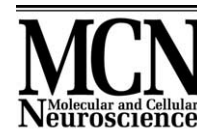




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## Two distinct forms of desensitization of G- protein coupled inwardly rectifying potassium currents evoked by alkaloid and peptide $\mu$ -opioid receptor agonists

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### Abstract

$\mu$ -Opioid receptors (MORs) activate G-protein coupled inwardly rectifying potassium (GIRK) channels. The peptide agonist [D-Ala<sup>2</sup>, NMe-Phe<sup>4</sup>, Gly<sup>5</sup>-ol]enkephalin (DAMGO), but not the alkaloid morphine (MS), leads to acute desensitization of this response. Furthermore, DAMGO, as opposed to MS, triggers rapid internalization of MORs. Given this dichotomy, we probed the relationship between receptor internalization and GIRK current desensitization in neurons of the locus coeruleus (LC) using acute rat brain slices. Interfering with MOR recycling by selective impairment of dynamin-dependent endocytosis left GIRK current desensitization unchanged. Conversely, coapplication of MS with a low concentration of DAMGO, a cocktail reported to enhance MOR internalization, revealed competition between the two agonists and normal desensitization. We also examined the case of methadone (MD), an alkaloid that has been reported to strongly trigger endocytosis. Interestingly, MD and other alkaloids agonists did induce GIRK current desensitization, but only at suprasaturating concentrations. Furthermore, responses to alkaloids were blunted in LC neurons and the same agonists inhibited GIRK currents in cells that do not express MORs. Our results indicate that two distinct forms of MOR-evoked GIRK current desensitization exist. Peptide agonists induce receptor-mediated desensitization while alkaloid agonists lead to apparent desensitization by receptor-independent inhibition of GIRK channels.

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### Introduction

Repetitive applications of opioid alkaloids such as morphine (MS), heroin, and methadone (MD) invariably induce physical dependence, which is defined by a stereotypical withdrawal syndrome following the termination of application. The induction of dependence needs the activation of  $\mu$ -opioid receptors (MORs), as mice with the targeted disruption of this receptor do not show any withdrawal syndrome when challenged with naloxone after chronic morphine administration (Matthes et al., 1996). The cellular and molecular mechanisms of dependence, although still elu-

sive, are believed to be associated with a form of neuroadaptation of MOR -signaling (Berke and Hyman, 2000; Nestler and Aghajanian, 1997), particularly in response to alkaloid agonists.

The prototypical agonist to induce dependence is the alkaloid morphine, whereas peptide agonists like [D-Ala<sup>2</sup>, NMe-Phe<sup>4</sup>, Gly<sup>5</sup>-ol]enkephalin (DAMGO) are claimed to be much less efficient (Noble et al., 1994; Stevens and Yaksh, 1989). These two groups of agonists also lead to very different responses when G-protein coupled inwardly rectifying potassium (GIRK) currents are monitored. Whereas Met-enkephalin (ME), endomorphine-1, and DAMGO at saturating concentrations elicit maximal GIRK currents that desensitize substantially within 15 min, the alkaloid morphine gives rise to smaller, but sustained currents (Blanchet and Lüscher, 2002). This observation has received considerable attention, since DAMGO, but not MS

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Table 1  
Maximal amplitude and desensitization of GIRK currents are unchanged by various kinase inhibitors.

	Control	Heparin (100 $\mu$ M)	Zinc (1 mM)	Staurosporine (10 $\mu$ M)	Genistein (30 $\mu$ M)	Wortmanin (0.5 $\mu$ M)
$I_{\max}$ (pA)	292 $\pm$ 25	318 $\pm$ 53	193 $\pm$ 50	402 $\pm$ 35	236 $\pm$ 34	208 $\pm$ 52
$P$ (compared to control)		0.70	0.10	0.04	0.21	0.17
Res.amplitude (%)	66 $\pm$ 2	68 $\pm$ 3	60 $\pm$ 5	64 $\pm$ 3	67 $\pm$ 3	64 $\pm$ 5
$P$ (compared to control)		0.41	0.09	0.41	0.59	0.85
$N$	19	5	5	5	5	5

Note. For further explanations see text. All results are expressed as mean  $\pm$  SEM.

triggers endocytosis of MORs heterologously expressed in mammalian cells within 15–30 min (Keith et al., 1996). It may therefore be possible that internalization could protect neurons from overstimulation and thus prevent adaptive processes eventually leading to dependence (reviewed in Kieffer and Evans, 2002). This possibility is at the origin of the RAVE (relative activity versus endocytosis) model, which predicts abuse-liability of a given agonist by the ratio of the relative efficacy and its capability to trigger receptor internalization (Whistler et al., 1999). Here, we submit this hypothesis to further scrutiny by monitoring GIRK responses of neurons in acute slices of the locus coeruleus (LC) elicited by the alkaloid agonists MD and fentanyl (Ftn). We also searched for functional roles of rapid receptor redistribution on MOR-activated GIRK currents by interfering with endocytosis of clathrin-coated vesicles. Our results suggest that MOR-induced desensitization of GIRK currents is independent of receptor redistribution. Furthermore, we provide evidence that alkaloids lead to an apparent form of desensitization through a MOR-independent inhibition of GIRK channels.

