

Flupirtine shows functional NMDA receptor antagonism by enhancing Mg²⁺ block via activation of voltage independent potassium channels

Rapid Communication

J. Kornhuber¹, S. Bleich¹, J. Wiltfang¹, M. Maler¹, and C. G. Parsons²

¹Department of Psychiatry, University of Göttingen, and

²Department of Pharmacology, Frankfurt am Main,
Federal Republic of Germany

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Summary. The spectrum of action of flupirtine includes analgesia, muscle relaxation and neuroprotection. N-methyl-D-aspartate (NMDA) receptor antagonism has been discussed as a possible mechanism of action of this compound with little direct evidence. The objective of the present study was to develop a plausible model to explain flupirtine's spectrum of action. A four-stage strategy was selected for this purpose: Firstly, the serum concentration of flupirtine under therapeutic conditions was determined on the basis of the current literature. The second stage involved assessing the known in-vitro effects in light of the therapeutic active concentration. Using whole cell patch clamp recordings from cultured rat superior colliculus neurones interactions between flupirtine and NMDA receptors were assessed. Only very high concentrations of flupirtine antagonized inward currents to NMDA (200 μM) at -70 mV with an IC₅₀ against steady-state responses of 182.1 ± 12.1 μM. The effects of flupirtine were voltage-independent and not associated with receptor desensitization making actions within the NMDA receptor channel or at the glycine modulatory site unlikely. NMDA receptor antagonism probably has little relevance for the clinical efficacy of flupirtine as the concentrations needed were far higher than those achieved in clinical practice. However, the activation of a G-protein-regulated inwardly rectifying K⁺ channel was identified as an interesting molecular target site of flupirtine. In the next stage, the central nervous spectrum of action of experimental K⁺ channel openers (PCO) was considered. As far as they have been studied, experimental K⁺ channel openers display a spectrum of action comparable to that of flupirtine. In the final stage, a global model was developed in which flupirtine stabilizes the resting membrane potential by activating inwardly rectifying K⁺ channels,

thus indirectly inhibiting the activation of NMDA receptors. The model presented here reconciles the known functional NMDA receptor antagonism of flupirtine with the activation of K⁺ channels that occurs at therapeutic concentrations, thus providing an understanding of flupirtine's spectrum of action. This makes flupirtine the prototype of a clinically applicable substance group with analgesic, muscle-relaxant and neuroprotective properties.

Keywords: Potassium channel, inwardly rectifying potassium channel, GIRK, flupirtine, N-methyl-D-aspartate receptor antagonist, patch clamp, superior colliculus culture.

Flupirtine (ethyl-N-[2-amino-6-(4-fluorophenylmethylamino)pyridin-3-yl] carbamate) has been in clinical use for many years as a centrally active analgesic with muscle-relaxant properties (Friedel and Fitton, 1993). In preclinical and preliminary clinical studies, neuroprotective (Schuster et al., 1999), antiepileptic (Sheridan et al., 1986) and antiparkinsonian (Schwarz et al., 1996) effects were additionally found. The mechanism of action of flupirtine has not been clear up to now. Although flupirtine does not have relevant affinity for any known recognition site on the NMDA receptor complex in binding studies (Osborne et al., 1996, 1998), antagonism of this receptor has recently been discussed at length as a possible mechanism of action of this compound (Osborne et al., 1994; Perovic et al., 1994; Schwarz et al., 1994, 1995; Timmann et al., 1995). This assumption is the expression of a misleading tendency to propose a precise mechanism of action at the receptor level purely on the basis of results from behavioral studies or neurotoxicity studies in vitro with inadequate pharmacological characterization of the interactions observed. Such speculations obviously neglect the complexity of neuronal networks, interactions between neuronal systems and the importance of processes up- and downstream of receptor activation in mediating end point parameters such as cell death. In view of this it seemed pertinent to test for possible interactions between flupirtine and NMDA receptors using whole cell patch clamp recordings from cultured neurones. This approach is better suited for the detection of direct antagonists or agonists at any site on the NMDA receptor and thus circumvents the need to propose yet another modulatory site if no interaction is observed.

