intervals) given at the end of a long depolarization (3 min at 0 mV) (Chen *et al.* 1999; Martina *et al.* 2004). The pairing protocol was induced after 10–12 min of baseline in the absence or presence of drugs. This protocol induced an increase of the synaptic responses lasting for more than 40 min.

Data were collected using software pCLAMP 9 (Axon Instrument). Analyses were performed off-line with the software IGOR (WaveMetrics Inc., Lake Oswego, OR, USA). Statistical significance of the results was determined with unpaired *t* tests (two-tailed). All values are expressed as means  $\pm$  s.e.m.

All drugs were obtained from Sigma-Aldrich, with the exception of CGP 52432, NBQX and ryanodine (Tocris, Bristol, UK). (+)-Cinnamyl-1-phenyl-1-*N*-methyl-*N*-cyclopropylamine (igmesine or JO 1784) was a kind gift from Dr Guy Debonnel (McGill University, Canada). Stock solutions of haloperidol (1 mM), ryanodine (10 mM) and cyclopiazonic acid (CPA; 30 mM) were obtained dissolving the pharmacological agents in dimethyl sulfoxide (DMSO). A 1 mM of stock solution of nicardipine was made using HCl (1 N). A 1 mM solution of (+)-pentazocine was prepared by warming 2.85 mg of (+)-pentazocine in 2 ml of HCl (0.1 N) with shaking. When all solids were dissolved, the solution was cooled to room temperature and diluted to 10 ml with a buffer at pH 7. The solutions were freshly prepared daily and kept at room temperature. The other drugs were dissolved in water to obtain stock solutions of 10 mM. All the drugs stock solutions were kept at  $-20^\circ\text{C}$ .

## Results

### $\sigma$ R-1 activation increases NMDAR currents

To study the effect of  $\sigma$ R-1 activation on NMDAR response, we measured the effect of (+)pentazocine ( $1 \mu\text{M}$ ), a high-affinity and selective  $\sigma$ R-1 agonist (Hayashi & Su, 2004), on isolated NMDAR currents recorded from CA1 pyramidal cells on rat hippocampal slices using the patch-clamp whole-cell technique. To evoke postsynaptic glutamatergic currents (PSCs), the Schaffer collaterals were stimulated with a bipolar

electrode. The NMDAR-mediated component of the PSCs was pharmacologically isolated in a low- $\text{Mg}^{2+}$  ACSF containing NBQX, picrotoxin, CGP 52432 and strychnine to block AMPA-,  $\text{GABA}_A$ -,  $\text{GABA}_B$ - and glycine receptor-mediated responses, respectively. We recorded NMDAR currents with a potassium-based solution in the recording pipette (see Methods) and applied (+)pentazocine. (+)Pentazocine (Steinfels *et al.* 1988) is most probably the more highly selective and potent  $\sigma$ R-1 agonist (Quirion *et al.* 1992) and it has been widely used by many groups in different experimental protocols (mainly *in vivo*; for review see Hayashi & Su, 2004) where it dose-dependently potentiates the iontophoretic application of NMDA (Monnet *et al.* 1990; Bergeron *et al.* 1997). We used a concentration of (+)pentazocine of  $1 \mu\text{M}$  because at this concentration the NMDAR currents were consistently and stably increased. The rise, decay, weighted time constants ( $\tau_{\text{mean}}$ ) as well as the relative portions ( $A_f$  and  $A_s$ ) of decay time constants of NMDAR currents were calculated (Table 1). (+)Pentazocine significantly increased the amplitude of NMDAR currents by  $56.5 \pm 4.87\%$  (from an averaged amplitude of  $20.24 \pm 2.07 \text{ pA}$  in control to  $31.83 \pm 3.73 \text{ pA}$  in (+)pentazocine;  $n = 12$ ;  $P < 0.05$ ; Fig. 1A and B, while having no effect on their kinetic properties (Table 1).

To test the specificity of the  $\sigma$ R-1 activation, we observed the effect of haloperidol, a well-known and potent  $\sigma$ R-1 antagonist (Hayashi & Su, 2004), when applied with (+)pentazocine. The addition of haloperidol ( $1 \mu\text{M}$ ) completely reversed the effect of (+)pentazocine ( $1 \mu\text{M}$ ), returning the NMDAR currents to  $98.4 \pm 3.7\%$  of its initial amplitude ( $n = 3$ ; Fig. 2). To rule out any effect of haloperidol on the NMDAR currents, we recorded NMDAR currents and applied haloperidol ( $1 \mu\text{M}$ ) alone. Haloperidol reduced the NMDAR currents by  $15.85 \pm 5.43\%$  ( $n = 3$ ; Supplementary Fig. 2). This value is significantly smaller ( $P < 0.05$ ) than that obtained in the presence of (+)pentazocine. Overall these results demonstrate the specificity of the  $\sigma$ R-1 activation in enhancing the amplitude of NMDAR currents.

To rule out a presynaptic effect of (+)pentazocine, we performed experiments using paired pulses. If the enhancement of the NMDAR currents by (+)pentazocine were due to a presynaptic increase in the transmitter release probability then (+)pentazocine would be expected to reduce paired pulse facilitation. Paired pulses were delivered with an interpulse interval of 50 ms, the second response showing facilitation in CA1 pyramidal cells. Consistent with a postsynaptic effect of (+)pentazocine, stimuli-induced NMDAR currents showed similar ratios (peak 2/peak 1;  $P > 0.05$ ) in the absence (control,  $2.74 \pm 0.34$ ;  $n = 6$ ) and in the presence of (+)pentazocine ( $2.93 \pm 0.46$ ;  $n = 6$ ; Supplementary Fig. 3).

**Table 1. Rise and decay time constants of NMDAR currents in CA1 pyramidal cells recorded in the absence and presence of (+)pentazocine (1  $\mu\text{M}$ ) at  $-65$  mV**

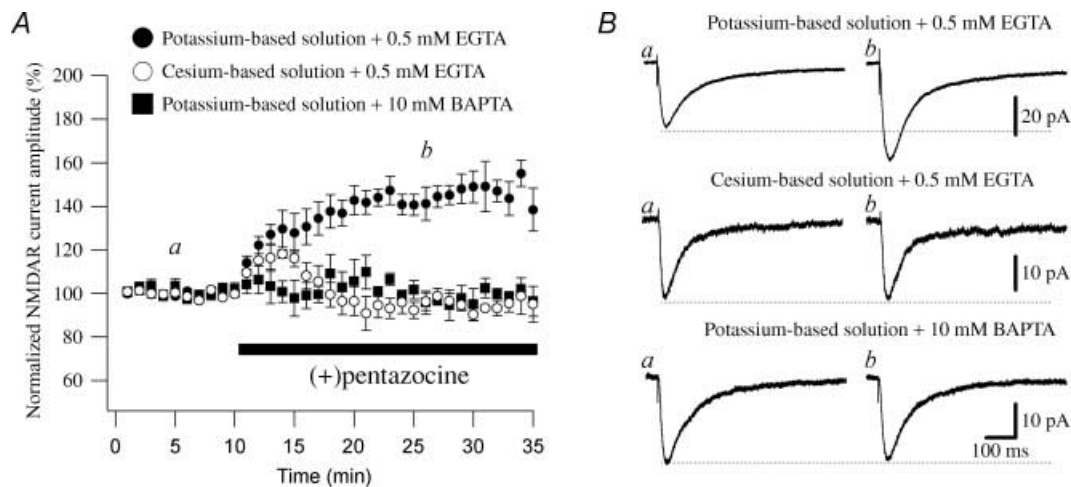
| Control   |                          |                        |                        |                           |                    |                    | (+)-Pentazocine (1 $\mu\text{M}$ ) |                          |                        |                        |                           |                    |                    |
|---|--------------------------|------------------------|------------------------|---------------------------|--------------------|--------------------|------------------------------------|--------------------------|------------------------|------------------------|---------------------------|--------------------|--------------------|
| Ampl (pA)   | $\tau_{\text{act}}$ (ms) | $\tau_{\text{f}}$ (ms) | $\tau_{\text{s}}$ (ms) | $\tau_{\text{mean}}$ (ms) | $A_{\text{f}}$ (%) | $A_{\text{s}}$ (%) | Ampl (pA)                          | $\tau_{\text{act}}$ (ms) | $\tau_{\text{f}}$ (ms) | $\tau_{\text{s}}$ (ms) | $\tau_{\text{mean}}$ (ms) | $A_{\text{f}}$ (%) | $A_{\text{s}}$ (%) |
| Intracellular solution: potassium-based solution + 0.5 mM EGTA ( $n = 12$ ) |                          |                        |                        |                           |                    |                    |                                    |                          |                        |                        |                           |                    |                    |
| 20.24   | 10.6                     | 85.0                   | 728.7                  | 177.2                     | 82                 | 18                 | 31.83*                             | 11.4                     | 95.3                   | 795.5                  | 201.6                     | 82                 | 18                 |
| $\pm 2.07$  | $\pm 1.47$               | $\pm 3.93$             | $\pm 96.6$             | $\pm 15.3$                | $\pm 2.68$         | $\pm 2.68$         | $\pm 3.73$                         | $\pm 1.26$               | $\pm 6.72$             | $\pm 115$              | $\pm 25.2$                | $\pm 2.52$         | $\pm 2.52$         |
| Intracellular solution: caesium-based solution + 0.5 mM EGTA ( $n = 9$ )    |                          |                        |                        |                           |                    |                    |                                    |                          |                        |                        |                           |                    |                    |
| 37.52   | 7.49                     | 75.4                   | 494.4                  | 166.0                     | 79                 | 21                 | 34.90                              | 7.51                     | 74.4                   | 424.0                  | 158.3                     | 77                 | 23                 |
| $\pm 7.02$  | $\pm 0.57$               | $\pm 8.25$             | $\pm 73.0$             | $\pm 21.3$                | $\pm 1.46$         | $\pm 1.46$         | $\pm 7.45$                         | $\pm 0.23$               | $\pm 9.10$             | $\pm 65.3$             | $\pm 38.1$                | $\pm 2.92$         | $\pm 2.92$         |
| Intracellular solution: potassium-based solution + 10 mM BAPTA ( $n = 5$ )  |                          |                        |                        |                           |                    |                    |                                    |                          |                        |                        |                           |                    |                    |
| 18.06   | 9.10                     | 93.3                   | 782.9                  | 178.2                     | 82                 | 18                 | 18.52                              | 11.5                     | 76.4                   | 546.3                  | 157.2                     | 82                 | 18                 |
| $\pm 3.12$  | $\pm 0.70$               | $\pm 13.4$             | $\pm 229$              | $\pm 31.2$                | $\pm 3.28$         | $\pm 3.28$         | $\pm 3.73$                         | $\pm 2.55$               | $\pm 17.9$             | $\pm 115$              | $\pm 22.5$                | $\pm 3.29$         | $\pm 3.29$         |