## Results

### Normal desensitization with impaired endocytosis

In LC neurons, DAMGO-elicited GIRK responses desensitized substantially within 15 min while morphine-induced responses did not (Blanchet and Lüscher, 2002). Interestingly, this dissociation for desensitization parallels the reported capability of these MOR agonists to drive internalization through clathrin-coated vesicles (Whistler et al., 1999), suggesting that desensitization may in fact be caused by a decreasing number of MORs at the cell surface, due to activity-driven endocytosis after receptor phosphorylation.

We therefore loaded the cells with the GPCR kinase (GRK) inhibitors heparin (100  $\mu$ M) or Zn (1 mM) through the patch pipette. Neither of the two compounds significantly altered desensitization of DAMGO-elicited currents (see Table 1). Reasoning that, in the case of MORs, other kinases may trigger the initial steps of internalization, we also studied DAMGO-elicited responses in cells dialyzed

with staurosporine (10  $\mu$ M, a concentration reported to block PKC and PKA (Tamaoki et al., 1986) or during extracellular application of the tyrosine kinase inhibitor genistein or the phosphatidylinositol-3-kinase inhibitor wortmanin (30 and 0.5  $\mu$ M, respectively; with 10–15 min preapplication). Again, in agreement with previous observations (Fiorillo and Williams, 1996; Harris and Williams, 1991; Osborne and Williams, 1995), no significant effect was observed (Table 1), suggesting that desensitization of MOR-elicited GIRK currents in LC neurons may be a phosphorylation-independent mechanism.

However, since concomitant same cell positive controls for kinase inhibitors to our knowledge are not available, we specifically blocked endocytosis of clathrin-coated vesicles with a peptide of 15 amino acids (D15, see Experimental Methods) coding for the interaction site of dynamin with amphiphysin (Wigge and McMahon, 1998) to directly address the role of MOR internalization in GIRK current desensitization. D15 was dialyzed into cells through the patch pipette and its efficiency at blocking endocytosis was assessed, after 20 min of recording, by the slowly developing increase of fast inhibitory postsynaptic currents elicited by stimulation of afferent fibers of LC neurons in the presence of the AMPAR blocker CNQX (193  $\pm$  30% versus 111  $\pm$  8% without D15,  $n$  = 5,  $P$  < 0.02, Fig. 1A and inset). Similar results were obtained by monitoring excitatory postsynaptic currents (not shown). In either case the developing increase of the amplitude of the synaptic current has been reported to reflect an increased number of receptors at the synapse (Kittler et al., 2000; Lüscher et al., 1999). In the presence of rapid constitutive recycling, a block of internalization leads to receptor accumulation at the surface as exocytosis continues unabated. However, despite this control of D15 efficacy in LC neurons of acute slices, subsequent GIRK current desensitization induced by DAMGO (1  $\mu$ M, 67  $\pm$  2%,  $n$  = 12) or ME (30  $\mu$ M, 54  $\pm$  2%,  $n$  = 5) was not different compared to control experiments without the peptide (67  $\pm$  2%,  $n$  = 19, and 52  $\pm$  2%,  $n$  = 15, respectively,  $P$  > 0.05; Fig. 1B). These results suggest that desensitization and MOR internalization are two independent neuroadaptive processes. This is in agreement with evidence that GIRK current desensitization may be mediated by a G-protein-dependent process interfering with GIRK channel activation (Blanchet and Lüscher, 2002).

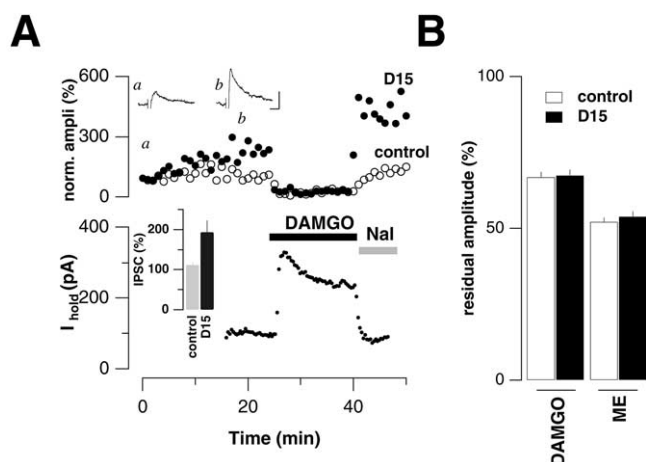


Fig. 1. Disruption of endocytosis does not prevent desensitization. A. Intracellular dialysis of the dominant negative D15 peptide (see Experimental Methods) effectively disrupted endocytosis as assessed by the increase in evoked GABA<sub>A</sub>-mediated inhibitory postsynaptic current (IPSC) amplitudes during the first 20 min of whole-cell recording (filled symbols, top) compared to the superimposed control experiment obtained in a different cell without D15 loading (open symbols). The subsequent application of DAMGO (1  $\mu$ M) elicited a GIRK current that still desensitized (bottom). These experiments were performed in the continuous presence of CNQX (10  $\mu$ M) to block AMPA-mediated EPSCs and DAMGO-elicited responses were reverted with naloxone (Nal; 1  $\mu$ M). Note that DAMGO-induced presynaptic inhibition of GABA<sub>A</sub>-mediated IPSCs did not desensitize (Blanchet and Lüscher, 2002). Insets: sample traces of evoked IPSCs in a D15 loaded cell at referred time (a, b) and normalized IPSC amplitude after 20 min with D15 compared to control. Scale bars for sample traces: 50 pA, 10 ms. (B) Bar graph representation of the mean ( $\pm$  SEM) normalized residual response after 15-min application of 1  $\mu$ M DAMGO (left) or 30  $\mu$ M Met-enkephalin (ME, right) under control conditions (empty bars,  $n = 19$  and 15, respectively) and in the presence of intracellular D15 (1–2 mM, filled bars;  $n = 12$  and 5, respectively). No significant difference between control and D15 conditions was found for any agonist.