Patch clamp recordings from cultured superior colliculus neurones were performed as described previously (Parsons et al., 1993a). In brief, superior colliculi were isolated from embryonic rats (E20–21) and maintained in culture for 11–14 days in NaHCO₃/HEPES-buffered minimum essential medium supplemented with 5% foetal calf serum and 5% horse serum (Gibco) and incubated at 37°C with 5% CO₂ (95% humidity). The superior colliculus culture was chosen for these experiments as it provides very stable recording conditions which are an absolute prerequisite for voltage-dependency and kinetic experiments. Moreover, the relatively small neurones (soma 15–20 μm Ø) are ideally suited to minimise problems of buffered diffusion for concentration clamp experiments. Finally, our own unpublished data indicate that

the somatic NMDA receptors expressed in cultured hippocampal and cortical neurones are similar.

Patch clamp recordings were made from these neurones with polished glass electrodes (4–5 M Ω) in the whole cell mode at room temperature (20–22°C) with the aid of an EPC-7 amplifier (List). Test substances were applied by switching channels of a custom made fast superfusion system with a common outflow (<10 ms exchange times). The contents of the intracellular solution were as follows (mM): CsCl (120), TEACl (20), EGTA (10), MgCl₂ (1), CaCl₂ (0.2), Glucose (10), ATP (2), cAMP (0.25); pH was adjusted to 7.3 with CsOH or HCl. The extracellular solutions had the following basic composition (mM): NaCl (140), KCl (3), CaCl₂ (0.2), glucose (10), HEPES (10), sucrose (4.5), tetrodotoxin (0.3 μ M), glycine (1 μ M), (pH 7.3). Flupirtine maleate was a generous gift of G. Pergande, ASTA Medica, Frankfurt. All other compounds were obtained from Sigma.

Very high concentrations of flupirtine antagonized inward current responses to NMDA (200 μ M) at -70 mV (Fig. 1A) with an IC₅₀ against steady-state responses of 182.1 ± 12.1 μ M (Fig. 1B). Flupirtine did not enhance glycine-dependent desensitization in the continuous presence of the non-saturating concentration of glycine (1 μ M), as evidence by the similar potency against the peak component of NMDA-induced currents (IC₅₀ = 228.6 ± 8.9 μ M). This probably excludes antagonistic actions at the strychnine-insensitive glycine modulatory (glycine_B) site of the NMDA receptor complex (Mayer et al., 1989; Parsons et al., 1993b) as previous observations indicate that most moderate to low affinity full glycine_B antagonists are three to ten times more potent against steady-state than against peak currents. The effects of flupirtine (300 μ M) were not voltage-dependent (Fig. 1C) and were apparently not use-dependent making channel blockade an unlikely candidate as the mechanism of NMDA receptor antagonism (Parsons et al., 1995). The Hill coeff. for NMDA receptor antagonism was close to unity and does not give any indication for cooperativity. Flupirtine alone did not evoke any measurable inward or outward current which also excludes direct agonistic actions at inhibitory GABA_A or classical glycine_A receptors as a possible mechanism of action as both of these receptors are expressed in these cultures and can be activated by their respective agonists under the conditions used (Parsons et al., 1993a).

The further intention of the present contribution was to provide an understanding of the mechanism of action. A four-stage strategy was selected to this end. The first stage was to determine the therapeutic serum concentration on the basis of the published data. In a second stage, the in-vitro effects of flupirtine were assessed to determine whether they are relevant under therapeutic conditions. In the next stage, flupirtine's spectrum of action was compared with that of experimental K⁺ channel openers. Finally, a global model was developed to explain the clinical spectrum of action. In this contribution, we show that the spectrum of action of flupirtine can be understood within the context of the opening of inwardly rectifying K⁺ channels. Flupirtine is thus the prototype of a new clinically applicable substance group with analgesic, muscle-relaxant and neuroprotective properties.