Values are mean  $\pm$  s.e.m. Electrically evoked NMDAR currents were recorded in a low- $\text{Mg}^{2+}$  ACSF (0.1 mM) in the presence of NBQX (20  $\mu\text{M}$ ), picrotoxin (50  $\mu\text{M}$ ), CGP 52432 (10  $\mu\text{M}$ ) and strychnine (0.5  $\mu\text{M}$ ). The fast and slow decay components are designated by  $\tau_{\text{f}}$  and  $\tau_{\text{s}}$ , and the weighted time constant by  $\tau_{\text{mean}}$ . The amplitude of the NMDAR currents is also shown. Electrically evoked NMDAR currents were recorded in the absence (Control) and presence of (+)pentazocine (1  $\mu\text{M}$ ). \*Significant difference between two values ( $P < 0.05$ ).

### The effect of the $\sigma\text{R-1}$ agonist (+)pentazocine on NMDAR currents depends on a $\text{K}^+$ conductance

Since several  $\sigma\text{R}$  ligands modulate and interact with  $\text{K}^+$  channels (Wilke *et al.* 1999; Aydar *et al.* 2002), we tested the implication of a  $\text{K}^+$  conductance in the potentiation of the NMDAR responses by the  $\sigma\text{R-1}$  agonist. We examined the effect of blocking postsynaptic  $\text{K}^+$  currents by replacing

intracellular  $\text{K}^+$  with caesium ions (see Methods). With this caesium-based internal solution, the application of (+)pentazocine (1  $\mu\text{M}$ ) did not increase ( $P > 0.05$ ) the amplitude (from  $37.52 \pm 7.02$  to  $34.90 \pm 7.45$  pA;  $n = 9$ ; Fig. 1A and B) of the NMDAR currents (Table 1). The kinetics of the NMDAR currents recorded with this solution in the absence or presence of (+)pentazocine were similar to those recorded with the potassium-based



**Figure 1. The effect of the  $\sigma\text{R-1}$  agonist (+)pentazocine on NMDAR currents is dependent on a  $\text{K}^+$  conductance and intracellular  $\text{Ca}^{2+}$  concentration**

A, normalized NMDAR current amplitudes (%) are plotted as a function of time. Each point (one every minute; mean  $\pm$  s.e.m.) is the average of 6 points (stimulations every 10 s). The application of the  $\sigma\text{R-1}$  agonist (+)pentazocine (1  $\mu\text{M}$ ) caused an increase in the amplitude of the NMDAR currents when the CA1 pyramidal cells are recorded with a potassium-based solution including 0.5 mM EGTA ( $\bullet$ ;  $n = 12$ ). When a caesium-based solution including 0.5 mM EGTA ( $\circ$ ;  $n = 9$ ) or potassium-based solution including 10 mM BAPTA ( $\blacksquare$ ;  $n = 5$ ) is used (+)pentazocine had no effect on the NMDAR current amplitude. B, examples of traces of NMDAR currents measured at the time points indicated in A (a and b) are shown. Each trace is an average of 20 traces.

solution (Table 1). This finding strongly suggests the involvement of a  $K^+$  conductance in the potentiating effect observed on NMDAR currents following the application of (+)pentazocine.

### Intracellular $Ca^{2+}$ is required for the $\sigma$ R-1 agonist to exert its effect on NMDAR currents

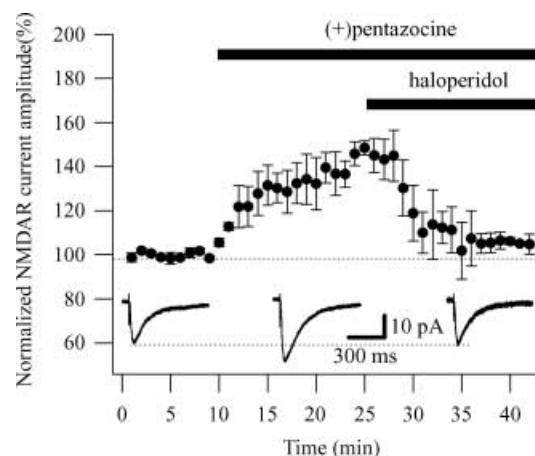
$\sigma$ Rs-1 have been described to regulate intracellular  $Ca^{2+}$  concentration *via* the inositol 1,4,5-triphosphate ( $IP_3$ ) receptor on the endoplasmic reticulum (Hayashi *et al.* 2000) and voltage-dependent  $Ca^{2+}$  channels (Zhang & Cuevas, 2002). To evaluate whether intracellular  $Ca^{2+}$  is required for the  $\sigma$ R-1 agonist to exercise its action on NMDAR currents, we recorded CA1 pyramidal cells with a potassium-based solution with the addition of 10 mM BAPTA in the recording pipette to buffer cytosolic  $Ca^{2+}$ . We found that in the cells recorded with this solution, the application of (+)pentazocine (1  $\mu$ M) did not increase the amplitude of NMDAR currents (from  $18.03 \pm 3.12$  to  $18.52 \pm 3.73$  pA;  $n = 5$ ;  $P > 0.05$ ; Fig. 1A and B) or change their kinetics (Table 1), suggesting that intracellular  $Ca^{2+}$  is required for the  $\sigma$ R-1 agonist to exert its effect on NMDAR currents. The lack of effect of (+)pentazocine in experiments where a caesium-based intracellular solution including 0.5 mM EGTA instead of 10 mM BAPTA was used, ruled out any effect of the  $\sigma$ R-1 agonist on the concentration of  $Ca^{2+}$  alone in its action on NMDAR currents.

### $Ca^{2+}$ influx through NMDARs is required for $\sigma$ R-1 agonist modulation of NMDAR currents

The  $Ca^{2+}$  and  $K^+$  dependence of the enhancing effect of  $\sigma$ R-1 activation on NMDAR currents suggests the involvement of a  $Ca^{2+}$ -activated  $K^+$  conductance. It has been reported that NMDAR-mediated rise in  $Ca^{2+}$  concentration results in the activation of  $Ca^{2+}$ -activated  $K^+$  channels (Shah & Haylett, 2002; Ngo-Anh *et al.* 2005). It has also been found that  $\sigma$ Rs-1 modulate  $Ca^{2+}$  release from intracellular  $Ca^{2+}$  storage sites (Hayashi *et al.* 2000) and voltage-dependent  $Ca^{2+}$  channels (Zhang & Cuevas, 2002).