#### DAMGO and morphine evoked currents: individual effects and coapplication

A recent study reported that a massive facilitation of MOR internalization occurs when morphine is coapplied with a low concentration of DAMGO, through a process of “pharmacological dragging” (He et al., 2002). However, the effects of this dragging on GIRK current desensitization have not been examined. We therefore examined whether coapplication of morphine and DAMGO enhanced desensitization.

Compared to high concentrations (1–10  $\mu$ M), DAMGO at 0.1  $\mu$ M led to modest desensitization (residual amplitude  $87 \pm 3\%$ ,  $n = 7$ , Fig. 2A and D). Similarly, currents induced by a saturating concentration of the partial agonist morphine remained almost stable during the application ( $92 \pm 5\%$ ,  $n = 5$ , Fig. 2B and D). Finally, coapplication of 10  $\mu$ M morphine plus 0.1  $\mu$ M DAMGO did not significantly increase desensitization of the induced current when compared to each agonist alone ( $83 \pm 4\%$ ,  $n = 6$ ,  $P > 0.05$ , Fig. 2C and D). Only competitive interactions between the two

agonists might have functional consequences since, when added on top of an established DAMGO response, morphine led to a reduction of the current amplitude, while in the reverse order, DAMGO led to an increase of the response (Fig. 2E and F).

Taken together, these observations indicate that the functional consequence of agonist coapplication is a competition between the full agonist DAMGO and the partial agonist morphine and that pharmacological dragging does not seem to enhance desensitization of GIRK currents, at least at the time scale of minutes.

#### Alkaloid agonists inhibit GIRK channels independent of MORs

Based on the parallelism of the efficacy to induce GIRK current desensitization and to trigger internalization for a

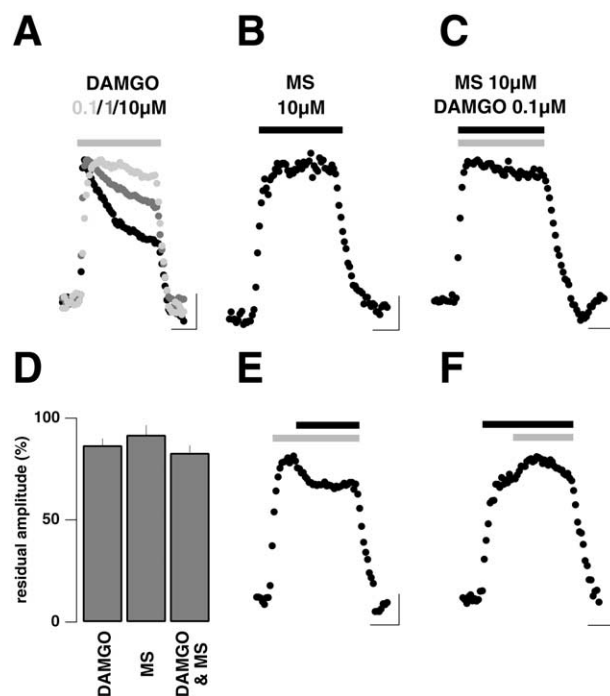


Fig. 2. Competition of morphine with a low concentration of DAMGO. A. Applications for 15 min of DAMGO (gray bar) at increasing concentrations induced increasingly desensitizing GIRK currents. Responses at 1 and 10  $\mu$ M (gray and black traces, scale bar 22%/5 min) were scaled to maximal amplitude of the trace obtained with 0.1  $\mu$ M (light-gray trace, 50 pA/5 min). B. In contrast morphine (MS; black bar), even at saturating doses, elicited nondesensitizing responses. C. Coapplication of 0.1  $\mu$ M DAMGO with 10  $\mu$ M morphine led to a slightly desensitizing response. D. Bar graph representation of normalized residual amplitude after 15-min application of 0.1  $\mu$ M DAMGO, 10  $\mu$ M MS, and 0.1  $\mu$ M DAMGO plus 10  $\mu$ M MS. Group data for the three cases did not show any significant differences. E and F. Competitive interaction between DAMGO (0.1  $\mu$ M, gray bar) and MS (10  $\mu$ M, black bar). When added on top of a response to DAMGO, MS inhibits the response (E), while in the reverse order, DAMGO increases the response (F). All data are expressed as means ( $\pm$  SEM),  $n = 5$ , scale bars 50 pA/5 min. All responses were antagonized with naloxone (1  $\mu$ M).

