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## Extensive Overlap of Mu-Opioid and Nicotinic Sensitivity in Cortical Interneurons

**We studied  $\mu$ -opioid transmission in acute slices of rat neocortex using whole-cell recordings and single-cell reverse transcription-polymerase chain reaction. The  $\mu$ -opioid receptor (MOR) was found in  $\gamma$ -aminobutyric acidergic (GABAergic) interneurons that were either layer I cells frequently expressing neuropeptide Y or layers II-V cells expressing vasoactive intestinal peptide and enkephalin (Enk). We found that  $\mu$ -opioid agonists inhibit these interneurons that are selectively excited by nicotinic agonists. The extensive overlap of  $\mu$ -opioid and nicotinic responsiveness allowed  $\mu$ -opioid agonists to inhibit nicotinic excitation of responsive interneurons and of their GABAergic output onto pyramidal cells. Finally, nicotinic stimulation resulted in a dynamic sequence where GABAergic transmission was first enhanced and then depressed below its baseline. This latter disinhibitory effect was prevented by a  $\mu$ -opioid antagonist, indicating that excitation of nicotinic-responsive interneurons induced the release of endogenous Enk, which in turn led to MOR activation. Our results suggest that neocortical  $\mu$ -opioid transmission acts as an inhibitory feedback onto nicotinic-responsive interneurons, which may change network excitability and inhibition patterns during cholinergic excitation.**

**Keywords:** enkephalin, GABAergic interneuron,  $\mu$ -opioid receptor, neocortex, nicotinic receptor

### Introduction

The neocortical network integrates afferent inputs through a heterogeneous neuronal population comprising excitatory pyramidal cells and interneurons (Peters and Jones 1984; Connors and Gutnick 1990; Thomson and Deuchars 1994; Somogyi et al. 1998; Dantzer and Callaway 2000). These interneurons transmute incoming excitatory signals into local  $\gamma$ -aminobutyric acidergic (GABAergic) inhibition. They also constitute a rich source of neuropeptides whose explicit contributions to neocortical integration are largely undefined (Papadopoulos et al. 1987; DeFelipe 1993; Kubota et al. 1994; Cauli et al. 1997; Kaneko et al. 1998; Gallopin et al. 2005; Toledo-Rodriguez et al. 2005).

$\mu$ -Opioid agonists have wide behavioral implications, and it is established that in the hippocampus, they reduce GABAergic transmission by inhibiting interneurons (Zieglansberger et al. 1979; Madison and Nicoll 1988; Capogna et al. 1993), thereby increasing network excitability (disinhibition). However, little is known about their function in the neocortex. It is well documented that enkephalin (Enk) is the only endogenous  $\mu$ -opioid agonist in the neocortex (Rossier et al. 1977; Gee et al. 1983; Giraud et al. 1983). In this structure, the immunocytochemical distributions of Enk and of the  $\mu$ -opioid receptor (MOR) largely overlap and are restricted to GABAergic interneurons (Taki et al. 2000). The coexpression of Enk and MOR

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suggests that  $\mu$ -opioid transmission acts as an inhibitory feedback loop onto interneurons to disinhibit the neocortical network.

A functional link between  $\mu$ -opioid and cholinergic transmission is suggested by behavioral evidence of interactions between nicotine and opioids (Pomerleau 1998; Malin 2001; Mathieu-Kia et al. 2002) and by the reduction of nicotine effects in mice lacking MOR or Enk (Berrendero et al. 2002, 2005). In the neocortex, multiple neuronal types mediate the effects of acetylcholine (McCormick and Prince 1985; Kawaguchi 1997; Xiang et al. 1998; Porter et al. 1999; Christophe et al. 2002). Among these cell types, interneurons expressing vasoactive intestinal peptide (VIP) constitute a major target of nicotinic agonists and express receptor channels with high affinity for nicotine (Porter et al. 1999). The observation that the majority of MOR-expressing interneurons also expresses VIP (Taki et al. 2000) suggests that  $\mu$ -opioid transmission might contribute to the neocortical integration of cholinergic nicotinic signals.

In the present study, we combined patch-clamp recordings and single-cell reverse transcription-polymerase chain reaction (scPCR; Lambollez et al. 1992) in rat neocortical slices to identify and characterize MOR- and Enk-expressing neurons. The results show that in the neocortex,  $\mu$ -opioid transmission selectively inhibits interneuron populations that respond to nicotinic agonists and controls the GABAergic contribution of these interneurons to the network.

### Materials and Methods

#### *Slice Preparation*

All experiments were carried out in accordance with the guidelines published in the European Communities Council Directive of 24 November 1986 (86/609/EEC). Young Wistar rats (14–21 postnatal days old) were decapitated, the brains were quickly removed, and 300- $\mu$ m-thick parasagittal sections of cerebral cortex were prepared as described (Cauli et al. 1997). The slices were incubated at room temperature (20–25   C) in artificial cerebrospinal fluid (ACSF) containing (in mM) NaCl, 126; KCl, 5; NaH<sub>2</sub>PO<sub>4</sub>, 1.25; CaCl<sub>2</sub>, 2; MgCl<sub>2</sub>, 1; NaHCO<sub>3</sub>, 26; Glucose, 20; and pyruvate 5 that was bubbled with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

#### *Whole-Cell Recordings*

Slices were transferred to a chamber and perfused at 1.5 mL/min with ACSF at room temperature. Patch pipettes (5–7 M $\Omega$ ) pulled from borosilicate glass were filled with 8  $\mu$ L of internal solution containing (in mM) K-gluconate, 144; MgCl<sub>2</sub>, 3; ethyleneglycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 0.5; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10; and biocytin, 2 mg/mL. The pH was adjusted to 7.2 and osmolarity to 285/295 mosm. Whole-cell patch-clamp recordings were made from neocortical neurons identified under infrared videomicroscopy with Nomarski optics (Stuart et al. 1993) and using a patch-clamp amplifier (Axopatch 200A, Axon Instruments,

Foster City, CA). All membrane potentials were corrected for junction potential (-11 mV). Resting membrane potential was measured just after passing into whole-cell configuration, and only cells with a resting membrane potential more hyperpolarized than -50 mV were selected. After characterization of the recorded neurons according to their firing behavior, effects of different drugs were investigated using current-clamp or voltage-clamp methods (VH, -71 mV). The series resistances were not compensated but were monitored throughout the experiments. The signals were filtered at 5 kHz, digitized at 10 kHz, saved to a PC, and analyzed off-line with pCLAMP8 software (Axon Instruments).

Drugs and chemicals were obtained from Sigma (Saint Louis, MO) except tetrodotoxin (TTX) that was purchased from Latoxan (Valence, France). All drugs were applied in the bath perfusion. The nicotinic receptor agonist 1-1-dimethyl-4-phenyl-piperazinium iodide (DMPP) was applied in the presence of the muscarinic receptor antagonist atropine (5  $\mu$ M). Reproducible responses to DMPP- and to the MOR-selective agonist [D-Ala<sup>2</sup>, N-Me-Phe<sup>4</sup>, Gly<sup>5</sup>-ol]-enkephalin (DAMGO) were obtained with 6- and 13-min intervals between applications, respectively. Somatodendritic responses to drug applications are presented as mean  $\pm$  standard deviation.