To rule out the possibility that a rise in  $Ca^{2+}$  concentration originates from voltage-dependent  $Ca^{2+}$  channels, we examined the effect of (+)pentazocine (1  $\mu$ M) on NMDAR currents in the presence of blockers of the different types of  $Ca^{2+}$  channels: mibefradil dihydrochloride hydrate (mibefradil; T-type  $Ca^{2+}$  channel blocker), nifedipine (L-type  $Ca^{2+}$  channel blocker),  $\omega$ -conotoxin GVIA (N-type  $Ca^{2+}$  channel blocker) and  $\omega$ -agatoxin IVA (P/Q-type  $Ca^{2+}$  channel blocker). When (+)pentazocine was applied after mibefradil (10  $\mu$ M), the amplitude of the NMDAR currents was significantly

increased by  $44.94 \pm 3.26\%$  ( $n = 5$ ;  $P < 0.05$ ; from  $18.93 \pm 2.37$  pA in control to  $27.50 \pm 3.63$  pA; Fig. 3A and B). In the same way, the application of (+)pentazocine after nifedipine (5  $\mu$ M) increased the amplitude of the NMDAR currents by  $68.15 \pm 3.46\%$  ( $n = 4$ ;  $P < 0.05$ ; from  $18.68 \pm 5.89$  pA in control to  $30.97 \pm 9.18$  pA; Fig. 3A and B). Since, N- and P/Q-type  $Ca^{2+}$  channels are the predominant species of  $Ca^{2+}$  channels in presynaptic nerve terminals (Westenbroek *et al.* 1992, 1995) and that these channels couple physically to proteins that form the release machinery for synaptic vesicles (Stanley, 1997), to test the  $\omega$ -conotoxin GVIA (N-type  $Ca^{2+}$  channel blocker) and  $\omega$ -agatoxin IVA (P/Q-type  $Ca^{2+}$  channel blocker) we first observed these drugs alone on the NMDAR currents and then applied (+)pentazocine. Following 10 min of  $\omega$ -conotoxin GVIA (3  $\mu$ M) and  $\omega$ -agatoxin IVA (200 nM) application, the amplitude of NMDAR currents was reduced by  $13.85 \pm 4.85\%$  ( $n = 3$ ) and  $19.21 \pm 3.79\%$  ( $n = 3$ ), respectively. The additional application of (+)pentazocine significantly increased the NMDAR currents by  $46.7 \pm 9.18\%$  ( $n = 3$ ) and  $41.08 \pm 4.66\%$  ( $n = 3$ ), respectively (Supplementary Fig. 4). These values were similar to that obtained when (+)pentazocine was applied alone ( $56.5 \pm 4.87\%$ ,  $n = 12$ ; Fig. 3C). Overall, these results ruled out a role for  $Ca^{2+}$  entering through voltage-dependent  $Ca^{2+}$  channels in the enhancing effect of the  $\sigma$ R-1 agonist on NMDAR currents.



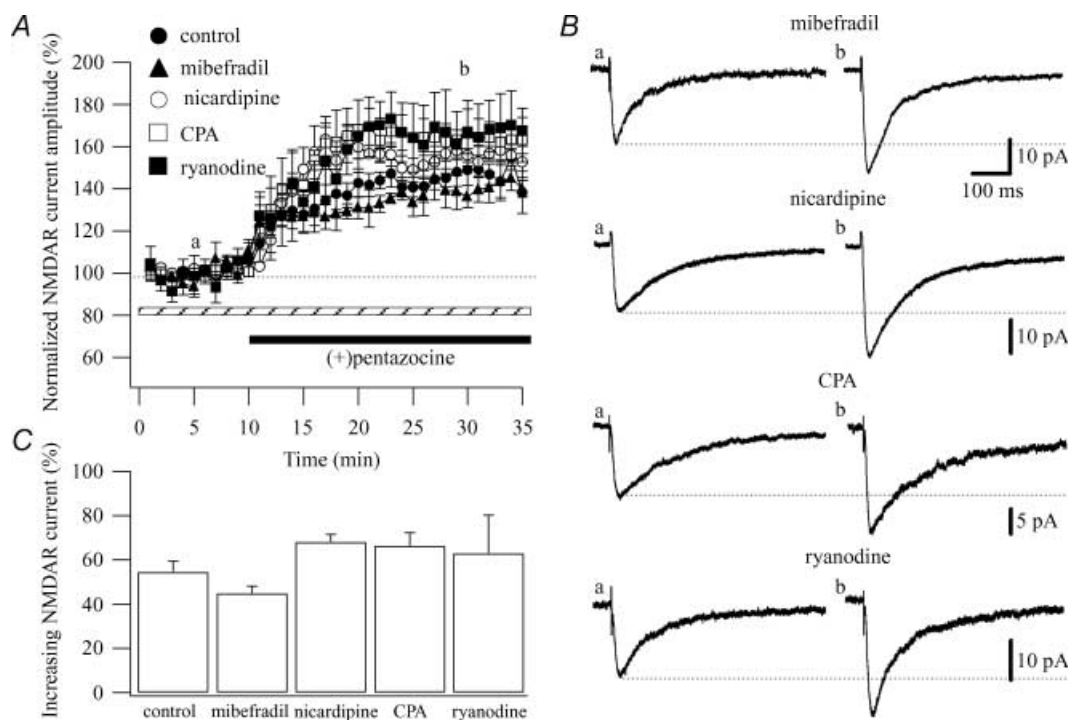
**Figure 2. Haloperidol reverts the effect of (+)pentazocine on NMDAR currents**

Normalized NMDAR current amplitudes (%) are plotted as a function of time. Each point (one every minute; mean  $\pm$  s.e.m.) is the average of 6 points (stimulations every 10 s). The application of the  $\sigma$ R-1 agonist (+)pentazocine (1  $\mu$ M) caused an increase in the amplitude of the NMDAR currents ( $n = 3$ ). The subsequent addition of haloperidol (1  $\mu$ M) completely reversed the effect of (+)pentazocine (1  $\mu$ M), returning the NMDAR currents to their initial amplitude ( $n = 3$ ). Insets show examples of traces of the NMDAR currents measured in absence (right) and presence of (+)pentazocine (centre) and (+)pentazocine plus haloperidol (left), respectively. Each trace is an average of 20 traces.

To rule out the possibility that a rise in  $\text{Ca}^{2+}$  concentration originates from intracellular  $\text{Ca}^{2+}$  storage sites, we tested the effect of (+)pentazocine ( $1\ \mu\text{M}$ ) on NMDAR currents in presence of two  $\text{Ca}^{2+}$  ATPase inhibitors that cause the depletion of intracellular  $\text{Ca}^{2+}$  stores (cyclopiazonic acid (CPA) and ryanodine). When (+)pentazocine was applied after CPA ( $30\ \mu\text{M}$ ) and ryanodine ( $10\ \mu\text{M}$ ), the amplitude of the NMDAR currents was significantly increased by  $66.36 \pm 6.01\%$  ( $n=4$ ;  $P < 0.05$ ; from  $15.26 \pm 2.37\ \text{pA}$  in control to  $25.14 \pm 3.58\ \text{pA}$ ; Fig. 3A and B) and  $63.03 \pm 17.21\%$  ( $n=4$ ;  $P < 0.05$ ; from  $19.10 \pm 2.29\ \text{pA}$  in control to  $29.99 \pm 2.64\ \text{pA}$ ; Fig. 3A and B), respectively. These data were similar ( $P > 0.05$ ) to those observed with (+)pentazocine alone, ruling out a role for  $\text{Ca}^{2+}$  freed from intracellular  $\text{Ca}^{2+}$  stores in the potentiating effect of the  $\sigma\text{R-1}$  agonist on NMDAR currents (Fig. 3C). Experiments with CPA and ryanodine also ruled out the involvement of the  $\sigma\text{R-1}$  action on the  $\text{Ca}^{2+}$  store in the regulation of NMDAR currents.

To test whether  $\text{Ca}^{2+}$  influx through post-synaptic NMDARs is required for (+)pentazocine to exert the enhancement of the NMDAR currents, we measured the effect of (+)pentazocine ( $1\ \mu\text{M}$ ) on the response to local application of NMDA ( $100\ \mu\text{M}$ ). NMDA ( $100\ \mu\text{M}$ ) was applied in a low  $\text{Mg}^{2+}$  ACSF ( $0.1\ \text{mM}$ ) in the presence of tetrodotoxin (TTX,  $0.5\ \mu\text{M}$ ). The amplitude of the currents in response to NMDA application was  $18.32 \pm 4.46\ \text{pA}$  ( $n=5$ ; Fig. 4B). Application of (+)pentazocine significantly ( $P < 0.05$ ) enhanced the response evoked by NMDA by  $67.29 \pm 12.68\%$  ( $n=5$ ; Fig. 4A). Application of AP-5 ( $50\ \mu\text{M}$ ) completely abolished the responses ( $n=4$ ; data not shown).