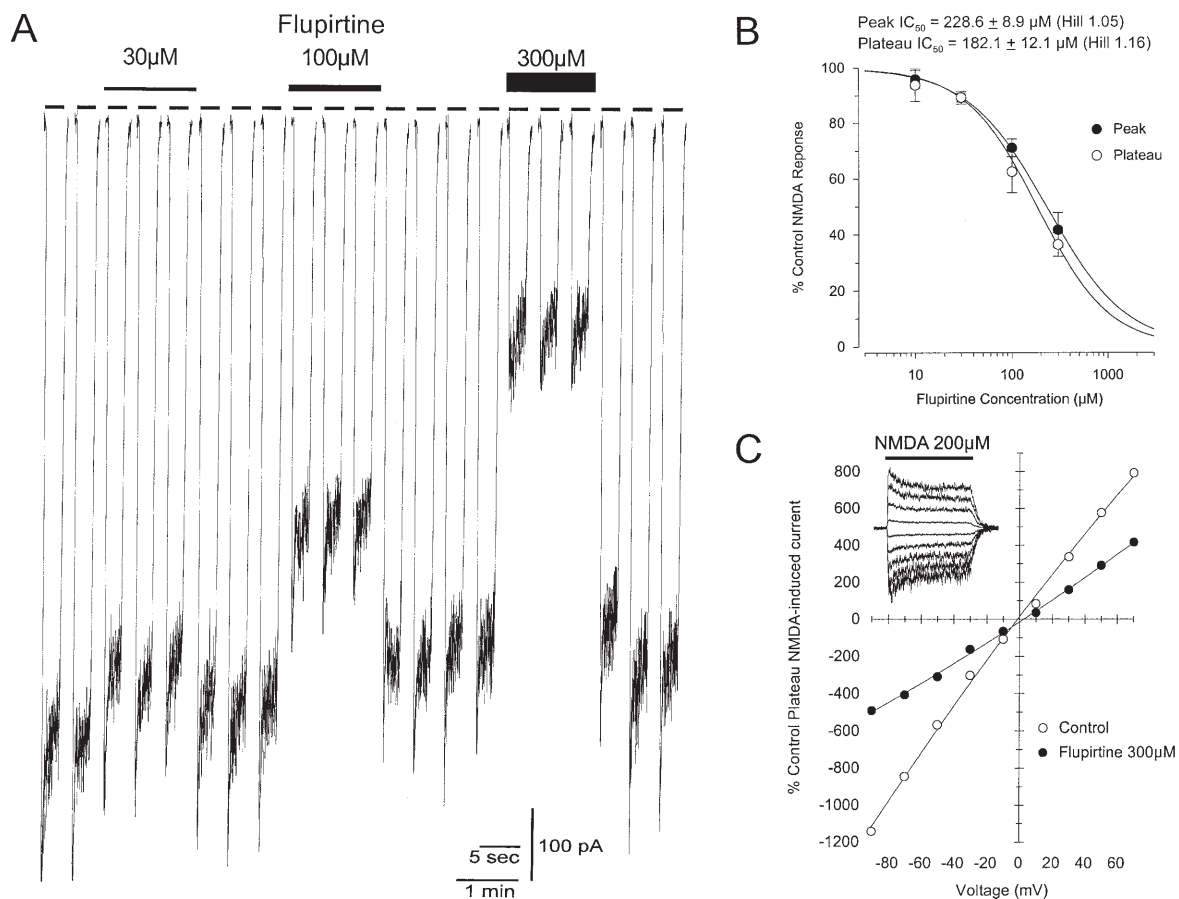


Fig. 1. **A** Concentration-dependence of the blockade of NMDA receptors by flupirtine on a single superior colliculus neurone. NMDA (200 μ M) was applied for 2.5 seconds every 30 seconds in the continuous presence of glycine (1 μ M) and at a constant membrane potential of -70 mV. The inter-response interval has been omitted to allow better resolution of the kinetics of individual responses (blank spaces in trace). Flupirtine (30, 100 and 300 μ M) was continuously present for 1.5 minutes as indicated by the bars. **B** Concentration-dependence of the blockade of NMDA receptors by flupirtine. Peak and plateau (steady state) NMDA current response were normalised to control levels and plotted as means (\pm SEM) against flupirtine concentration (10 μ M $n = 3$, 30 μ M $n = 7$, 100 μ M $n = 7$, 300 μ M $n = 5$). Estimation of IC_{50} s and curve fitting were made according to the 4 parameter logistic equation (Grafit, Erithacus Software). **C** Voltage-independence of the blockade of NMDA receptors by flupirtine. NMDA (200 μ M) was applied for 2.5 seconds every 30 seconds in the continuous presence of glycine (1 μ M) at various membrane potentials. Plateau NMDA current responses in the absence and presence of flupirtine (300 μ M) have been plotted as means against membrane potential ($n = 2$). The upper left insert shows original data for the i.v. curve in the presence of flupirtine (300 μ M)

Therapeutically relevant concentrations of flupirtine

The therapeutically relevant concentrations of flupirtine are of decisive importance for assessing its molecular mechanisms of action. Of the various experimentally demonstrated effects of flupirtine (Table 1), only those that occur within a concentration range achieved under