To record inhibitory postsynaptic currents (IPSCs), 27 mM of K-glucuronate was replaced by KCl in the internal solution, resulting in a theoretical chloride reversal potential of -40.4 mV. IPSCs were thus recorded as inward currents in our conditions. Recordings were performed in the presence of ( $\pm$ )-2-amino-5-phosphonovaleric acid (AP-5, 20  $\mu$ M) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10  $\mu$ M) to prevent glutamatergic transmission. In this set of experiments, DMPP was bath applied in the presence of atropine (5  $\mu$ M) and the selective antagonist for the  $\alpha$ 7 subtype of nicotinic receptors, methyllycaconitine (10 nM). The GABAergic nature of the DMPP-induced postsynaptic events in pyramidal neurons was confirmed by applying DMPP in the absence of glutamate receptor antagonists and in the presence of bicuculline (10  $\mu$ M), using an internal solution containing lidocaine N-ethyl bromide (QX-314, 5 mM). In these conditions, DMPP did not induce any change in postsynaptic event amplitude or frequency ( $n = 3$ ).

Postsynaptic currents were analyzed off-line using Mini Analysis software (Synaptosoft, Decatur, GA). Signals <7 pA were excluded from the measurements. Average DMPP effects on IPSCs amplitude and frequency in different conditions are presented as mean  $\pm$  standard error of mean.

### Single-Cell Reverse Transcription-PCR

Cytoplasm harvesting of the recorded neurons and reverse transcription were performed as described (Lambolez et al. 1992). At the end of the recording, as much as possible of the cell's content was aspirated into the recording pipette by application of a gentle negative pressure while maintaining the tight seal. The pipette was then delicately removed to allow outside-out patch formation. Next, the content of the pipette was expelled into a test tube and reverse transcription was performed in a final volume of 10  $\mu$ L. Two steps of multiplex polymerase chain reaction (PCR) were performed essentially as described (Cauli et al. 1997). The cDNAs present in the 10  $\mu$ L reverse transcription reaction were first amplified simultaneously using all the primer pairs described in Table 1 (for each primer pair, the sense and antisense primers were positioned on 2 different exons). Taq polymerase (2.5 units, Qiagen GmbH, Hilden, Germany) and 20 pmole of each primer were added to the buffer supplied by the manufacturer (final volume 100  $\mu$ L), and 20 cycles (94  $^{\circ}$ C, 30 s; 60  $^{\circ}$ C, 30 s; 72  $^{\circ}$ C, 35 s) of PCR were run. Second rounds of PCR were then performed using 2  $\mu$ L of the first PCR product as template. In this second round, each cDNA was individually amplified using its specific primer pair by performing 35 PCR cycles (as described above). Ten microliters of each individual PCR reaction were then run on a 2% agarose gel using  $\phi \times 174$  digested by Hae III as molecular weight marker and stained with ethidium bromide. MOR scPCR was initially performed with sense and antisense primers A (see Table 1) located in exons 1 and 2, respectively. Primer pair B (exons 2 and 3) was used subsequently due to the existence of exon 1 alternative splicing in mouse (Pan et al. 2001). Both primer pairs were equally effective in detecting MOR expression in single cells, suggesting that exon 1 is present in a significant proportion of rat neocortical MOR mRNAs.

**Table 1**  
PCR primers and FRET probes

	Size	PCR primers	FRET probes
<b>MOR # NM_013071</b>	236	Sense A, 75: ACCTGGCTCCTGGCTCAACT Antisense A, 290: TGGTGGCAGTCTCATTTTGG Sense B, 486: CGTGGACCGCTACATTGCTG Antisense B, 711: CGGCATGATGAAAGCGAAGATA	3' FITC, 340: AACGACAGCCTGTGCCCTCA 5' R705, 361: ACCGGAGCCCTCCATG Nested sense, 498: CATTGCTGTCTGCCACCCA Nested antisense, 687: AAGACACAGATTTTGAGCAGGTTC
<b><math>\delta</math>-opioid R # NM_012617</b>	508	Sense, 33: GCAGTTTTGGCTCCTCGC Antisense, 520: CCCAACACCTGAAGCCAAGAC	3' FITC, 307: CCCTTCAGAGCGCCCAAGT 5' R705, 327: CCTGATGGAACCTGGCCG
<b><math>\kappa</math>-opioid R # NM_017167</b>	326	Sense, 590: GAGGCACCAAGTCAAGGAA Antisense, 898: GCTGTGGGAGGTGCTGCC	3' FITC, 733: TGCTACACCTGATGATCCTGC 5' R705, 756: CTTGAAGAGTGTCCGGCTCCTC
<b>Enk # M28263</b>	345	Sense, 451: GCCAACTCCTCGACCTGCT Antisense, 772: GCCTCCGATCTTTTTCCATCTC	3' FITC, 588: TGAAGACAGCACGAGCAAGAGGT 5' R705, 612: TGGGGGCTTCATGAGAGGC
<b>Dynorphin # NM_019374</b>	286	Sense, 65: ACTGCCTGTCTGTGTTCCCT Antisense, 329: GAGACGCTGGTAAGGAGTTGGC	3' FITC, 113: CCCATCCATCAACCCCT 5' R705, 133: ATTTGCTCCCTGGATGCCAG mRNA not detected in this study
<b>POMC # K01878</b>	217	Sense, 105: GGACCTCACCACGAAAGCA Antisense, 304: CCGTCTCTTCTCCGCAC	
<b>Gad65 # M72422</b>	391	Sense <sup>a</sup> , 713: TGTTTTCTCCTGGTGGTGCC Antisense <sup>a</sup> , 1085: CCCAAGCAGCATCCACAT	3' FITC <sup>c</sup> 848: TTTCTCTCAAGAAGGGAGCTGCA 5' R705 <sup>c</sup> 872: CCTTGGGGATCGGAACAGACA
<b>Gad67 # M76177</b>	600	Sense <sup>b</sup> , 713: TACGGGGTTCGCACAGGTC Antisense <sup>b</sup> , 1294: same as Gad65	3' FITC <sup>c</sup> 930: CCTGGGGAGCCATATCCAA 5' R705 <sup>c</sup> 951: ATGTACAGCATCATGGCGGCTC
<b>CB # M27839</b>	432	Sense <sup>b</sup> , 134: AGGCACGAAAGAAGGCTGGAT Antisense <sup>b</sup> , 544: TCCCACACATTTTGATCCCTG	3' FITC <sup>c</sup> 263: TCCTGTCTCTTTTCGATGCC 5' R705 <sup>c</sup> 285: GCAACTGAAAGTCTGGCAGGAA
<b>CR # X66974</b>	309	Sense <sup>b</sup> , 142: CTGGAGAAGGCAAGGAAAGGT Antisense <sup>b</sup> , 429: AGGTTTCATCATAGGACGGTTG	3' FITC <sup>c</sup> 248: TTGAGATGGCAGAGCTGGCC 5' R705 <sup>c</sup> 269: AGATCCTGCCAACCGAAGAGAAT
<b>NPY # M15880</b>	359	Sense <sup>b</sup> , -45: GCCCAGAGCAGAGCACCC Antisense <sup>b</sup> , 292: CAAGTTTCATTTCCCATCCCA	3' FITC <sup>c</sup> 17: AACGAATGGGGCTGTGTGGA 5' R705 <sup>c</sup> 38: TGACCTCGCTCTATCCCTGC
<b>SOM # K02248</b>	209	Sense <sup>b</sup> , 43: ATCGTCTGGCTTTGGGC Antisense <sup>b</sup> , 231: GCCTCATCTGCTCTGTCTCA	3' FITC <sup>c</sup> 105: GCAGAAGTCTGTGGCGGCTG 5' R705 <sup>c</sup> 126: CACCGGAAACAGGAACTGGC
<b>VIP # X02341</b>	286	Sense <sup>b</sup> , 167: TGCCTTAGCGGAGAATGACA Antisense <sup>b</sup> , 434: CCTCACTGCTCTTCCCA	3' FITC <sup>c</sup> 199: TGTGTCAGAAATGCCAGGCA 5' R705 <sup>c</sup> 221: GCTGATGGAGTTTTCCACGAGCG
<b>CCK # K01259</b>	216	Sense <sup>b</sup> , 174: CGCACTGCTAGCCCGATACA Antisense <sup>b</sup> , 373: TTTCTCATTCGCCCTCCTCC	3' FITC <sup>c</sup> 198: CCAGCAGTCCGCAAGCT 5' R705 <sup>c</sup> , 218: CCTCTGGCCGATGTGCC