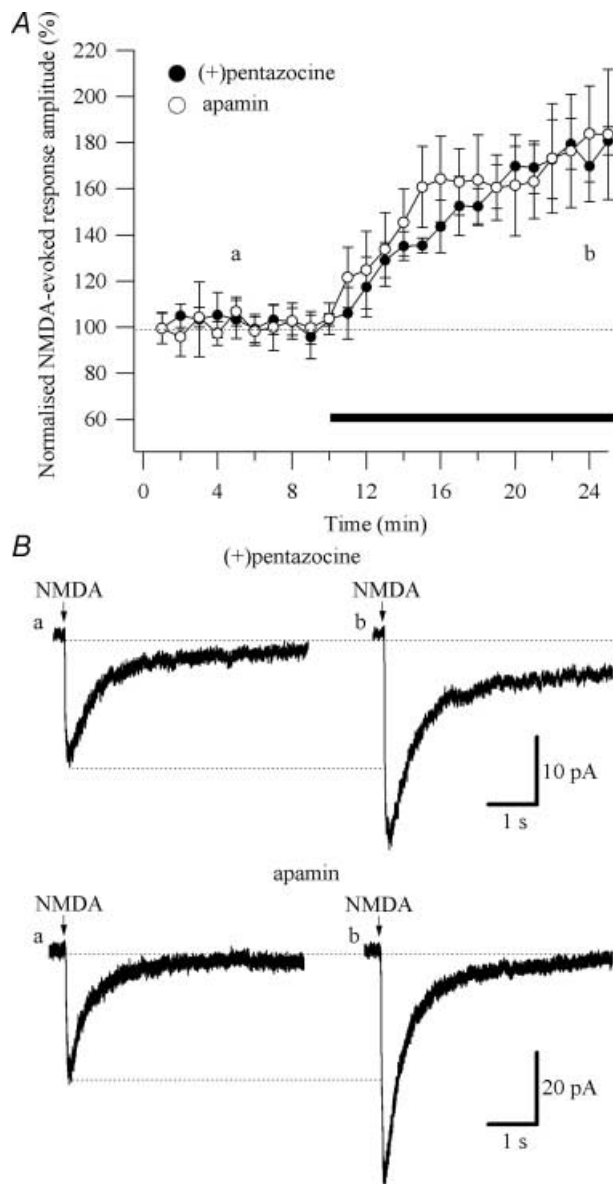
NMDARs are highly permeable to  $\text{Ca}^{2+}$  and are blocked by  $\text{Mg}^{2+}$  at resting membrane potential in a voltage-dependent manner (Nowak *et al.* 1984). Their activation requires binding of glutamate and its coagonist glycine, as well as membrane depolarization (Cull-Candy & Leszkiewicz, 2004). If the  $\sigma\text{R-1}$  activation potentiated NMDAR currents through its action on  $\text{Ca}^{2+}$ -activated



**Figure 3.**  $\sigma\text{R-1}$  agonist modulation of NMDAR currents does not depend on  $\text{Ca}^{2+}$  influx via T- and L-type  $\text{Ca}^{2+}$  channels or  $\text{Ca}^{2+}$  release from the intracellular stores

A, normalized NMDAR current amplitudes (%) are plotted as a function of time. Each point (one every minute; mean  $\pm$  s.e.m.) is the average of 6 points (stimulations every 10 s). The application of (+)pentazocine ( $1\ \mu\text{M}$ ; black bar) caused an increase in the amplitude of the NMDAR currents when the CA1 pyramidal cells were recorded in presence of mibefradil ( $10\ \mu\text{M}$ ;  $\blacktriangle$ ;  $n=5$ ), nicardipine ( $5\ \mu\text{M}$ ;  $\circ$ ;  $n=4$ ), CPA ( $30\ \mu\text{M}$ ;  $\square$ ;  $n=4$ ) and ryanodine ( $10\ \mu\text{M}$ ;  $\blacksquare$ ;  $n=4$ ), dotted bar. B, examples of traces of the NMDAR currents measured at the time points indicated in A (a and b) are shown for mibefradil, nicardipine, CPA and ryanodine. Each trace is an average of 20 traces. C, histogram showing the average of the enhancing effect of (+)pentazocine on NMDAR currents when the CA1 pyramidal cells are recorded in control (no additional drug;  $n=12$ ), in presence of mibefradil ( $n=5$ ), nicardipine ( $n=4$ ), CPA ( $n=4$ ) and ryanodine ( $n=4$ ). All the values are means  $\pm$  s.e.m.

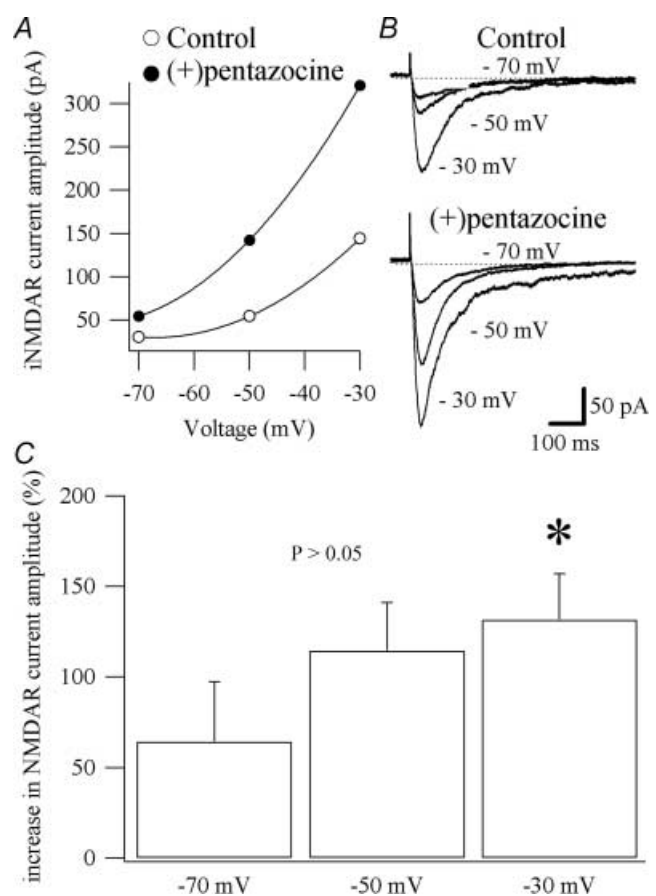
$K^+$  channels activated by  $Ca^{2+}$  entering the cells through the NMDARs, then the effect of (+)pentazocine would be greater at more depolarized potentials where the  $Mg^{2+}$  block is relieved and the driving force for the  $K^+$  ion larger.



**Figure 4. Effect of (+)pentazocine and apamin on the responses evoked by local pressure application of NMDA**  
NMDA ( $100 \mu\text{M}$ ) was applied through a patch pipette positioned directly above the proximal dendrites. TTX ( $0.5 \mu\text{M}$ ) was present throughout the experiments. The cells were voltage-clamped at  $V_m = -65 \text{ mV}$ . **A**, normalized amplitudes of responses evoked by NMDA (%) are plotted as a function of time. Each point (one every minute; mean  $\pm$  s.e.m.) is the average of 2 points (pressure application every 30 s). The application of (+)pentazocine ( $1 \mu\text{M}$ ;  $\bullet$ ;  $n = 5$ ) and apamin ( $100 \text{ nM}$ ;  $\circ$ ;  $n = 6$ ) caused an increase in the amplitude of the responses evoked by NMDA. The black bar indicates the duration of the drugs application. **B**, examples of responses evoked by NMDA observed in low- $Mg^{2+}$  ACSF (**a**) and during application of (+)pentazocine and apamin (**b**). Each trace is an average of 10 traces.

To test this hypothesis, we recorded CA1 pyramidal cells at different potentials ( $-30$ ,  $-50$  and  $-70 \text{ mV}$ ; Fig. 5A). The NMDAR currents were pharmacologically isolated using a normal ACSF (normal  $Mg^{2+}$ ; see Methods) containing NBQX, picrotoxin, CGP 52432 and strychnine (Fig. 5B). The amplitude of NMDAR currents was significantly more increased ( $P < 0.05$ ) by (+)pentazocine ( $1 \mu\text{M}$ ) at a holding potential of  $-30 \text{ mV}$  ( $131.6 \pm 25.3\%$ ;  $n = 6$ ) compared with  $-70 \text{ mV}$  ( $64.3 \pm 33.06\%$ ;  $n = 6$ ; Fig. 5A and C).

Overall these results indicate that  $Ca^{2+}$  influx through post-synaptic NMDARs is required for the  $\sigma$ R-1 activation to exert the enhancement of the NMDAR currents.



**Figure 5. Voltage-dependent effect of (+)pentazocine on NMDAR currents**

**A**, current-voltage relationships for the NMDAR currents shown in **B**. The application of (+)pentazocine ( $1 \mu\text{M}$ ) caused a voltage-dependent enhancement of the NMDAR currents. **B**, examples of traces of NMDAR currents recorded in voltage clamp obtained from CA1 pyramidal cells held at  $-70$ ,  $-50$  and  $-30 \text{ mV}$ . Each trace is an average of 6 traces. **C**, histogram showing the average of the enhancing effect of (+)pentazocine application on NMDAR currents at different holding potential in 6 CA1 pyramidal cells (mean  $\pm$  s.e.m.). \*Significant difference between the percentages of augmentation of the NMDAR currents recorded at  $-70$  and  $-30 \text{ mV}$ .