therapeutic conditions are relevant. Animal experiments show higher values in the brain than in the plasma 15min after intravenous administration. After oral administration, comparable concentrations in the plasma and brain are measured within 30min (Obermeier et al., 1985). Under therapeutic conditions, the plasma concentration is up to 6.5µM; 2.5µM are still detectable 12h after the last intake (Hlavica and Niebch, 1985). The elimination half-life of

Table 1. A selection of the in-vitro effects of flupirtine

Effect investigated	Assay	Potency (µM)	Reference
NMDA channel	[³ H]MK-801 binding, cortex	>10	*
NMDA channel	Patch clamp, hippocampus	>>1	results presented here and Jakob and Krieglstein, 1997
α ₁ receptor	[³ H]Prazosin binding, brain	>10	Szelenyi et al., 1989
α ₂ receptor	[³ H]Clonidine binding, brain	>10	Szelenyi et al., 1989
5HT ₁ receptor	[³ H]5-HT binding, frontal cortex	>100	Szelenyi et al., 1989
5HT ₂ receptor	[³ H]Mesulergine binding, frontal cortex	75	Szelenyi et al., 1989
µ-opiate receptor	[³ H]Oxymorphone binding	>10	Nickel, 1987
δ-opiate receptor	[³ H]D-Ala ² -D-Leu ⁵ -enkephalin binding	>>10	Nickel, 1987
κ-opiate receptor	[³ H]Diprenorphine binding, sequential blocking	>>10	Nickel, 1987
µ/δ/κ-opiate receptor	[³ H]Diprenorphine binding	>>10	Nickel, 1987
µ/δ/κ-opiate receptor	[³ H]Naloxone binding	>10	Nickel, 1987
µ/δ/κ-opiate receptor	[³ H]Etorphine binding	>10	Nickel et al., 1985
Benzodiazepine receptor	[³ H]Flunitrazepam binding	>10	Nickel et al., 1990b
Basal prostaglandin I ₂ production	Aorta specimen of the rat	5–50	Darius and Schrör, 1985
Inhibition of arachidonic acid-induced thromboxane production	Platelets, human	>23	Darius and Schrör, 1985
Inwardly rectifying K ⁺ channel	Patch-clamp, hippocampus	1	Jakob and Krieglstein, 1997