Position 1: First base of the start codon.

<sup>a</sup>Bochet et al. (1994).

<sup>b</sup>Cauli et al. (1997).

<sup>c</sup>Ferezou et al. (2002).

## Identification of the PCR Products and Test of the scPCR

### Protocol

PCR-generated fragments obtained from each cell were analyzed by fluorescence resonance energy transfer (FRET) between 2 adjacent oligoprobes (Table 1, purchased from Prologo, Paris, France) internal to the amplified sequence. The upstream probe was fluorescein isothiocyanate labeled at the 3' end (donor, excitation 470 nm) and the downstream probe Red705 labeled at the 5' end (acceptor, emission, 710 nm). FRET between the 2 fluorophores, which can only occur when both probes are hybridized to their cognate PCR fragment, was measured with a LightCycler instrument (Roche Diagnostics GmbH, Mannheim, Germany) as described (Ferezou et al. 2002). The identity of PCR fragments obtained with MOR primer pair B was confirmed using a nested PCR with primers described in Table 1. The reverse transcription-PCR protocol was tested on 500 pg of total RNA purified from rat neocortex (Chomczynski and Sacchi 1987). All the transcripts were detected from 500 pg of neocortical RNA except for the pro-opiomelanocortin (POMC) mRNA, which was detected from 4 ng of whole brain RNA. The sizes of the PCR-generated fragments were as predicted by the mRNA sequences (see Table 1), and their identity was confirmed by FRET between adjacent oligoprobes (as described above).

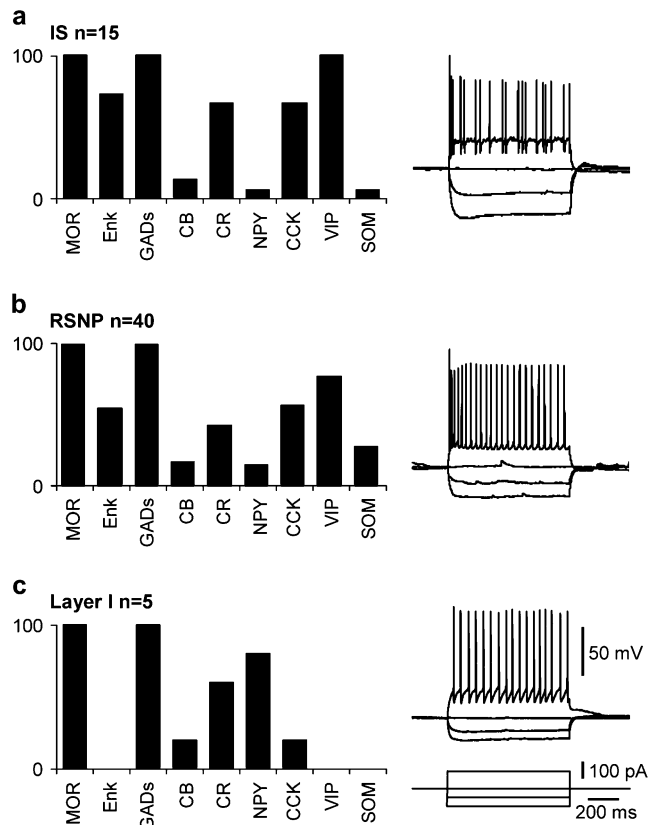
### Intracellular Labeling

Identification of the recorded neurons was regularly confirmed by histochemical revelation of intracellular biocytin, performed using the ABC elite kit (Vector Laboratories, Burlingame, CA).

## Results

### Characterization of MOR-Expressing Neurons

With the aim of characterizing MOR-expressing neurons, 360 neurons from cortical layers I-V were electrophysiologically characterized and analyzed by scPCR. Pyramidal cells ( $n = 98$ ) and interneurons ( $n = 262$ ) were selected according to the shape of their soma and proximal dendrites as seen with infrared videomicroscopy. Cells were classified as regular spiking nonpyramidal (RSNP,  $n = 174$ ), irregular spiking (IS,  $n = 39$ ), fast spiking ( $n = 38$ ), or pyramidal neurons according to their action potential firing behavior upon application of depolarizing current pulses (McCormick et al. 1985; Kawaguchi 1993, 1995; Cauli et al. 1997). The scPCR protocol was designed to probe for the expression of mRNAs encoding MOR and its endogenous agonist Enk, in addition to 8 interneuron markers (GABA synthesizing enzymes: GAD65, GAD67; calcium binding proteins: calretinin [CR] and calbindin [CB]; and neuropeptides: neuropeptide Y [NPY], somatostatin [SOM], cholecystokinin [CCK], and VIP). MOR mRNA was detected in a small subset of neocortical neurons ( $n = 60$  of 360), which all expressed GAD65 and/or GAD67 mRNAs and exhibited IS or RSNP firing patterns. This confirms that MOR expression is restricted to inhibitory GABAergic interneurons in the neocortex (Taki et al. 2000). Within neocortical layers II-V, MOR-expressing IS ( $n = 15$ ) and RSNP ( $n = 40$ ) interneurons exhibited similar molecular profiles (Fig. 1*a,b*). Indeed, they frequently expressed VIP (IS: 100%, RSNP: 78%), CCK (IS: 67%, RSNP: 58%), Enk (IS: 73%, RSNP: 55%), and CR (IS: 67%, RSNP: 43%) and showed a low occurrence of CB (IS: 13%, RSNP: 18%), SOM (IS: 7%, RSNP: 28%), and NPY (IS: 7%, RSNP: 15%). It has been shown previously that VIP-expressing IS and RSNP interneurons form a relatively homogenous cell population despite their different firing patterns (Porter et al. 1999; Cauli et al. 2000; Ferezou et al. 2002). The present data indicate that MOR and Enk expression is another shared property of VIP-expressing interneurons and are consistent with a previous immunocytochemical report



**Figure 1.** MOR-expressing neurons. The scPCR analyses of 360 neocortical neurons, including 98 pyramidal cells and 262 GABAergic interneurons, revealed MOR expression in 60 neurons including 15 IS (*a*), 40 RSNP (*b*), and 5 layer I interneurons (*c*). All MOR-positive neurons expressed GAD67 and/or GAD65 (GADs). MOR-expressing IS and RSNP interneurons exhibited similar molecular profiles characterized by a high expression level of VIP, CCK, Enk, and CR and a low expression level of CB, NPY, and SOM (*a-b*). MOR-expressing layer I interneurons presented a high expression level of NPY and CR, a low expression level of CB and CCK, and did not express Enk, VIP, and SOM (*c*). The current-clamp recordings shown on the right of each panel illustrate the voltage responses exhibited by an IS (*a*), a RSNP (*b*), and a layer I (*c*) MOR-expressing interneurons induced by hyperpolarizing and depolarizing current injections.

showing that most MOR-positive cells coexpress VIP (Taki et al. 2000).