### (+)Pentazocine enhancement of NMDAR currents is similar to that of apamin

Ca<sup>2+</sup> influx through NMDARs can open Ca<sup>2+</sup>-activated K<sup>+</sup> channels in hippocampal slices and cultured neurons (Zorumski *et al.* 1989; Shah & Haylett, 2002; Ngo-Anh *et al.* 2005). In CA1 pyramidal cells, these channels are small-conductance voltage-insensitive Ca<sup>2+</sup>-activated K<sup>+</sup> channels (SK channels; Shah & Haylett, 2002), sensitive to apamin (Sah, 1996). In these cells, action potentials are followed by a rise in intracellular Ca<sup>2+</sup> that leads to an after-hyperpolarization (AHP) of the membrane. The AHP is comprised of three components, fast AHP, medium AHP and slow AHP, reflecting the activation of different K<sup>+</sup> currents (Storm, 1987, 1990). The fast AHP is due to large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels (BK channels). The type of current that contributes to the medium AHP in CA1 pyramidal cells is a matter of controversy. Several groups have observed an apamin-sensitive component of the medium AHP in CA1 pyramidal neurons (Stocker *et al.* 1999; Empson & Jefferys, 2001; Oh *et al.* 2003; Kramar *et al.* 2004), suggesting that the medium AHP is underlain by the opening of SK channels (see Sah, 1996; Stocker *et al.* 1999). Recently, it has been proposed that SK channels, although available for activation, are not activated by spike train in CA1 pyramidal cells, and consequently do not affect the medium AHP or spike frequency adaptation (Gu *et al.* 2005), excluding the contribution of SK channel to the medium AHP (Storm, 1989; Gu *et al.* 2005). The slow AHP is underlain by an apamin-, tetraethylammonium (TEA)- and 4-aminopyridine-insensitive Ca<sup>2+</sup>-activated K<sup>+</sup> conductance (Sah, 1996). It has been shown that apamin-sensitive SK channels respond to rapid increases in Ca<sup>2+</sup> concentration and reduce the amplitude of NMDAR currents (Ngo-Anh *et al.* 2005). To test whether the effect of the  $\sigma$ R-1 agonist was mimicked by the block of SK channels, we recorded CA1 pyramidal cells with a potassium-based solution and compared the effect of two SK channel blockers (apamin and bicuculline methiodide) to that of (+)pentazocine on NMDAR currents (Fig. 6B). The percentage increment of NMDAR currents amplitude caused by (+)pentazocine (1  $\mu$ M; 56.5  $\pm$  4.87%;  $n$  = 12; Fig. 6A and C) was similar ( $P$  > 0.05) to that induced by apamin (100 nM; 50.7  $\pm$  5.60%,  $n$  = 8; Fig. 6A and C) and bicuculline methiodide (10  $\mu$ M; 52.5  $\pm$  12.94%,  $n$  = 5; Fig. 6A and C). Contrary to this, the application of a low concentration of TEA (1 mM), which blocks BK channels, did not increase the amplitude of NMDAR currents (0.05  $\pm$  2.43%,  $n$  = 3; Fig. 6A and C).

To test whether (+)pentazocine was able to occlude the effect of apamin on NMDAR currents, we recorded CA1 pyramidal cells with a potassium-based solution and measured the effect of apamin (100 nM) on the amplitude of NMDAR currents after the application

of (+)pentazocine (1  $\mu$ M). The NMDAR currents were pharmacologically isolated from EPSCs elicited by stimulation of Schaffer collaterals using a low-Mg<sup>2+</sup> ACSF containing NBQX, picrotoxin, CGP 52432 and strychnine (see Methods). The application of (+)pentazocine increased the amplitude of the NMDAR currents by 65.14  $\pm$  9.88% ( $P$  < 0.05;  $n$  = 4). The subsequent application of apamin did not change the amplitude of the NMDAR currents (35.0  $\pm$  9.29 pA in (+)pentazocine and 34.7  $\pm$  8.66 pA in (+)pentazocine plus apamin;  $n$  = 4; supplementary Fig. 5A and B).

To further test whether another  $\sigma$ R-1 ligand with a chemical structure unrelated to (+)pentazocine can occlude the effect of apamin, we recorded CA1 pyramidal cells with a potassium-based solution and measured the effect of apamin (100 nM) on the amplitude of NMDAR currents after the application of (+) - cinnamyl - 1-phenyl-1-*N*-methyl-*N*-cyclopropylene (igmesine or JO 1784; 1  $\mu$ M). Igmesine is a potent and selective ligand for rat and mouse sigma sites (Roman *et al.* 1990). Igmesine has an IC<sub>50</sub> = 39  $\pm$  8 nM (Roman *et al.* 1990), consequently a concentration of 1  $\mu$ M was used to ensure the activation of all the  $\sigma$ R-1. It has also been shown that igmesine enhanced the neuronal activation induced by NMDA application *in vivo* (Monnet *et al.* 1990). As for (+)pentazocine, the application of igmesine (1  $\mu$ M) significantly and consistently increased the amplitude of the NMDAR currents by 77.52  $\pm$  16.22% ( $P$  < 0.05;  $n$  = 5). The additional application of apamin did not change the amplitude of the NMDAR currents (26.5  $\pm$  5.91 pA in igmesine and 26.2  $\pm$  5.60 pA in igmesine plus apamin;  $n$  = 5; Supplementary Fig. 5C and D).

Overall, these results strongly suggest that  $\sigma$ R-1 activation with (+)pentazocine or igmesine prevents SK channels opening.

To test whether SK channels inhibitors mimicked the effect of (+)pentazocine on currents evoked by NMDA, we measured the effect of apamin (100 nM) on the response to local application of NMDA (100  $\mu$ M; Fig. 4). NMDA (100  $\mu$ M) was applied in a low-Mg<sup>2+</sup> ACSF (0.1 mM) in presence of tetrodotoxin (TTX, 0.5  $\mu$ M). The amplitude of the currents in response to NMDA application was 18.54  $\pm$  2.37 pA ( $n$  = 6; Fig. 4B). Application of apamin significantly ( $P$  < 0.05) enhanced the currents evoked by NMDA by 85.13  $\pm$  38.7% ( $n$  = 6; Fig. 4A and B). To test if (+)pentazocine was able to occlude the effect that apamin had on currents evoked by NMDA, we measured the effect of apamin (100 nM) on the response to local application of NMDA (100  $\mu$ M) after the application of (+)pentazocine (1  $\mu$ M). The application of (+)pentazocine increases the amplitude of the currents evoked by NMDA by 88.77  $\pm$  16.81% ( $P$  < 0.05;  $n$  = 3). The additional application of apamin did not change the amplitude of the currents evoked

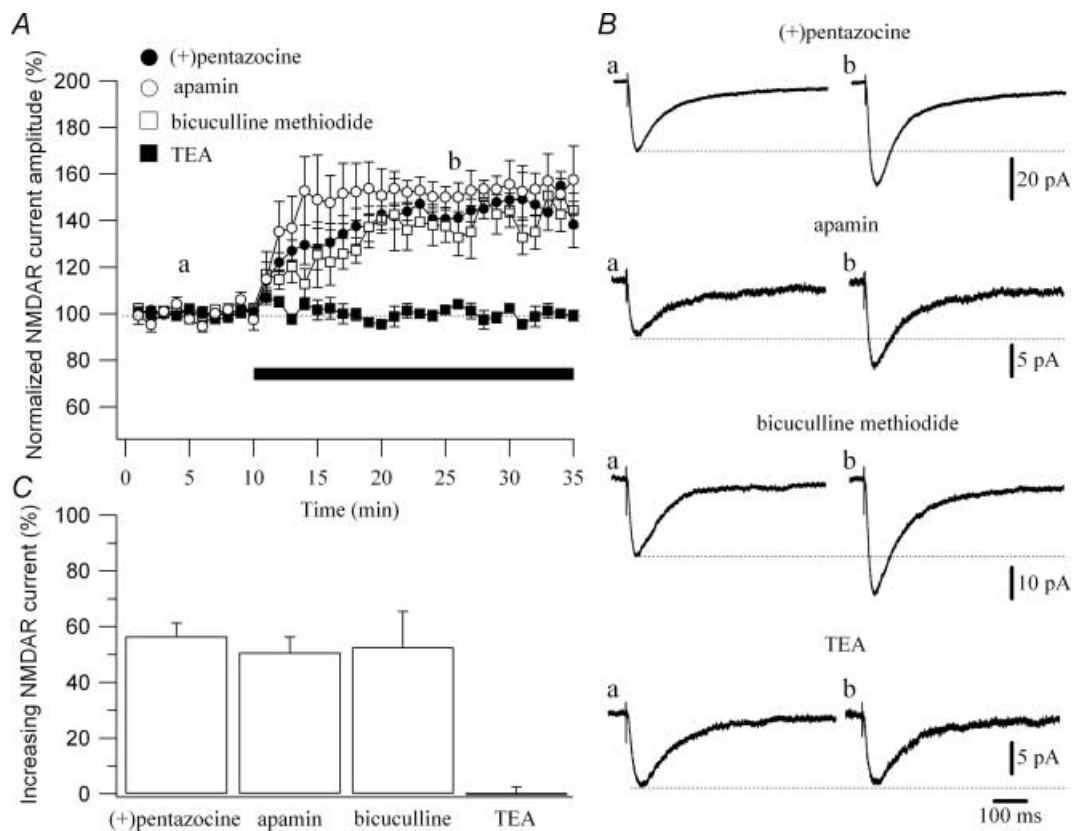


by NMDA ( $40.31 \pm 13.91$  pA in (+)pentazocine and  $44.80 \pm 16.87$  pA in (+)pentazocine plus apamin;  $n = 3$ ; Supplementary Fig. 6).