number of agonists, it has been argued that the two processes are closely linked (Alvarez et al., 2002). The case of MD is of particular interest, because it is an alkaloid that induces dependence, but also readily triggers MOR endocytosis. However, the degree of desensitization induced by MD is under debate. While with 1  $\mu\text{M}$  MD nondesensitizing responses were reported (Blanchet and Lüscher, 2002), higher concentrations (10–30  $\mu\text{M}$ ) led to desensitization (Alvarez et al., 2002). This prompted us to reevaluate this question by testing increasing concentrations of MD and other alkaloids in acute slices of the LC. At concentrations reported to saturate MORs (1  $\mu\text{M}$  MD, 0.1  $\mu\text{M}$  Ftn, 10  $\mu\text{M}$  MS; (Raynor et al., 1994)), alkaloid agonists elicited nondesensitizing currents (Fig. 3A, left; Fig. 2B). Beyond these concentrations, however, responses apparently desensitized to various extents (Fig. 3A and B). To account for cell-to-cell variability of response amplitudes, we normalized the maximal MOR-mediated currents to  $\alpha_2$  adrenergic receptor ( $\alpha_2\text{AR}$ ) mediated responses (known to activate the same population of GIRK channels; (North and Williams, 1985)). This revealed saturation of the MOR alkaloid agonist induced response because peak responses increased only slightly, or even decreased, at the high concentration range. In addition, we observed that MOR responses to alkaloids developed very slowly and never fully activated maximal GIRK currents even at high concentrations when compared to saturating DAMGO-mediated responses (compare traces in Figs. 3A and 2A). We also observed that reversal by naloxone of alkaloid-evoked currents revealed a downward shift of the baseline (Fig. 3A), which reversed polarity close to the equilibrium potential of K. This shift of the baseline may in fact reflect an inhibition of constitutively active GIRK currents (Lüscher et al., 1997) given that, at this concentration (1  $\mu\text{M}$ ), naloxone did not have any effect in the absence of MOR stimulation (not shown). Taken together, these observations raise the possibility that alkaloids may directly inhibit GIRK channels, as reported in heterologous systems (Ulens et al., 1999). To further test this possibility in the acute slice preparation, we elicited sustained  $\alpha_2\text{AR}$  mediated GIRK currents and applied MD (10  $\mu\text{M}$ ) in the presence of naloxone (1  $\mu\text{M}$ ), which caused a  $25 \pm 5\%$  inhibition of the current ( $n = 3$ , data not shown). However, the interpretation of this result is difficult as we cannot exclude unforeseen interactions at the MORs, particularly because the alkaloid naloxone may also affect GIRK channels (Ulens et al., 1999). We therefore directly tested the possibility of GIRK inhibition by MOR agonists in midbrain dopaminergic cells that do not express this receptor (Johnson and North, 1992). Indeed, DAMGO at 1–10  $\mu\text{M}$  did not have any effect on holding current ( $n = 5$ , data not shown) nor on GIRK currents elicited by the GABA<sub>B</sub> receptor agonist baclofen. In contrast, all alkaloid agonists reliably inhibited GIRK currents (Fig. 4). Interestingly, this receptor-independent GIRK channel inhibition was observed to a similar extent at the same alkaloid con-