In contrast, MOR-expressing interneurons from layer I ( $n = 5$  RSNP cells) expressed neither Enk nor VIP (Fig. 1*c*) and showed a low occurrence of CCK (20%) and a high occurrence of NPY (80%). The occurrences of CR, CB, and SOM (60, 20, and 0%, respectively) in layer I MOR-expressing interneurons were similar to those of layers II-V ( $P > 0.56$ ,  $z$ -test). These properties were shared by all layer I neurons analyzed ( $n = 11$ , including the 5 MOR-positive neurons), for which Enk, VIP, CCK, and NPY expression levels (0, 0, 18, and 55%, respectively, data not shown) were significantly different from those of MOR-expressing neurons from layer II to V ( $P \leq 0.05$ ,  $z$ -test). Altogether, these results disclose striking similarities between the distribution of MOR expression and the reported sensitivity to nicotinic agonists, mainly found in VIP-expressing (Porter et al. 1999) and layer I (Christophe et al. 2002) neocortical interneurons. This indicates that  $\mu$ -opioid and nicotinic agonists may share the same neuronal targets in the neocortex.

The distribution of the Enk mRNA was broader than expected from immunocytochemical detection of this neuropeptide,

essentially observed in MOR-positive neurons (Taki et al. 2000). Indeed, the Enk mRNA was detected in all MOR-negative layer II-V neuronal populations, including in 32% of pyramidal neurons, albeit at lower levels than in VIP-expressing neurons (60%).

A subset of 166 neurons was additionally tested for the expression of the  $\delta$ - and  $\kappa$ -opioid receptors (DOR and KOR, respectively) and the endogenous opioid peptides POMC and dynorphin (data not shown). Within MOR-expressing interneurons ( $n = 28$ ), the KOR mRNA was detected in 3 cells, whereas the DOR, POMC, and dynorphin mRNAs were not detected. Within MOR-negative neurons ( $n = 138$ ), DOR and KOR mRNAs were detected in 2 and 6 GABAergic interneurons, respectively. The dynorphin mRNA was detected in 16 GABAergic interneurons characterized by a high occurrence of SOM (94%). The POMC mRNA was never detected, confirming that Enk is the only  $\mu$ -opioid endogenous agonist in the neocortex, as previously established (Rossier et al. 1977; Gee et al. 1983; Giraud et al. 1983).

### MOR Is Coupled with Potassium Channels in Neocortical Interneurons

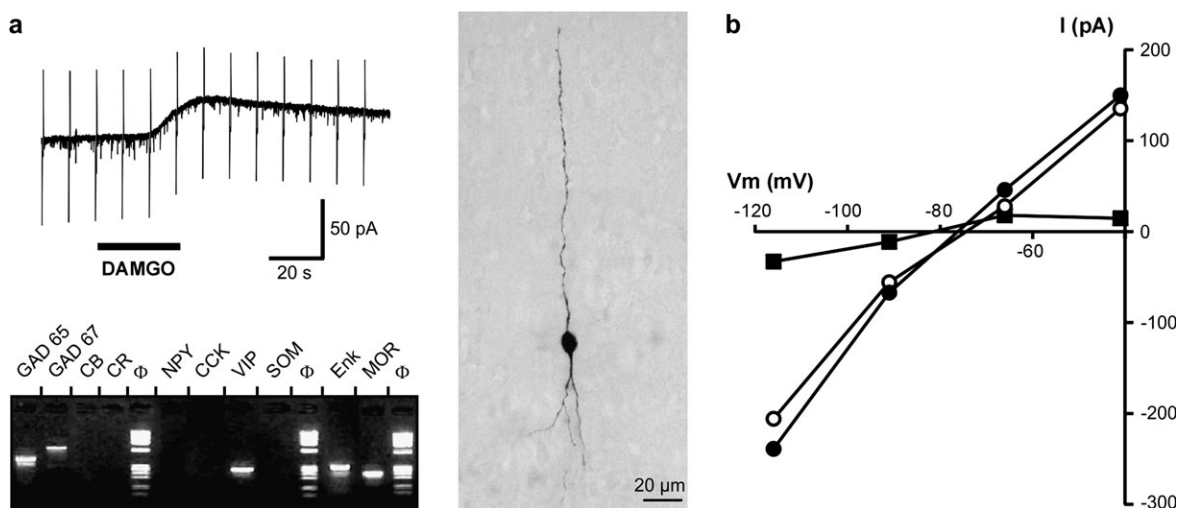
The functional effect of MOR activation was investigated by applying the MOR-selective agonist DAMGO (2.5  $\mu$ M) on 169 neocortical neurons recorded in voltage-clamp or current-clamp conditions. The presence of the MOR mRNA was tested in a subset of these neurons by scPCR. DAMGO responses, observed in 86 interneurons, consisted of a slow hyperpolarization ( $-9.3 \pm 3.4$  mV,  $n = 17$ ) due to the activation of an outward current ( $15.5 \pm 6.2$  pA,  $n = 69$ ) associated with a significant decrease in membrane resistance ( $-187.6 \pm 145.8$  M $\Omega$ ,  $n = 86$ ;  $P < 0.001$ , Wilcoxon signed rank test). The DAMGO-induced current persisted in the presence of TTX (1  $\mu$ M,  $n = 10$ ) and was prevented by the selective MOR antagonist D-Pen-Cys-Tys-D-Trp-Orn-Thr-Pen-Thr-NH<sub>2</sub>: (CTOP,  $n = 4$ , not shown). The MOR mRNA was detected in 20 out of 32 DAMGO-responsive

neurons. Conversely, all MOR-positive neurons responded to DAMGO application, indicating that the occurrence of the MOR mRNA was underestimated by scPCR. Indeed, although we detected the MOR mRNA in 5 out of 11 layer I interneurons, we observed that all layer I interneurons tested responded to DAMGO application ( $n = 10$ ). An example of a MOR-positive neuron is illustrated in Figure 2*a*. The expression of VIP was observed in 21 out of 22 DAMGO-responsive neurons found in layers II-V, confirming the frequent coexpression of VIP and MOR observed by scPCR.