### $\sigma$ R-1 activation prevents SK channels opening

To test the effect of  $\sigma$ R-1 activation on SK channels, we recorded the  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  currents underlain by SK channels in voltage clamp. These currents were evoked in voltage clamp by giving a 100 ms, 50 mV step from a holding potential of  $-50$  mV in low- $\text{Mg}^{2+}$  ACSF. This voltage step evoked unclamped  $\text{Ca}^{2+}$  spikes (Pedarzani & Storm, 1993; Stocker *et al.* 1999; Gu *et al.* 2005) followed by outward tail currents (Fig. 7A and B). In CA1 pyramidal cells, the SK channel blocker apamin (100 nM) abolished the early part of the outward tail current ( $n = 8$ ; Fig. 7A; see also Stocker *et al.* 1999; Sailer *et al.* 2002;

Gu *et al.* 2005). Similarly to apamin, the  $\sigma$ R-1 activation with (+)pentazocine ( $1 \mu\text{M}$ ) blocked the early part of the outward tail current ( $n = 11$ ; Fig. 7B). The blocked currents were extracted by subtraction of the current before and after application of apamin or (+)pentazocine (Fig. 7A and B). These currents decayed with a similar ( $P > 0.05$ ) time constant:  $132.7 \pm 42.5$  ms ( $n = 8$ ) for apamin and  $130.1 \pm 29.2$  ms ( $n = 11$ ) for (+)pentazocine (Fig. 7A, B and C). The currents had a peak amplitude of  $101.0 \pm 35.0$  pA ( $n = 8$ ) and  $114.1 \pm 21.3$  pA ( $n = 11$ ) for apamin and (+)pentazocine, respectively (Fig. 7C). These values were not significantly different ( $P > 0.05$ ). In three CA1 pyramidal cells, apamin (100 nM) was applied after (+)pentazocine ( $1 \mu\text{M}$ ) to test if apamin blocked an additional current. The blocked currents were extracted by subtraction of the current before and after application of (+)pentazocine, and before the



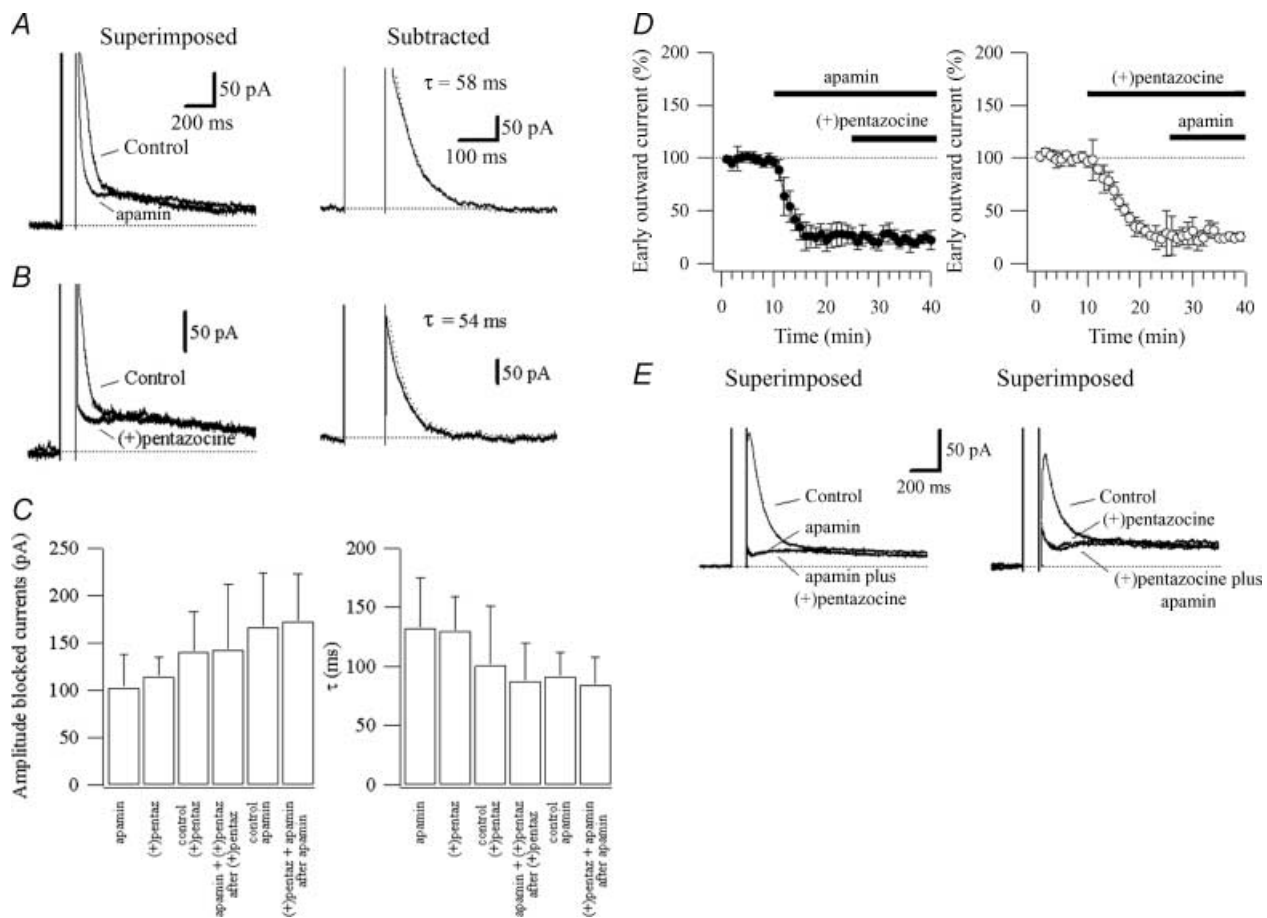
**Figure 6. (+)Pentazocine modulation of the NMDAR current is similar to that caused by blockers of SK channels**

A, normalized NMDAR current amplitudes (%) are plotted as a function of time. Each point (one every minute; mean  $\pm$  s.e.m.) is the average of 6 points (stimulations every 10 s). The application of drugs (black bar) caused an increase in the amplitude of the NMDAR currents when the CA1 pyramidal cells were recorded in presence of (+)pentazocine ( $1 \mu\text{M}$ ;  $\bullet$ ;  $n = 12$ ), apamin (100 nM;  $\circ$ ;  $n = 8$ ) and bicuculline methiodide ( $10 \mu\text{M}$ ;  $\square$ ;  $n = 5$ ). The application of TEA (1 mM;  $\blacksquare$ ;  $n = 3$ ) did not increase the amplitude of the NMDAR currents. B, examples of traces of the NMDAR currents measured at the time points indicated in A (a and b) are shown for (+)pentazocine, apamin, bicuculline methiodide and TEA. Each trace is an average of 20 traces. C, histogram showing the average of the enhancing effect of (+)pentazocine ( $n = 12$ ), apamin ( $n = 8$ ), bicuculline methiodide ( $n = 5$ ) and TEA ( $n = 3$ ) application on NMDAR currents.

application of (+)pentazocine and after the application of (+)pentazocine plus apamin. The currents blocked by  $\sigma$ R-1 activation with (+)pentazocine had an amplitude of  $140.66 \pm 42.2$  pA ( $n = 3$ ) and decayed with a time constant of  $100.9 \pm 49.7$  ms ( $n = 3$ ; Fig. 7C). The additional application of apamin did not significantly change the amplitude ( $143.0 \pm 68.6$  pA;  $n = 3$ ) nor the decay of the blocked current ( $87.7 \pm 32.6$  ms;  $n = 3$ ,  $P > 0.05$ ; Fig. 7C and E).

To test whether apamin was able to occlude the effect of (+)pentazocine on SK channels, we recorded the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  currents as described above and

measured the effect of (+)pentazocine ( $1 \mu\text{M}$ ) on the amplitude and kinetics of the currents blocked by apamin ( $100 \text{ nM}$ ). The blocked currents were extracted by subtraction of the current before and after application of apamin, and before the application of apamin and after the application of apamin plus (+)pentazocine. The currents blocked by apamin had an amplitude of  $166.92 \pm 56.7$  pA and decayed with a time constant of  $91.7 \pm 20.3$  ms ( $n = 5$ ). The additional application of (+)pentazocine did not significantly change the amplitude ( $172.28 \pm 50.8$  pA;  $n = 5$ ) nor the decay of the blocked current ( $84.4 \pm 23.9$  ms;  $n = 5$ ,  $P > 0.05$ ; Fig. 7C and E).



### Figure 7. Activation of $\sigma$ R-1 with (+)pentazocine prevents SK channels opening

Currents recorded in voltage clamp in response to a 100 ms step from a holding potential of  $-50$  mV. The currents recorded in absence (control) and presence of apamin ( $100 \text{ nM}$ ; A) or (+)pentazocine ( $1 \mu\text{M}$ ; B) are superimposed. The current obtained by subtraction is shown on the right for apamin (A) and (+)pentazocine (B). C, histograms summarizing the data for the amplitude (left) and decayed time constant (right;  $\tau$ ) of the currents blocked by apamin ( $n = 8$ ) and (+)pentazocine ( $n = 11$ ). Control (+)pentazocine ( $n = 3$ ) represent the currents blocked by (+)pentazocine for occlusion experiments of the effect of apamin on (+)pentazocine (apamin + (+)pentazocine after (+)pentazocine;  $n = 3$ ). Control apamin ( $n = 5$ ) represent the currents blocked by apamin for occlusion experiments of the effect of (+)pentazocine on apamin ((+)pentazocine + apamin after apamin;  $n = 5$ ). D, summary data showing the time course of the effect of apamin (left,  $n = 5$ ) and (+)pentazocine (right,  $n = 3$ ) on the early tail currents (normalized; control = 100%) and their respective occlusion effect. E, currents recorded in voltage clamp in response to a 100 ms step from a holding potential of  $-50$  mV. Left: currents recorded in absence (control) and presence of apamin and apamin plus (+)pentazocine. Right: currents recorded in absence (control) and presence of (+)pentazocine and (+)pentazocine plus apamin.