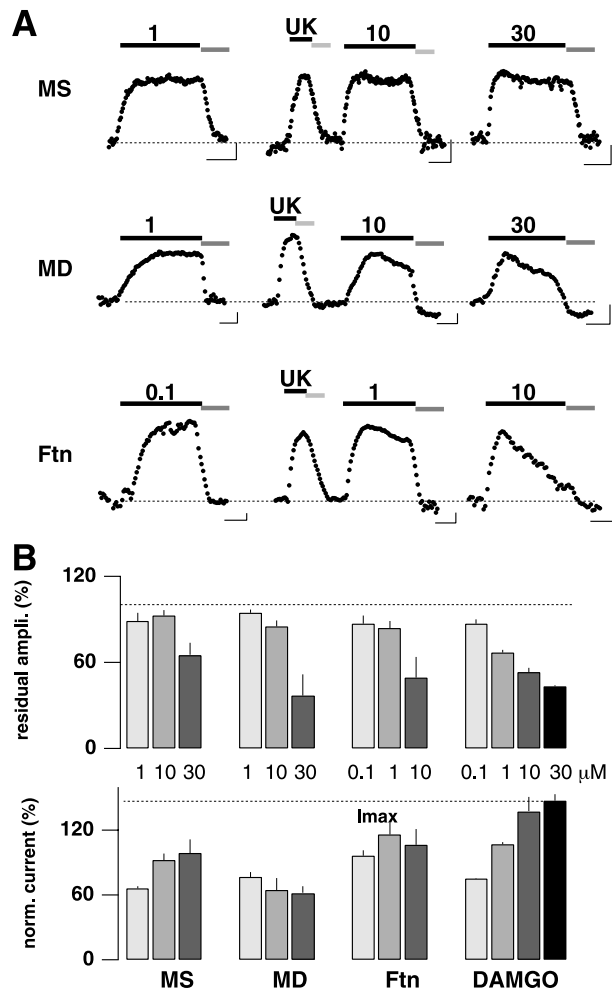


Fig. 3. Supramaximal concentrations of opioid alkaloids inhibit GIRK channels. A. Sustained applications (15 min) of MS, MD, and Ftn at increasing concentrations in a dose-dependent manner induced apparent desensitization of GIRK currents in cells of the LC. Examples of  $\alpha_2\text{AR}$  responses (UK14304, 3  $\mu\text{M}$ ), used to normalize MOR responses in B, are shown only on some traces for clarity. B. Bar graph representation of normalized residual amplitude after 15 min of MOR agonist application (top) and of maximal responses to MOR agonists normalized to maximal  $\alpha_2\text{AR}$ -mediated response (bottom). The dotted line in the lower panel represents the maximal current amplitude ( $I_{\text{max}}$ ) obtained with a saturating concentration of DAMGO. All data are expressed as means ( $\pm$  SEM),  $n = 3$ –10, scale bars 50 pA/5 min. All responses were antagonized with naloxone (1  $\mu\text{M}$ , gray bars) and yohimbine (10  $\mu\text{M}$ , light-gray bars), respectively.

centrations that induced apparent desensitization of MOR responses in LC neurons.

## Discussion

Desensitization of MOR-evoked GIRK currents, broadly defined as a decrease in the response in the continuous presence of an agonist, is a form of neuroadaptation and may therefore be associated with dependence. As alkaloid agonists are considered to have a higher abuse liability

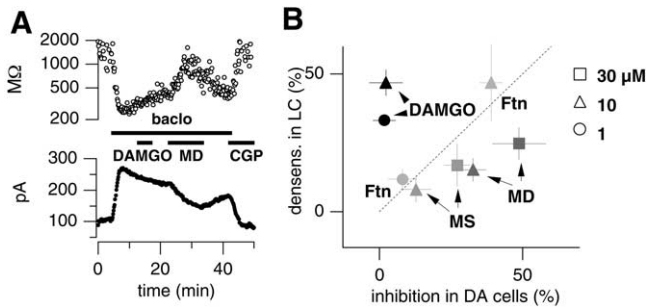


Fig. 4. Alkaloids inhibit GIRK currents. A. Example trace obtained from a midbrain dopaminergic cell that does not express MORs. MD ( $30 \mu\text{M}$ ) inhibited the baclofen (baclo)-induced current while DAMGO had no effect (lower trace). Increasing input resistance during alkaloid application confirmed channel closure. The baclofen-induced response was antagonized by CGP54626 (CGP;  $2 \mu\text{M}$ ). B. Apparent desensitization in LC neurons induced by alkaloid and peptide agonists plotted versus inhibition of GIRK currents in midbrain neurons at 8 min of MOR agonist application. Points representing alkaloid agonist fell close to the line of identity, whereas DAMGO in the presence of substantial desensitization in the LC had no effect on dopaminergic cells. All data are expressed as means ( $\pm$  SEM),  $n = 4-6$ .

compared to peptide agonists, any difference in signaling and desensitization may have implications for the understanding of dependence. Our results now indicate that desensitization occurs with both groups of agonists, but through two different mechanisms.

#### *MOR-dependent GIRK currents desensitize in response to alkaloid agonists through a receptor-independent inhibition of GIRK channels*

The alkaloid MOR agonists MS, Ftn, and MD tested in the present study led to sustained GIRK currents up to saturating concentrations. Beyond these concentrations, however, we observed strong desensitization, in line with recent data reported for MD (Alvarez et al., 2002). Moreover, the maximal amplitude, particularly in response to a full agonist MD, was blunted, which suggests a direct effect on the GIRK channels. This is supported by the observation that after the termination of the response the current fell below baseline, thus affecting constitutively active GIRK channels (Lüscher et al., 1997). Finally, even in cells that do not express MORs, GIRK currents evoked by another GPCR agonist were inhibited by concentrations of the alkaloids MS, MD, and Ftn that trigger apparent desensitization of the MOR-mediated response in LC neurons. The observation that GIRK channel inhibition in midbrain dopaminergic cells was typically larger than the apparent MOR-mediated response desensitization in LC neurons (Fig. 4D) as well as the generally slower onset of the alkaloid-mediated responses may be explained by concomitant GIRK activation and inhibition in these latter cell types, actually blunting the peak response. This is further supported by the observation that the agonist MD elicited peak responses that were significantly smaller than maximal

MOR responses as elicited with a saturating concentration of DAMGO and actually decreased with increasing agonist concentration (Fig. 3B). These results demonstrate a novel form of desensitization and imply that at least two distinct forms of desensitization exist: a receptor-independent form, where alkaloids may directly inhibit GIRK channels, and the receptor-induced desensitization triggered by peptide agonists. A similar inhibition has been described for GIRK1/GIRK2 containing channels expressed in oocytes (Ulens et al., 1999) with the exception of MS. MS, in our hands, led to receptor-independent desensitization, but to a lesser degree than the others alkaloids tested. This difference could be due to a different subunit constitution in the cells studied here. Indeed, LC neurons have been shown to express GIRK channels that also contain the GIRK3 subunit (Torrecilla et al., 2002). Interestingly, non-opioid alkaloids like bupivacaine and QX314 have also been reported to inhibit GIRK channels (Slesinger, 2001; Zhou et al., 2001), which raises the possibility that our observations may apply to a whole range of alkaloids.