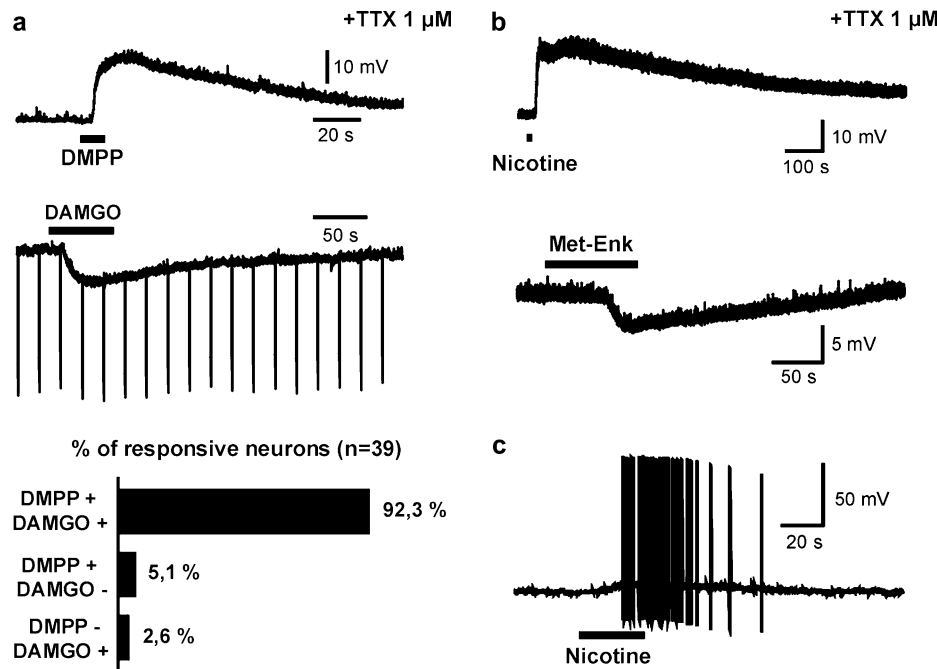
Altogether, these data confirmed that DAMGO responses were due to the activation of somatodendritic MORs in neocortical interneurons. DAMGO responses were not observed in pyramidal cells ( $n = 19$ ), consistent with the absence of the MOR mRNA in these neurons. To further characterize DAMGO responses, we determined current-voltage relationships in the presence of TTX (1  $\mu$ M), before and during DAMGO application (Fig. 2*b*). Voltage steps (2 s) were applied to responsive neurons in control condition and at the peak of the DAMGO response. The DAMGO-induced current reversed at  $-84.2 \pm 1.6$  mV ( $n = 4$ ), close to the equilibrium potential of potassium in our experimental conditions ( $-85$  mV). These results indicate that neocortical MOR activation leads to the opening of a potassium conductance, as described in other structures (Williams et al. 2001).

### Nicotinic and $\mu$ -Opioid Responsiveness Extensively Overlap in the Neocortex

In order to establish whether nicotinic receptors and MOR are expressed by the same neuronal population, we sequentially applied the nicotinic agonist DMPP (100  $\mu$ M) and the MOR-selective agonist DAMGO (2.5  $\mu$ M) on 61 neocortical interneurons visually identified from layers I to V. As exemplified in Figure 3*a*, the majority of responsive neurons (36 out of 39) responded to both DMPP and DAMGO applications, whereas 2 neurons responded only to DMPP and 1 responded only to DAMGO. All



**Figure 2.** MOR activation opens a potassium conductance. (*a*) DAMGO (2.5  $\mu$ M) induced a slow outward current with a peak amplitude of 32 pA (upper left panel, steps of  $-5$  mV were applied every 10 s) in a neuron recorded in voltage-clamp mode. Molecular analysis of this DAMGO-responsive neuron showed the expression of MOR together with GAD65, GAD67, VIP, and Enk (lower left panel,  $\phi$ : molecular weight marker). Intracellular labeling of the same neuron by biocytin (right panel) revealed a bipolar/bifurcated morphology typical of VIP-expressing interneurons (pial surface is upward). (*b*) Current-voltage plots obtained, in the presence of TTX (1  $\mu$ M), from another cell in control conditions (open circles, input resistance: 133.16 M $\Omega$ ) and at the peak of the DAMGO response (filled circles). The third plot (filled squares) shows the DAMGO-induced current obtained by subtracting control values from those measured during DAMGO application. The reversal potential for the DAMGO-induced current ( $-81.9$  mV) was close to equilibrium potential of potassium ( $-85$  mV in our experimental conditions).



**Figure 3.** Nicotinic and  $\mu$ -opioid agonists act on the same neocortical interneurons. Current-clamp recordings in (a, upper panels) show the responses of a neuron to sequential applications of the nicotinic agonist DMPP and the MOR-selective agonist DAMGO in the presence of TTX. In this neuron, DMPP (100  $\mu$ M) induced a fast depolarization (19.2 mV), whereas DAMGO (2.5  $\mu$ M) induced a hyperpolarization (-9.71 mV, steps of -50 pA were applied every 20 s). The lower panel shows the extensive overlap of nicotinic- and  $\mu$ -opioid-responsive populations. The example of responses to nicotine (100  $\mu$ M) and Met-Enk (30  $\mu$ M) shown in (b) illustrates their similarity with those induced by DMPP and DAMGO, respectively. In this neuron, sequential nicotine and Met-Enk application induced a 21.6 mV depolarization and a 4.9 mV hyperpolarization, respectively. As illustrated by the current-clamp recording in (c), application of nicotine at low concentration (1  $\mu$ M) resulted in a depolarization leading to a sustained action potential discharge in responsive interneurons.

layer I interneurons tested responded to both DMPP and DAMGO ( $n = 10$ ). Both DMPP and DAMGO responses persisted in the presence of TTX (1  $\mu$ M,  $n = 10$ ). As previously reported (Xiang et al. 1998; Porter et al. 1999; Christophe et al. 2002), nicotinic-responsive interneurons, found in all layers, were depolarized by DMPP applications ( $19.2 \pm 1.1$  mV,  $n = 10$ ). In contrast, DAMGO responses consisted of slow hyperpolarizations ( $-10.4 \pm 1.9$  mV,  $n = 10$ ). DMPP and DAMGO effects were mimicked by nicotine (100  $\mu$ M,  $n = 3$ ) and the endogenous MOR agonist methionine-enkephalin (Met-Enk, 30  $\mu$ M,  $n = 6$ ), respectively (Fig. 3b). Consistent with the expression of the high affinity  $\alpha 4$ ,  $\alpha 5$ , and  $\beta 2$  subunits (Ramirez-Latorre et al. 1996; Marubio and Changeux 2000) in nicotinic-responsive neocortical interneurons (Porter et al. 1999; Christophe et al. 2002), a low dose of nicotine (1  $\mu$ M) also depolarized these neurons and triggered action potentials ( $n = 3$ , Fig. 3c).