Figure 7D shows the time course of the effect of apamin ( $n = 5$ ) and (+)pentazocine ( $n = 3$ ) and their respective occlusion effects on the early tail currents.

These data strongly suggest that the current blocked by apamin and that blocked by  $\sigma$ R-1 activation with (+)pentazocine is the same. The fitting values of the decays were similar to that previously described (Stocker *et al.* 1999; Faber & Sah, 2002; Sailer *et al.* 2002), suggesting that the activation of  $\sigma$ R-1 with (+)pentazocine prevents SK channels opening.

### $\sigma$ R-1 activation enhances LTP

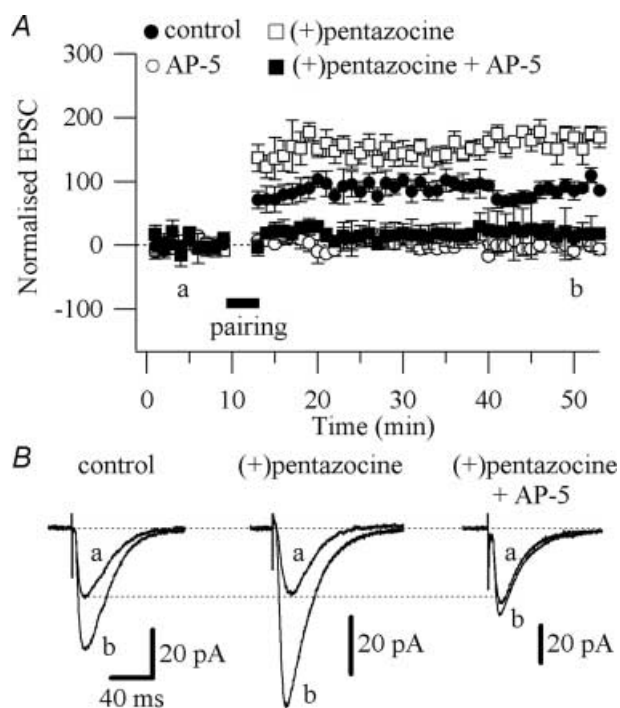
Finally, we sought to examine the functional role of  $\sigma$ R-1 activation in regulating the synaptic transmission in the CA1 region of the hippocampus. Since in CA1 pyramidal cells application of apamin enhances LTP (Behnisch & Reymann, 1998; Stackman *et al.* 2002; Ngo-Anh *et al.* 2005; Hammond *et al.* 2006), then, if we assume that activation of  $\sigma$ R-1 with (+)pentazocine prevents SK channels opening, (+)pentazocine should enhance LTP.

We recorded CA1 pyramidal cells and induced LTP with a pairing protocol comprised of three brief high-frequency tetani (50 pulses at 100 Hz, 4 s intervals) given at the end of a 3 min long depolarization at 0 mV. This protocol induced a  $90.9 \pm 11.9\%$  (control,  $n = 13$ ;  $P < 0.005$ ; Fig. 8A and B) increase above baseline of the synaptic responses, lasting for more than 40 min. This LTP was NMDAR dependent since it was prevented by application of AP-5 ( $50 \mu\text{M}$ ;  $-9.50 \pm 8.77\%$  above baseline;  $n = 4$ ;  $P > 0.05$ ; Fig. 8A). LTP in the presence of (+)pentazocine ( $1 \mu\text{M}$ ) was significantly enhanced ( $154.26 \pm 17.4\%$  above baseline;  $n = 5$ ;  $P < 0.05$ ; Fig. 8A and B) compared with control conditions, and was abolished by the concomitant application of (+)pentazocine ( $1 \mu\text{M}$ ) and AP-5 ( $50 \mu\text{M}$ ;  $8.82 \pm 15.6\%$  above baseline;  $n = 5$ ; Fig. 8A and B). Since the increase of NMDAR currents would primarily affect the induction of LTP, we analysed the summation during the tetanic stimulation in the absence (control) and presence of (+)pentazocine. We found that in the presence of (+)pentazocine the summation was significantly larger ( $P < 0.05$ ) compared with control (tetanus 1:  $50.51 \pm 97.26$  pA,  $n = 6$  in control *versus*  $394.4 \pm 83.86$  pA,  $n = 4$  in (+)pentazocine; tetanus 2:  $6.19 \pm 63.10$  pA,  $n = 6$  in control *versus*  $167.54 \pm 50.07$  pA,  $n = 4$  in (+)pentazocine; tetanus 3:  $-20.61 \pm 50.68$  pA,  $n = 6$  in control *versus*  $113.80 \pm 52.48$  pA,  $n = 4$  in (+)pentazocine; Supplementary Fig. 7). Overall these results suggest that the enhanced LTP observed in the presence of (+)pentazocine was probably the result of the enhanced  $\text{Ca}^{2+}$  influx through the NMDAR due to the prevention of SK channels activation.

## Discussion

In this paper we show that the potentiation of NMDAR currents following the activation of the  $\sigma$ R-1 by (+)pentazocine is due to the prevention of SK channel activation.  $\text{Ca}^{2+}$  entering the cells through the NMDAR activates a  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current, underlain by SK channels, which in turn shunts the NMDAR responses. Consequently, the prevention of SK channel opening by  $\sigma$ R-1 activation increases the NMDAR response and LTP.

The existence of a functional interaction between  $\sigma$ R and NMDARs has been suggested by several studies using biochemical, neuroendocrinological and behavioural models (Su & Hayashi, 2003). In the hippocampus,  $\sigma$ R-1 have been shown to play a role in the modulation of the glutamatergic neurotransmission *via* NMDARs (Monnet *et al.* 1990; Bergeron *et al.* 1997). Several  $\sigma$ R-1 ligands have also been shown to increase the NMDAR response (Bergeron *et al.* 1993; Yamamoto *et al.* 1995; Bergeron *et al.* 1996; Karasawa *et al.* 2002). In addition,  $\sigma$ R have been described to modulate the excitability of peptidergic nerve terminals in the neurohypophysis by inhibiting voltage-dependent  $\text{K}^+$  channels (Wilke *et al.* 1999). The activation of  $\sigma$ R by a variety of ligands reduces current



**Figure 8. Effect of (+)pentazocine on LTP**

A, pooled data of the effect of AP-5 ( $50 \mu\text{M}$ ;  $\circ$ ;  $n = 4$ ), (+)pentazocine ( $1 \mu\text{M}$ ;  $\square$ ;  $n = 5$ ) and (+)pentazocine ( $1 \mu\text{M}$ ) plus AP-5 ( $50 \mu\text{M}$ ;  $\blacksquare$ ;  $n = 5$ ) on the LTP, compared with the LTP in absence of drugs (control;  $\bullet$ ;  $n = 13$ ). All values are mean  $\pm$  s.e.m. B, examples of traces of EPSCs measured at the time points indicated in A (a and b) are shown for control, (+)pentazocine and (+)pentazocine plus AP-5. Each trace is an average of 20 traces.

flow through two distinct  $K^+$  channel types: the A current channel and the  $Ca^{2+}$ -activated  $K^+$  channel (Wilke *et al.* 1999). It has also been found that  $\sigma$ Rs and voltage-gated  $K^+$  channels have a protein–protein interaction and that ligands binding to  $\sigma$ Rs modulate channel activity through this interaction (Aydar *et al.* 2002). Indeed, none of the classical mechanisms of ion channel modulation, such as G protein or phosphorylation, were found to be involved in the mechanism of transduction through which the  $\sigma$ Rs modulate  $K^+$  channels (Lupardus *et al.* 2000).