#### *MOR internalization does not cause desensitization of MOR-induced GIRK currents*

A further difference between alkaloids and peptide MOR agonists has been described in heterologous systems (Keith et al., 1996) and myenteric neurons in vivo (Sternini et al., 1996), where prolonged DAMGO but not morphine application induces MOR internalization (Keith et al., 1996; Whistler and von Zastrow, 1998); for reviews see (Law et al., 2000; Tsao and Zastrow, 2000). This occurs after receptor phosphorylation through clathrin- and dynamin-dependent endocytosis (Ferguson, 2001). It is therefore tempting to assume that internalization is closely linked to or even causes desensitization (Alvarez et al., 2002). There is, however, an obvious exception to this rule. MD has been shown to trigger internalization (Whistler et al., 1999) at concentrations that do not induce receptor dependent desensitization (Blanchet and Lüscher, 2002). Moreover, our attempts to reduce desensitization of DAMGO and ME-elicited responses by preventing receptor phosphorylation and inhibiting internalization were not successful. The results obtained in expression systems implicating receptor phosphorylation and internalization (Kovoor et al., 1997, 1998; Whistler et al., 1999) may in part be explained by the high receptor density, which may enhance endocytotic removal of functional receptors from the cell surface and lead to fast apparent desensitization. This would also explain why desensitization occurs faster in heterologous systems (Kovoor et al., 1995), while it takes minutes in neurons of acute brain slices. Based on the results of the present study, we favor a model where receptor-dependent desensitization and internalization are molecularly distinct processes. The characteristics of receptor-dependent desensitization are provided in our previous work (Blanchet and Lüscher, 2002) and

implicate a distinct MOR-dependent inhibitory pathway responsible for GIRK current desensitization.

Coapplication of morphine with a low concentration of DAMGO has been shown to facilitate internalization of MORs. This phenomenon, coined pharmacological dragging, was supposed to depend on oligomerization of morphine-bound with DAMGO-bound MORs (He et al., 2002). It is therefore plausible that pharmacological dragging could lead to desensitization through receptor internalization, even if endocytosis is normally not required. Our observations that morphine partially inhibits DAMGO-induced GIRK current, while DAMGO enhances the response to morphine, demonstrate that pharmacological dragging does not enhance MOR-dependent desensitization and that the functional consequence of coapplication of morphine and DAMGO on GIRK currents is competitive rather than cooperative.

#### *Possible functional consequences of MOR-induced desensitization*

An intriguing observation is that agonists that readily induce dependence like MS, MD, and Ftn do not cause receptor-dependent desensitization and induce receptor-independent desensitization only at supersaturating concentrations. It therefore seems unlikely that dependence results from desensitization. The low affinity of the receptor-independent desensitization may be indicative of an important role for this process in the context of opioid toxicity (e.g., overdose). Contrary to initial assumptions, receptor-dependent desensitization may in fact protect neurons from the overstimulation that causes the induction of dependence. Absence of receptor-dependent desensitization may therefore be used as a predictor of dependence, which implies that *sustained* receptor signaling is necessary for the induction of dependence. Taking desensitization rather than internalization (RAVE hypothesis) as the predictive parameter has the advantage that it would also apply to MD, which is considered an exception to the RAVE hypothesis as it triggers endocytosis yet induces strong dependence (Ling et al., 1984).

In the model proposed here, alkaloids would lead to sustained receptor activation even at very high concentrations, where GIRK currents desensitize through direct channel inhibition, but receptor signaling to other effectors continues unabated. It is this sustained signaling, which eventually may induce dependence, for example, through neuroadaptation of the adenylyl cyclase pathway (Nestler, 2001).

## **Experimental methods**

### *Electrophysiology in acute slices*

Horizontal pontine slices (300  $\mu\text{m}$  thick, VT1000 vibratome, Leica) were prepared from P10–P21 Sprague–

Dawley rat brains (after decapitation according to the guidelines of the Cantonal Veterinary Office of Geneva) in cooled artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 119, KCl 2.5,  $\text{MgCl}_2$  1.3,  $\text{CaCl}_2$  2.5,  $\text{NaH}_2\text{PO}_4$  1.0,  $\text{NaHCO}_3$  26.2, and glucose 11, and continuously bubbled with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . Slices were progressively warmed up to 32–34°C, left to recover for at least 1 h, and then transferred to the recording chamber superfused (2 ml/min) with ACSF. The visualized whole-cell voltage-clamp recording technique was used to measure holding currents and synaptic responses of LC adrenergic neurons. To evoke stable synaptic currents, we delivered stimuli (0.1-ms duration) at 0.05 Hz through bipolar stainless-steel electrodes positioned just caudal to the LC. The internal solution contained (in mM): K-gluconate 140, NaCl 4,  $\text{MgCl}_2$  2, EGTA 1.1, Hepes 5,  $\text{Na}_2\text{ATP}$  2,  $\text{Na}_2$ -creatine-phosphate 5, and  $\text{Na}_3\text{GTP}$  0.6, pH adjusted to 7.3, with 7–8 mM KOH. In some experiments, heparin (100  $\mu\text{M}$ ),  $\text{ZnCl}_2$  (1 mM), staurosporine (10  $\mu\text{M}$ ), or a 15-amino-acid peptide (D15) coding for the interaction site of dynamin with amphiphysin (position 828–42, PPPQVPSRPNRAPPG 1–2 mM, custom synthesized at the Institute of Biochemistry, Univ. Zurich, Switzerland) were added to the internal solution. Currents were amplified (Visual patch 500, Bio-logic, France and Axopatch ID, Axon Instruments, USA), filtered at 1 kHz, and digitized at 5 kHz (National Instruments Board PCI-MIO-16E4, NI-DAQ Igor Software, Wave Metrics, Lake Oswego, OR, USA) and stored on a hard disk. Cells were clamped at either  $-63$  or  $-73$  mV and elementary recordings consisted of sweeps of 1.2–3 s every 10–20 s. In each sweep, multiple steps were imposed with 10-mV increments up to  $-113$  mV to measure cell membrane and access resistance and test for electrotonic coupling of LC neurons with other cells of the LC via gap junctions (Alvarez-Maubecin et al., 2000; Christie et al., 1989). Desensitization in coupled neurons on average was slightly smaller (<5%). This difference, however, did not reach significance under any experimental condition and therefore data from apparently coupled and uncoupled LC neurons were compiled. Voltage shifts due to liquid junction potential ( $-13$  mV) were corrected.