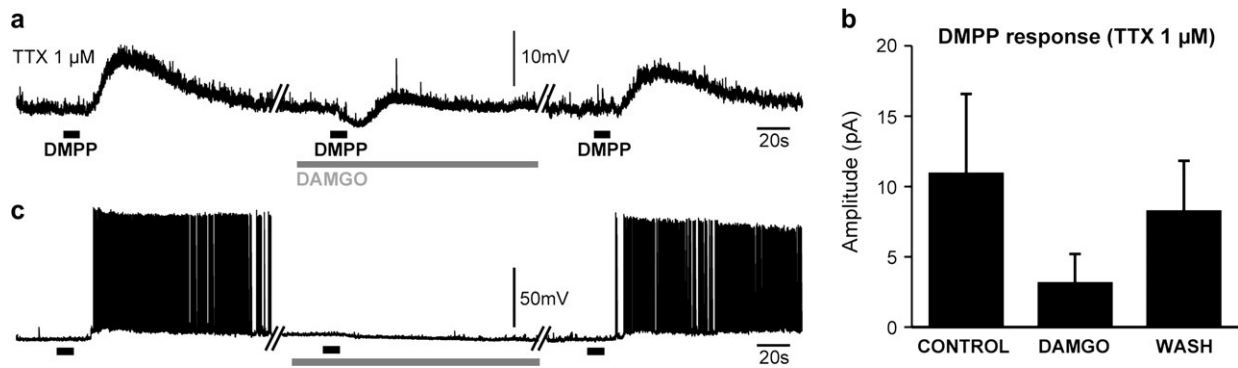
The almost complete overlap between nicotinic and  $\mu$ -opioid responses indicates that interactions between corresponding neurotransmitter pathways or their related drugs occur at the cellular level in the neocortex.

### Integration of Nicotinic and $\mu$ -Opioid Signals

Consequences of nicotinic receptor and MOR colocalization in neocortical interneurons were next investigated. Nonpyramidal neurons were recorded in current-clamp mode and exposed to the nicotinic agonist DMPP (100  $\mu$ M), in the absence or in the presence of DAMGO (2.5  $\mu$ M, Fig. 4). In the presence of TTX (1  $\mu$ M, see example in Fig. 4a), DMPP elicited a depolarization of responsive interneurons ( $10.9 \pm 5.7$  mV), which was reduced to 28% of control during DAMGO application ( $3.1 \pm 2.1$  mV) and recovered after 15 min of DAMGO washout ( $8.2 \pm 3.6$  mV,  $n = 5$ ,

Fig. 4b). In the absence of TTX, DMPP application elicited a sustained action potential discharge (see example in Fig. 4b). This DMPP response was prevented by DAMGO and recovered after DAMGO washout ( $n = 4$ ). These results indicate that interneurons integrate nicotinic and  $\mu$ -opioid signals at the somatodendritic level.

IPSCs elicited by cholinergic agonists in neocortical neurons are TTX sensitive (Xiang et al. 1998) and thus stem from somatodendritic excitation of GABAergic interneurons leading to action potential discharge. In order to evaluate the influence of nicotinic and  $\mu$ -opioid interactions on neocortical network activity, IPSCs were recorded in pyramidal cells that constitute one of the postsynaptic targets of both VIP-expressing interneurons (Hajos et al. 1988; Peters and Harriman 1988; Peters 1990) and layer I interneurons (Chu et al. 2003). DMPP applications (100  $\mu$ M) were performed in the absence or in the presence of DAMGO (2.5  $\mu$ M), whereas IPSCs were recorded in voltage-clamp mode. This set of experiments was performed in the presence of D-AP5 (20  $\mu$ M) and CNQX (10  $\mu$ M) to prevent glutamatergic transmission. DMPP stimulation of nicotinic-responsive interneurons induced, in the pyramidal neuron shown in Figure 5a,b, a large increase in IPSC frequency and amplitude. This DMPP effect was suppressed in the presence of DAMGO and recovered after DAMGO washout. Similar effects of DAMGO on the DMPP-induced increase of IPSCs were observed in 6 additional pyramidal cells and are summarized in Figure 5c. Mean inter-IPSC interval obtained from these 7 neurons during DMPP stimulation varied from  $0.17 \pm 0.0$  s for control DMPP application, to  $0.62 \pm 0.04$  s in the presence of DAMGO, and to  $0.22 \pm 0.01$  s following DAMGO washout (Mean inter-IPSC interval preceding DMPP application



**Figure 4.** Interneurons integrate nicotinic and  $\mu$ -opioid responses. (a) In the presence of TTX, DMPP (100  $\mu$ M) elicited a membrane depolarization that was decreased in the presence of DAMGO (2.5  $\mu$ M) and recovered after DAMGO washout. In this interneuron, the summation of DAMGO-induced hyperpolarization and of DMPP-induced depolarization resulted in a minimal deviation from the preset membrane potential. (b) In the presence of TTX, the amplitude of DMPP-induced depolarization was reversibly decreased by the application of DAMGO ( $n = 5$ ). (c) In the absence of TTX, DMPP elicited an action potential discharge that was prevented in the presence of DAMGO and recovered after DAMGO washout. In this interneuron, the depolarizing effect of DMPP was virtually abolished in the presence of DAMGO.

in control conditions:  $1.08 \pm 0.10$  s). Mean IPSC amplitudes varied from  $17.49 \pm 0.27$  pA for control DMPP application, to  $12.56 \pm 0.31$  pA in the presence of DAMGO, and to  $15.90 \pm 0.33$  pA after DAMGO washout (Mean IPSC amplitude preceding DMPP application in control conditions:  $12.23 \pm 0.39$  pA). These results show that modulation of interneuron activity via MOR and nicotinic receptors influences neocortical network activity. Furthermore, they confirm that expression patterns of these receptors overlap extensively and allow the selective integration of nicotinic and  $\mu$ -opioid stimuli by a single GABAergic interneuron population.

#### Nicotinic Activation of Endogenous $\mu$ -Opioid Transmission

The expression of the MOR endogenous agonist Enk in a large proportion of MOR-expressing neurons suggests that nicotinic stimulation of these neurons can induce the release of Enk and consequently activate MORs. To test this assumption, we investigated the effects of the MOR-selective antagonist CTOP on DMPP-induced GABAergic postsynaptic currents recorded in pyramidal cells. The changes in IPSC frequency elicited by prolonged DMPP applications (100  $\mu$ M, 30 s application), either in the absence (control,  $n = 11$ ) or in the continuous presence of CTOP (1  $\mu$ M,  $n = 11$ ), are illustrated in Figure 6. In control conditions, DMPP application elicited a biphasic response consisting of an initial increase of IPSC frequency, followed by a depression of GABAergic inhibitory inputs (Fig. 6a). Indeed, the mean IPSC interval decreased to  $42.49 \pm 0.04\%$  of its initial value (pre:  $1.48 \pm 0.49$  s, Fig. 6b) during the first phase and subsequently increased to  $222.4 \pm 0.07\%$  of the pre-DMPP level during the second period. In the presence of CTOP, the first phase was potentiated. Then, in contrast with control conditions, the IPSC frequency subsequently returned to the pre-DMPP value without undershooting the baseline (Fig. 6a). In CTOP conditions, the mean inter-IPSC interval first decreased to  $28.78 \pm 0.02\%$  of its initial value ( $1.05 \pm 0.40$  s) and subsequently returned to its initial level ( $98.93 \pm 0.03\%$ , Fig. 6b).