Very recently it has been demonstrated that in dendritic spines of hippocampal CA1 pyramidal neurons,  $Ca^{2+}$  entry after synaptic activation opens SK channels that act to limit the amplitude of synaptic potentials and reduce  $Ca^{2+}$  influx through NMDARs (Ngo-Anh *et al.* 2005). It has also been established that  $Ca^{2+}$  influx through NMDARs could open  $Ca^{2+}$ -activated  $K^+$  channels in several systems. In hippocampal slices, glutamate-evoked membrane depolarization could be followed by a  $Ca^{2+}$ -dependent and  $K^+$ -mediated AHP (Nicoll & Alger, 1981). Similarly, NMDA application evokes a  $Ca^{2+}$ -dependent  $K^+$  current in cultured hippocampal neurons (Zorumski *et al.* 1989). Evidence of an interaction between NMDARs and  $Ca^{2+}$ -activated  $K^+$  channels has been reported by Isaacson and Murphy in olfactory bulb granule cells (Isaacson & Murphy, 2001). They showed that NMDAR-mediated  $Ca^{2+}$  influx was coupled to large-conductance (BK)  $Ca^{2+}$ -activated  $K^+$  channels. However, in cultured hippocampal neurons, NMDA application was observed to activate SK channels, but not BK channels (Shah & Haylett, 2002). Indeed, the NMDAR-mediated rise in  $Ca^{2+}$  concentration results in the activation of an apamin-sensitive current (Shah & Haylett, 2002; Ngo-Anh *et al.* 2005). The  $Ca^{2+}$  dependence, as well as the blockade by intracellular caesium of the enhancing effect of  $\sigma$ R-1 activation on NMDAR currents, suggests the involvement of a  $Ca^{2+}$ -activated  $K^+$  conductance. The enhancing effect of (+)pentazocine on NMDAR currents was mimicked by apamin and bicuculline methiodide but not by a low concentration of TEA, suggesting the implication of SK channels in the modulation of the NMDAR currents. This hypothesis is further supported by the fact that the activation of  $\sigma$ R-1 with (+)pentazocine prevented SK channel activation. The voltage dependence of the effect of (+)pentazocine on the NMDAR currents strengthens the evidence for a relationship between the NMDAR-mediated rise in the  $Ca^{2+}$  concentration and  $K^+$  current. This was also supported by experiments using local application of NMDA confirming that the  $Ca^{2+}$  influx through postsynaptic NMDARs is required to activate  $Ca^{2+}$ -activated  $K^+$  channels. We ruled out a direct block of SK channels by (+)pentazocine because igmesine, a  $\sigma$ R-1 agonist structurally unrelated to

(+)pentazocine, also occluded the effect of apamin on NMDAR currents.

SK channels are activated by submicromolar concentration of intracellular  $Ca^{2+}$  and behave as high-affinity  $Ca^{2+}$  sensors that convert fluctuation of intracellular  $Ca^{2+}$  concentrations into changes in membrane potential (Xia *et al.* 1998). All the SK channel subtypes exhibit a similar  $Ca^{2+}$  dose–response relationship with  $Ca^{2+}$  concentration required for half-maximal activation ( $K_{0.5}$ ) of  $\sim 0.3 \mu\text{M}$  and an onset of the currents that commence within 1 ms with a time constant of 5–12 ms (Xia *et al.* 1998). The intracellular concentration of  $Ca^{2+}$  is maintained very low (10–100 nM) by channels, pumps and exchangers, allowing rapid metabolic response to  $Ca^{2+}$  changes. In the spines of CA1 pyramidal cells, the synaptic  $Ca^{2+}$  signals are primarily caused by  $Ca^{2+}$  influx through NMDARs (Kovalchuk *et al.* 2000), with a 20–80% rise time of the  $Ca^{2+}$  transient, evoked by uncaging of glutamate, of  $13 \pm 2$  ms (Ngo-Anh *et al.* 2005). In the spine of the CA1 pyramidal cells,  $Ca^{2+}$  signals (subthreshold  $Ca^{2+}$  signals) are generated by NMDARs that are not completely blocked by  $Mg^{2+}$  at resting membrane potential (subthreshold NMDARs). Consequently, when the glutamate is released from a single vesicle, it partially activates NMDARs (subthreshold NMDARs; Kovalchuk *et al.* 2000). Since the activation kinetics of the NMDAR currents recorded in our experiments is  $\sim 7$ –11 ms (Table 1) and a concentration of  $0.7 \mu\text{M}$  could be reached in the spine of CA1 pyramidal cells just after subthreshold NMDAR activation and/or single spontaneous synaptic vesicle release (Kovalchuk *et al.* 2000), the  $Ca^{2+}$  influx to the spine can easily activate SK channels (from the first millisecond) and affect the peak amplitude of the NMDAR currents (7–11 ms). We suggest that both subthreshold-activated NMDAR channels and evoked NMDAR currents (NMDAR currents evoked by electrical stimulation) may provide the source of  $Ca^{2+}$  that rapidly activates the SK channels and consequently influences the peak amplitude of NMDAR currents.

Time course experiments showed that the amplitude of NMDAR currents were stably increased by (+)pentazocine after 12–15 min of drug application, while apamin stably increased the amplitude of NMDAR currents after 5–7 min (Fig. 6). This is supported by the time course experiments on SK currents showing that apamin and (+)pentazocine stably blocked the currents after 5–7 min and 12–15 min, respectively (Fig. 7). Previous reports have shown that the amplitude of the current underlain by SK channels was stably blocked by the application of apamin after  $\sim 5$ –7 min (Sailer *et al.* 2002; Gu *et al.* 2005). The differences in time course between apamin and (+)pentazocine could be due to the difference in their mechanism of action in blocking or preventing the opening of SK channels.

$\sigma$ Rs-1 have been described to regulate  $\text{Ca}^{2+}$  release and signalling from intracellular  $\text{Ca}^{2+}$  storage sites via inositol 1,4,5-triphosphate ( $\text{IP}_3$ ) receptor on the endoplasmic reticulum (Hayashi *et al.* 2000). Indeed, the  $\sigma$ Rs-1 have been described to be localized on the endoplasmic reticulum. The effect of  $\sigma$ R-1 agonists on the  $\text{Ca}^{2+}$  store in regulating NMDAR currents is unlikely because of the inability of CPA and ryanodine, two  $\text{Ca}^{2+}$  ATPase inhibitors that cause the depletion of intracellular  $\text{Ca}^{2+}$  stores, to reverse the effect of (+)pentazocine on NMDAR currents.  $\sigma$ Rs-1 have also been described to regulate voltage-dependent  $\text{Ca}^{2+}$  channels (Zhang & Cuevas, 2002). There are multiple types of  $\text{Ca}^{2+}$  channels with unique physiological roles in the central nervous system. These have been classified by their distinct electrophysiological and pharmacological profiles into T-, N-, L-, Q-, P- and R-types (McCleskey, 1994). Mibefradil,  $\omega$ -conotoxin GVIA, nicardipine and  $\omega$ -agatoxin IVA, which block T-, N-, L- and P/Q-type  $\text{Ca}^{2+}$  channels, respectively, had no effect in abolishing the increasing effect caused by (+)pentazocine on NMDAR currents, excluding the implication of voltage-dependent  $\text{Ca}^{2+}$  channels in this effect. The lack of positive control experiments ensuring that CPA, ryanodine, mibefradil,  $\omega$ -conotoxin GVIA, nicardipine and  $\omega$ -agatoxin IVA inhibited SERCA pumps, ryanodine receptors and voltage-dependent T-, N-, L- and P/Q-type  $\text{Ca}^{2+}$  channels, respectively, limits our capacity to affirm that these sources of  $\text{Ca}^{2+}$  did not participate in the effect of  $\sigma$ R-1 activation on NMDAR currents. However, previous reports showing no effect of CPA, ryanodine and nicardipine in blocking the increasing effect of apamin on NMDAR EPSPs strongly support our hypothesis (Faber *et al.* 2005).

LTP of the Schaffer collateral synapses is NMDAR dependent and requires pre-synaptic activity and post-synaptic depolarization. The post-synaptic depolarization is necessary due to the properties of NMDARs, which require the relief of the  $\text{Mg}^{2+}$  block to open (Nowak *et al.* 1984). Once NMDARs are open,  $\text{Ca}^{2+}$  influx triggers synaptic plasticity (Bliss & Collingridge, 1993). Indeed, the competitive NMDAR antagonist AP-5 prevented the induction of LTP. Since (+)pentazocine enhances NMDAR currents through the prevention of SK channels activation, the enhanced LTP observed in the presence of the  $\sigma$ R-1 agonist is likely to be the result of the enhanced  $\text{Ca}^{2+}$  influx through the NMDAR. In the CA1 region of the hippocampus, the induction of LTP can also be influenced by the  $\text{Ca}^{2+}$  entering the cell through  $\text{Ca}^{2+}$  channels or by release from the intracellular  $\text{Ca}^{2+}$  stores (Chen *et al.* 1999; Kovalchuk *et al.* 2000). Since  $\sigma$ Rs-1 have been described to influence  $\text{Ca}^{2+}$  release and signalling from intracellular  $\text{Ca}^{2+}$  storage sites (Hayashi *et al.* 2000) as well as voltage-dependent  $\text{Ca}^{2+}$  channels (Zhang & Cuevas, 2002), the abolition of the LTP when AP-5 was applied with (+)pentazocine suggested that

(+)pentazocine did not influence the induction of LTP through other mechanisms than NMDARs. Our findings are also supported by previous reports showing that in CA1 pyramidal cells, application of apamin enhances LTP (Behnisch & Reymann, 1998; Stackman *et al.* 2002; Hammond *et al.* 2006) and that blocking SK channels facilitates the induction of LTP by enhancing NMDAR-dependent  $\text{Ca}^{2+}$  signals within dendritic spines (Ngo-Anh *et al.* 2005). The effect of  $\sigma$ R-1 activation on SK channels and NMDARs supports the role that  $\sigma$ Rs-1 play on synaptic transmission.

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## Supplemental material

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