* Kornhuber et al. (publication in preparation). Under therapeutic conditions with serum concentrations of up to about 5 µM, of the effects listed here, only the effect on the inwardly rectifying K⁺ channel is relevant

flupirtine is longer in older patients than in young normal subjects; this is accompanied by higher maximum serum concentrations in older patients (Abrams et al., 1988). The same probably applies for preclinical models where pronounced effects are seen with doses of around 1–20 mg/kg *in vivo* (Block et al., 1994; Carlsson and Jurna, 1987; Schwarz et al., 1994, 1995; Timmann et al., 1995) and 10–20 μ M *in vitro* (Nickel et al., 1990a; Osborne et al., 1994; Perovic et al., 1994; Rupalla et al., 1995). In summary, experimentally determined effects are only clinically relevant if they occur in the low micromolar range.

Pharmacologic effects at therapeutic concentrations

The pain-relieving effect of flupirtine does not appear to be achieved via central opiodergic mechanisms. For example, the analgesic effects of flupirtine are not antagonized by naloxone, and neither are they accompanied by tolerance or physical dependence of the opiate type (Nickel et al., 1985). Moreover, flupirtine does not show any relevant affinity to the opiate receptor system (Nickel, 1987; Nickel et al., 1985) (Table 1) and is also structurally markedly different from morphine. An action via benzodiazepine receptors could also be ruled out (Nickel et al., 1990b). The serotonin receptor antagonist cyproheptadine and the tryptophan hydroxylase inhibitor p-chlorophenylalanine do not inhibit the analgesic properties of flupirtine (Nickel et al., 1985), which also suggests that there is no influence on serotonergic mechanisms. Although no relevant affinities to the α_1 - or α_2 -adrenoreceptors have been found (Szelenyi et al., 1989), indirect evidence suggests a modulation of pain perception via the descending noradrenergic system (Szelenyi et al., 1989).

The central nervous system effects of flupirtine include analgesia, muscle relaxation and neuroprotection. Antiepileptic and antiparkinsonian properties are also to be found. These properties are only independent of each other at first sight: they are in fact the typical and classical effects of N-methyl-D-aspartate (NMDA) receptor antagonists (Zieglgänsberger and Tölle, 1993; Kornhuber and Weller, 1997). In various indirect studies, flupirtine displayed properties that are consistent with antagonism at the NMDA receptor (Block et al., 1994; Rupalla et al., 1995; Perovic et al., 1994, 1995; Osborne et al., 1994, 1996; Schwarz et al., 1994, 1995), and the recent review articles on flupirtine focus on the NMDA receptor as the main molecular target of flupirtine (Osborne et al., 1998; Schuster et al., 1999). In direct testing, however, it has not been possible to demonstrate a clear interaction with the previously known binding sites of the NMDA receptor (Table 1). In patch-clamp investigations on neuronal cell cultures, flupirtine has no influence on NMDA-induced ion currents (results presented here and Jakob and Krieglstein, 1997). In our own investigations (Kornhuber et al., publication in preparation), flupirtine did not show any relevant affinity to binding sites at the NMDA receptor in human post-mortem brain tissue. Other research groups have reported comparable negative results from binding studies (Osborne et al., 1996, 1998). Recently, it has been suggested that flupirtine interacts with

the redox binding site of the NMDA receptor (Osborne et al., 1998). But this is inconsistent with the negative findings from patch-clamp studies presented here. In summary, it can be stated that flupirtine acts like an NMDA receptor antagonist in functional investigations, although an action at the NMDA receptor could not be found in direct investigations. It is probable that a site of action "up- or downstream" of the NMDA receptor is influenced, leading to a functional NMDA receptor antagonism.