These results suggest that stimulation of nicotinic-responsive interneurons led to Enk release and subsequent MOR activation, which in turn resulted in the inhibition of these interneurons. Hence, the neocortical  $\mu$ -opioid system mediates an activity-

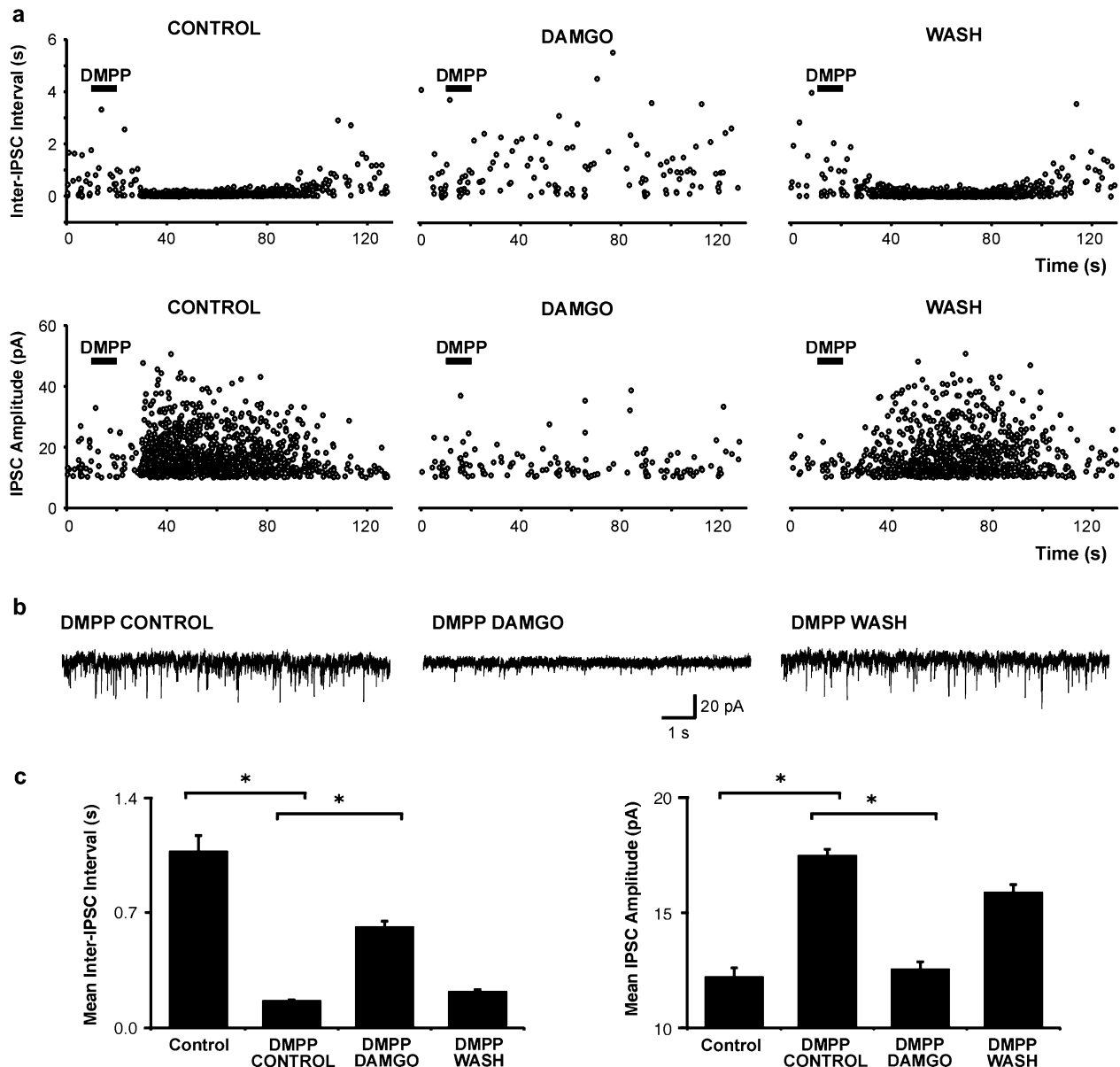
dependent, inhibitory feedback onto nicotinic-responsive interneurons.

#### Discussion

In the neocortex, we found a selective expression of MOR in interneurons responsive to nicotinic agonists throughout layers I-V. Enk was frequently expressed in MOR-expressing interneurons but was also observed in MOR-negative neurons. MOR agonists elicited a hyperpolarizing response that prevented the action potential discharge induced by nicotinic stimulation. MOR agonists also blocked the IPSC increase elicited in pyramidal neurons by application of a nicotinic agonist. Finally, nicotinic stimulation triggered a  $\mu$ -opioid inhibitory feedback on MOR-expressing interneurons through the release of endogenous Enk.

#### Expression Patterns of MOR and Enk

We found that in layers II-V, the MOR mRNA is primarily expressed in IS/RSNP VIP-positive interneurons, which frequently express CCK and CR and show a low occurrence of the SOM, NPY, and CB mRNAs. These results confirm a previous immunocytochemical report on MOR-expressing neurons (Taki et al. 2000) and are consistent with the known firing patterns and expression profile of VIPergic interneurons (Kawaguchi and Kubota 1996; Cauli et al. 1997, 2000; Porter et al. 1998, 1999). Although the detection of the MOR mRNA by scPCR was suboptimal, the detection of VIP in most DAMGO-responsive neurons in layers II-V confirms that MOR is primarily expressed in VIPergic interneurons in neocortical layers II-V. The MOR mRNA was additionally expressed in layer I interneurons characterized by a high occurrence of the NPY and CR mRNAs, which all responded to DAMGO. Layer I interneurons frequently express CR (Gonchar and Burkhalter 1999). Interestingly, these interneurons often exhibit neurogliaform morphology (DeFelipe and Jones 1988; Christophe et al. 2002) that correlates with the expression of NPY in the hippocampus (Price et al. 2005). Although layer I interneurons exhibit heterogeneity in their firing patterns and morphology (Zhou and Hablitz 1996a, 1996b; Chu et al. 2003), their extensive responsiveness to nicotinic agonists (Christophe



**Figure 5.** MOR controls the GABAergic output of nicotinic-responsive interneurons. DMPP-induced enhancement of spontaneous IPSCs in a pyramidal neuron was reduced in the presence of DAMGO and recovered after DAMGO washout. (a) Scatter plots showing the change in inter-IPSC interval (upper panel) and IPSC amplitude (lower panel) in response to DMPP application (100  $\mu$ M), in control condition (left panel), upon DAMGO application (2.5  $\mu$ M, middle panel) and after DAMGO washout (right panel). (b) Spontaneous IPSCs recorded in this neuron 30 s after the DMPP application in each condition. (c) Mean inter-IPSC interval and IPSC amplitude for data pooled from 7 cells in each condition. Asterisk,  $P \leq 0.05$ , Kruskal-Wallis one way analysis of variance on ranks followed by a Dunn's test.

et al. 2002) and to DAMGO indicates that almost all these neurons express both MOR and nicotinic receptors.