Compiled data are expressed as means  $\pm$  SEM. For statistical comparisons the nonparametric Mann–Whitney or Wilcoxon matched tests were used and the level of significance was taken at  $P = 0.05$ .

### *Drugs*

CNQX and balcofen was from Tocris, DAMGO, naloxone, ME, staurosporine, heparin, genistein, and wortmannin were from Sigma MS and MD were from Amino AG (Neuenhof, Switzerland), and Ftn was from Sintetica (Lugano, Switzerland).

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## References

- Alvarez, V.A., Arttamangkul, S., Dang, V., Salem, A., Whistler, J.L., Von Zastrow, M., Grandy, D.K., Williams, J.T., 2002.  $\mu$ -Opioid receptors: ligand-dependent activation of potassium conductance, desensitization, and internalization. *J. Neurosci.* 22, 5769–5776.
- Alvarez-Maubecin, V., Garcia-Hernandez, F., Williams, J.T., Van Bockstaele, E.J., 2000. Functional coupling between neurons and glia. *J. Neurosci.* 20, 4091–4098.
- Berke, J.D., Hyman, S.E., 2000. Addiction, dopamine, and the molecular mechanisms of memory. *Neuron* 25, 515–532.
- Blanchet, C., Lüscher, C., 2002. Desensitization of  $\mu$ -opioid receptor evoked potassium currents: initiation at the receptor, expression at the effector. *Proc. Natl. Acad. Sci. USA* 99, 4674–4679.
- Christie, M.J., Williams, J.T., North, R.A., 1989. Electrical coupling synchronizes subthreshold activity in locus coeruleus neurons in vitro from neonatal rats. *J. Neurosci.* 9, 3584–3589.
- Ferguson, S.S., 2001. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol. Rev.* 53, 1–24.
- Fiorillo, C.D., Williams, J.T., 1996. Opioid desensitization: interactions with G-protein-coupled receptors in the locus coeruleus. *J. Neurosci.* 16, 1479–1485.
- Harris, G.C., Williams, J.T., 1991. Transient homologous  $\mu$ -opioid receptor desensitization in rat locus coeruleus neurons. *J. Neurosci.* 11, 2574–2581.
- He, L., Fong, J., von Zastrow, M., Whistler, J.L., 2002. Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization. *Cell* 108, 271–282.
- Johnson, S.W., North, R.A., 1992. Opioids excite dopamine neurons by hyperpolarization of local interneurons. *J. Neurosci.* 12, 483–488.
- Keith, D.E., Murray, S.R., Zaki, P.A., Chu, P.C., Lissin, D.V., Kang, L., Evans, C.J., von Zastrow, M., 1996. Morphine activates opioid receptors without causing their rapid internalization. *J. Biol. Chem.* 271, 19021–19024.
- Kieffer, B.L., Evans, C.J., 2002. Opioid tolerance—in search of the holy grail. *Cell* 108, 587–590.
- Kittler, J.T., Delmas, P., Jovanovic, J.N., Brown, D.A., Smart, T.G., Moss, S.J., 2000. Constitutive endocytosis of GABAA receptors by an association with the adaptin AP2 complex modulates inhibitory synaptic currents in hippocampal neurons. *J. Neurosci.* 20, 7972–7977.
- Kovoor, A., Celver, J.P., Wu, A., Chavkin, C., 1998. Agonist induced homologous desensitization of  $\mu$ -opioid receptors mediated by G protein-coupled receptor kinases is dependent on agonist efficacy. *Mol. Pharmacol.* 54, 704–711.
- Kovoor, A., Henry, D.J., Chavkin, C., 1995. Agonist-induced desensitization of the  $\mu$  opioid receptor-coupled potassium channel (GIRK1). *J. Biol. Chem.* 270, 589–595.
- Kovoor, A., Nappey, V., Kieffer, B.L., Chavkin, C., 1997.  $\mu$  and  $\delta$  opioid receptors are differentially desensitized by the coexpression of beta-adrenergic receptor kinase 2 and beta-arrestin 2 in xenopus oocytes. *J. Biol. Chem.* 272, 27605–27611.
- Law, P.Y., Erickson, L.J., El-Kouhen, R., Dicker, L., Solberg, J., Wang, W., Miller, E., Burd, A.L., Loh, H.H., 2000. Receptor density and recycling affect the rate of agonist-induced desensitization of  $\mu$ -opioid receptor. *Mol. Pharmacol.* 58, 388–398.
- Ling, G.S., Tappe, N.S., Inturrisi, C.E., 1984. Methadone induced physical dependence in the rat. *Life Sci.* 34, 683–690.