Jakob and Kriegelstein (1997) found an activation of G-protein-regulated inwardly rectifying K^+ channels by flupirtine in therapeutically relevant concentration ranges. According to our current knowledge, this is the only mechanism known to be relevant in a therapeutic concentration range (Table 1). Inwardly rectifying K^+ channels represent a new family of K^+ channels and differ markedly from the classical voltage-dependent K^+ channels. The resting membrane potential is slightly above the K^+ equilibrium potential; a slight outflow of K^+ ions stabilizes the resting membrane potential close to the K^+ equilibrium potential. The overall effect on the cell is a stabilization of the resting membrane potential, e.g. in the case of slight depolarization by excitotoxic stimuli. The G-protein-activated inwardly rectifying K^+ channels (GIRK) are regulated via neurotransmitters, occur in various subtypes and are expressed differently according to the region of the brain involved (Karschin et al., 1994). K^+ channels also play an important role in the transmission of pain stimuli. The analgesic effects of opioids (Ocaña et al., 1990), α_2 -adrenergic agonists (Ocaña and Baeyens, 1993), 5-HT_{1A}-agonists (Robles et al., 1996) and other analgetic substances are mediated by receptor-mediated opening of K^+ channels and neuronal hyperpolarization. On the other hand, Substance P inhibits G-protein-dependent K^+ channels (Stanfield et al., 1985) and thus facilitates the transmission of pain stimuli.

Central nervous effects of flupirtine compared to those of experimental K^+ channel openers

Experimental K^+ channel openers like cromakalim display analgesic properties (Ocaña et al., 1996; Robles et al., 1996; Vergoni et al., 1992). Direct evidence of a neuroprotective action of openers of ligand-gated K^+ channels has been found in excitotoxic (Abele and Miller, 1990) and oxidative noxae (Goodman and Mattson, 1996). A study conducted by Lauritzen et al. (1997) shows that cromakalim prevents the glutamate-induced death of hippocampal neurons by counteracting the delayed increase in intracellular Ca^{2+} . An analogy to the effects of flupirtine can be seen here (Zimmer et al., 1998). A stabilization of the resting membrane potential by flupirtine would also be consistent with the initial evidence of antiepileptic properties (Sheridan et al., 1986). Antiepileptic properties have been shown for other K^+ channel openers (Gandolfo et al., 1989; Del Pozo et al., 1990; Popoli et al., 1991). In summary, a comparable central nervous spectrum of action is found for flupirtine and experimental K^+ channel openers. This can be interpreted as additional indirect evidence of an action of flupirtine via an activation of K^+ channels.

Global model and summary

The results obtained so far can be summarized as follows: Therapeutically relevant, analgesic plasma concentrations of flupirtine are in the low micromolar range. In direct investigations, no relevant affinities for α_1 , α_2 , 5HT₁, 5HT₂, dopamine, benzodiazepine, opiate, central muscarinic, or nicotinic receptors were found. The profile of preclinical and clinical actions (analgesic, muscle-relaxant, neuroprotective, antiepileptic and antiparkinsonian properties) suggests that the action of flupirtine is connected with the NMDA receptor. It has not been possible to convincingly demonstrate a direct action on the NMDA receptor to date. All previous experimental results could be mediated by an indirect influence on the NMDA receptor. Flupirtine acts functionally like an NMDA receptor antagonist. At a therapeutically relevant concentration, flupirtine activates neuronal inwardly rectifying G-protein-regulated K⁺ channels. The spectrum of action of the available experimental K⁺ channel openers, as far as they have been investigated to date, corresponds to that of flupirtine: These K⁺ channel openers also display analgesic, neuroprotective and anticonvulsant properties.

We present a new model to explain the spectrum of action of flupirtine: Flupirtine activates inwardly rectifying K⁺ channels and thus stabilizes the resting membrane potential. The Mg²⁺ block of the NMDA receptor remains in force; i.e. the NMDA receptor is indirectly inhibited (Fig. 2). This mechanism provides an explanation for the analgesia, muscle relaxation and neuroprotection. The model on the mechanism of action of flupirtine presented here links neuronal K⁺ channels with NMDA receptors via membrane excitability. This provides an understanding of the clinically observed profile of flupirtine's actions, with analgesic, muscle-relaxant and neuroprotective effects. The indirect inhibition of Ca²⁺ inflow into nerve cells, with plastic changes, e.g. in the sense of an increased response ("wind up"), is suppressed by substances like flupirtine. This counteracts the clinically corresponding chronification of pain. From a clinical point of view, flupirtine can be classified as the prototype of a new substance class with analgesic, muscle-relaxant and neuroprotective properties. While flupirtine is clinically used mainly as an analgesic substance, its neuroprotective properties will probably lead to new clinical applications, e.g. in chronic neurodegenerative diseases.