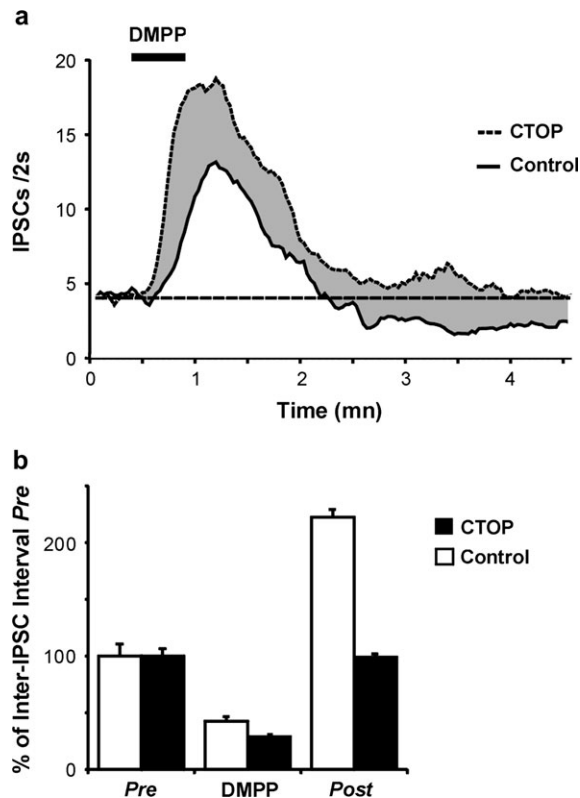
Consistent with a previous report (Taki et al. 2000), we found a high occurrence of the Enk mRNA in MOR-positive interneurons, with the notable exception of layer I cells. However, the presence of Enk in other neocortical neurons, including pyramidal cells, suggests that Enk expression is broader than appreciated by immunocytochemistry, as earlier observed by *in situ* hybridization (Harlan et al. 1987). Indeed, Enk is abundantly expressed in the neocortex where it is found at a concentration of 2.6 nmole/g protein as measured by radioimmunoassay (Rossier et al. 1977; Lindberg et al. 1979). For comparison (Crawley 1985), it is about 4 times more abundant than VIP (below 0.7 nmole/g protein), although immunocytochemistry reveals fewer cells positive for Enk than for VIP (Taki et al. 2000).

Furthermore, colchicine pretreatment allows Enk immunoreactivity to become detectable in pyramidal cells (McGinty et al. 1984). Hence, we propose that Enk is expressed in multiple neocortical neuronal types including pyramidal neurons, albeit at lower levels than in MOR-positive interneurons.

#### Neocortical $\mu$ -Opioid Transmission

Previous studies have established that  $\mu$ -opioid agonists inhibit the hippocampal network by inhibiting interneurons (Zieglansberger et al. 1979; Madison and Nicoll 1988; Capogna et al. 1993). Our observations indicate that in the neocortex,  $\mu$ -opioid agonists selectively inhibit interneurons excited by nicotinic agonists. Indeed, the overlap of nicotinic and  $\mu$ -opioid responsiveness encompassed a discrete interneuron population





**Figure 6.** Nicotinic stimulation activates the endogenous  $\mu$ -opioid system. Effect of DMPP (100  $\mu$ M, 30 s application) on IPSC frequency in pyramidal cells in the absence (control,  $n = 11$ ) or in the continuous presence of the MOR-selective antagonist CTOP (1  $\mu$ M,  $n = 11$ ). (a) Mean IPSC frequencies are expressed as the number of IPSCs per 2 s. DMPP increased IPSC frequency in both control and CTOP conditions. In control conditions, IPSC frequency subsequently fell below the baseline. This effect was prevented in the presence of CTOP. The gray area between the control and CTOP curves indicates the effect of MOR activation by endogenous Enk. (b) Same results presented as mean inter-IPSC interval measured before (pre, 0–30 s), during (DMPP, 50–100 s), and after (post, 180–280 s) the peak response to DMPP. Intervent intervals are expressed as percentages of the pre values. Note that post inter-IPSC interval differed significantly from pre values in control ( $P < 0.001$ ) but not in CTOP conditions ( $P = 0.88$ , 2-way analysis of variance completed by the Holm–Sidak method of comparison).

comprising cell types that reportedly exhibit nicotinic sensitivity (Porter et al. 1999; Christophe et al. 2002). In these interneurons,  $\mu$ -opioid agonists induced a hyperpolarizing response due to the opening of a potassium conductance, which was able to prevent action potential firing elicited by DMPP. Accordingly, DAMGO markedly decreased IPSCs evoked by DMPP in pyramidal cells. The present results further suggest that endogenous Enk released by nicotinic stimulation activates MOR, which in turn decreases IPSCs originating from nicotinic-responsive interneurons. Neuropeptide release requires a high level of activity and depends upon a sustained elevation of intracellular calcium (Zupanc 1996; Ludwig and Pittman 2003; Baraban and Tallent 2004). Upon nicotinic stimulation, the main source of Enk release in the neocortex is presumably VIP-expressing neurons. However, in addition to cholinergic inputs, VIP-expressing neurons integrate glutamatergic and serotonergic inputs that trigger intracellular calcium increase via ionotropic and metabotropic receptors (Staiger et al. 1996; Hajos et al. 1997; Porter et al. 1998; Cauli et al. 2000; Ferezou et al. 2002). In vivo, the release of Enk and thus the GABAergic contribution of MOR-expressing interneurons to the neocorti-

cal network are presumably controlled by summation of multiple local and afferent signals. The broad distribution of Enk suggests that other neocortical neuronal types participate in this process.

### Network Implications

The present results indicate that layer I and VIPergic interneurons are the main targets of  $\mu$ -opioid transmission in the neocortex. These interneuron populations influence the activity of pyramidal cells at all cortical layers (Hajos et al. 1988; Peters and Harriman 1988; Peters 1990; Kawaguchi and Kubota 1997; Zhu 2000; Larkum and Zhu 2002; Chu et al. 2003). However, because both interneuron types also innervate other interneurons (Hajos et al. 1988; Peters and Harriman 1988; Peters 1990; Kawaguchi and Kubota 1997; Christophe et al. 2002), the net effect of their activation or inhibition on pyramidal cell excitability is difficult to appreciate. Nonetheless, our findings suggest that sustained cholinergic stimulation of Layer I and VIP-expressing neurons results in a biphasic inhibitory/disinhibitory action on the network. During the disinhibitory phase,  $\mu$ -opioid transmission is able to reshape inhibition patterns and thus signal processing in the neocortical network by switching off the GABAergic output of Layer I and VIPergic interneurons. It is noteworthy that in addition to Enk, VIPergic interneurons also contain neurokinin B, corticotropin-releasing factor, and CCK, which selectively increase the excitability of pyramidal neurons (Gallopoin et al. 2005). The population of VIP-expressing neurons thus appears as an important source for the coordinated effects of several excitatory/disinhibitory neuropeptides in the neocortex.

### The $\mu$ -Opioid Involvement in Nicotine Effects

VIP-expressing and layer I interneurons coexpress the  $\alpha 4$ ,  $\alpha 5$ , and  $\beta 2$  nicotinic receptor subunits (Porter et al. 1999; Christophe et al. 2002) and MOR and thus constitute the primary target of both nicotine and opiates (Matthes et al. 1996; Marubio and Changeux 2000; Picciotto et al. 2000; Salas et al. 2003) in the neocortex. The present results suggest that, in this structure, nicotine intake is able to activate endogenous  $\mu$ -opioid transmission. Our findings thus provide a mechanistic explanation as to how  $\mu$ -opioid transmission can mediate part of nicotine effects and may help to explain the similarities between tobacco and opiate addiction (Pomerleau 1998; Mathieu-Kia et al. 2002).

### Notes

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