- Lüscher, C., Jan, L.Y., Stoffel, M., Malenka, R.C., Nicoll, R.A., 1997. G protein-coupled inwardly rectifying K<sup>+</sup> channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons. *Neuron* 19, 687–695.
- Lüscher, C., Xia, H., Beattie, E.C., Carroll, R.C., von Zastrow, M., Malenka, R.C., Nicoll, R.A., 1999. Role of AMPA receptor cycling in synaptic transmission and plasticity. *Neuron* 24, 649–658.
- Matthes, H.W., Maldonado, R., Simonin, F., Valverde, O., Slowe, S., Kitchen, I., Befort, K., Dierich, A., Le Meur, M., Dolle, P., et al., 1996. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the  $\mu$ -opioid-receptor gene. *Nature* 383, 819–823.
- Nestler, E.J., 2001. Molecular basis of long-term plasticity underlying addiction. *Nat. Rev. Neurosci.* 2, 119–128.
- Nestler, E.J., Aghajanian, G.K., 1997. Molecular and cellular basis of addiction. *Science* 278, 58–63.
- Noble, F., Coric, P., Turcaud, S., Fournie-Zaluski, M.C., Roques, B.P., 1994. Assessment of physical dependence after continuous perfusion into the rat jugular vein of the mixed inhibitor of enkephalin-degrading enzymes, RB 101. *Eur. J. Pharmacol.* 253, 283–287.
- North, R.A., Williams, J.T., 1985. On the potassium conductance increased by opioids in rat locus coeruleus neurons. *J. Physiol.* 364, 265–280.
- Osborne, P.B., Williams, J.T., 1995. Characterization of acute homologous desensitization of  $\mu$ -opioid receptor-induced currents in locus coeruleus neurons. *Br. J. Pharmacol.* 115, 925–932.
- Raynor, K., Kong, H., Chen, Y., Yasuda, K., Yu, L., Bell, G.I., Reisine, T., 1994. Pharmacological characterization of the cloned kappa-, delta-, and  $\mu$ -opioid receptors. *Mol. Pharmacol.* 45, 330–334.
- Slesinger, P.A., 2001. Ion selectivity filter regulates local anesthetic inhibition of G-protein-gated inwardly rectifying K<sup>+</sup> channels. *Biophys. J.* 80, 707–718.
- Sternini, C., Spann, M., Anton, B., Keith Jr., D.E., Bunnett, N.W., von Zastrow, M., Evans, C., Brecha, N.C., 1996. Agonist-selective endocytosis of  $\mu$  opioid receptor by neurons in vivo. *Proc. Natl. Acad. Sci. USA* 93, 9241–9246.
- Stevens, C.W., Yaksh, T.L., 1989. Magnitude of opioid dependence after continuous intrathecal infusion of  $\mu$ - and  $\delta$ -selective opioids in the rat. *Eur. J. Pharmacol.* 166, 467–472.
- Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., Tomita, F., 1986. Staurosporine, a potent inhibitor of phospholipid/Ca<sup>++</sup> dependent protein kinase. *Biochem. Biophys. Res. Commun.* 135, 397–402.
- Torreccilla, M., Marker, C.L., Cintora, S.C., Stoffel, M., Williams, J.T., Wickman, K., 2002. G-protein-gated potassium channels containing Kir3.2 and Kir3.3 subunits mediate the acute inhibitory effects of opioids on locus coeruleus neurons. *J. Neurosci.* 22, 4328–4334.
- Tsao, P., Zastrow, M., 2000. Downregulation of G protein-coupled receptors. *Curr. Opin. Neurobiol.* 10, 365–369.
- Ulens, C., Daenens, P., Tytgat, J., 1999. The dual modulation of GIRK1/GIRK2 channels by opioid receptor ligands. *Eur. J. Pharmacol.* 385, 239–245.
- Whistler, J.L., Chuang, H.H., Chu, P., Jan, L.Y., von Zastrow, M., 1999. Functional dissociation of  $\mu$  opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron* 23, 737–746.
- Whistler, J.L., von Zastrow, M., 1998. Morphine-activated opioid receptors elude desensitization by beta-arrestin. *Proc. Natl. Acad. Sci. USA* 95, 9914–9919.
- Wigge, P., McMahon, H.T., 1998. The amphiphysin family of proteins and their role in endocytosis at the synapse. *Trends Neurosci.* 21, 339–344.
- Zhou, W., Arrabit, C., Choe, S., Slesinger, P.A., 2001. Mechanism underlying bupivacaine inhibition of G protein-gated inwardly rectifying K<sup>+</sup> channels. *Proc. Natl. Acad. Sci. USA* 98, 6482–6487.