Various questions remain open. Different nerve cells often differ in their intrinsic electrophysiological properties as a result of the differential expression of specific ion channels and their different spatial distribution on the cell surface. In future investigations, it will have to be clarified which subtype of GIRK channels is influenced, in which regions of the brain this takes place, and whether specific receptors, G-proteins or K⁺ channels are influenced, and the precise molecular target site will have to be characterized. Cardiac side effects of flupirtine have not been reported to date. This can be interpreted as indirect evidence that flupirtine selectively influences neuronal K⁺ channels. This hypothesis must be examined in further investigations. If this hypothesis is confirmed, flupirtine is the prototype of a new substance class, the selective neuronal potassium channel openers (SNEPCO).

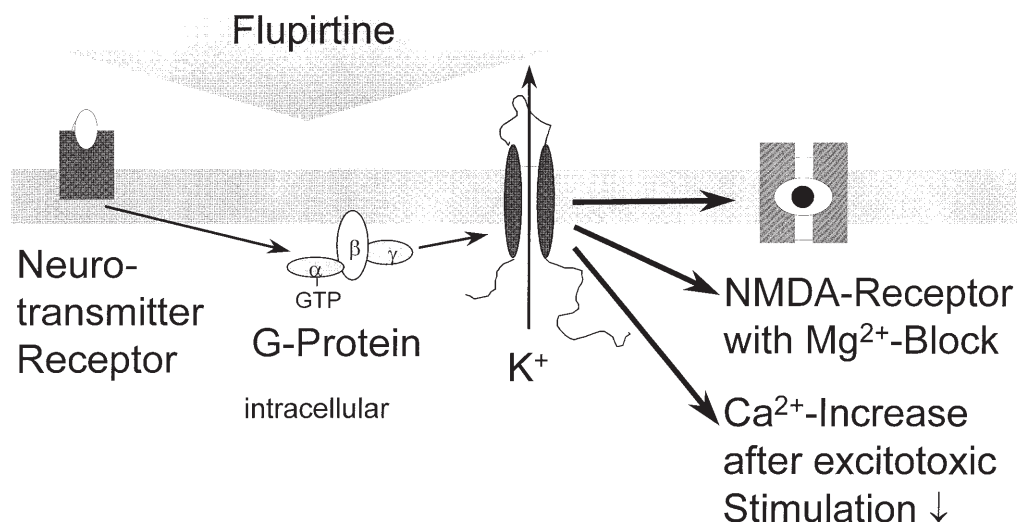


Fig. 2. Flupirtine activates G-protein-coupled inwardly rectifying K^+ channels. The changes under the influence of flupirtine are to be read from left to right. The resting membrane potential is stabilized, an activation of the cell membrane is inhibited. These processes are shown here in different grey tones of the cell membrane: The activated state of the cell membrane (left, dark) is brought into the resting state (right, light) by flupirtine via an activation of inwardly rectifying K^+ channels. An activation of NMDA receptors is prevented, since the Mg^{2+} block of the NMDA receptor is only relieved upon depolarization of the cell membrane. It is conceivable that flupirtine has additional effects that are independent of the NMDA receptor

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Authors' address: Prof. Dr. J. Kornhuber, Abteilung für Psychiatrie, Universität Göttingen, von-Siebold-Strasse 5, D-37075 Göttingen, Federal Republic of Germany, e-mail: jkornhu@gwdg.de