ELSEVIER

Neuroscience Letters 275 (1999) 109–112

Neuroscience Letters

www.elsevier.com/locate/neulet

Effects of etonitazene consumption and abstinence on the signal transmission of μ-opioid receptors in brain membranes of rats

Torsten May*, Folkert Juilfs, Jochen Wolffgramm

Abteilung für Klinische Neurobiologie, Psychiatrische Klinik und Poliklinik, Freie Universität Berlin, Ulmenallee 30/32, D-14050 Berlin, Germany

Received 31 May 1999; received in revised form 6 September 1999; accepted 7 September 1999

Abstract

Rats, for 8 weeks consuming the μ-opioid agonist etonitazene (forced and free choice conditions yielding high and low drug-consumers), were sacrificed after 2 days or 6 weeks lasting drug deprivation. Binding characteristics of membranes from the parieto-occipital cortex of these four groups were compared with those of drug-naive controls. In all five groups, 1 μM of the μ-opioid receptor agonist [D–Ala^2,N–MePhe^4,Gly^5–ol]enkephalin (DAMGO) increased the guanosine-5′-*O*-([^{35}S]3-thio)triphosphate ([^{35}S]GTPγS) binding activity on guanine nucleotide-binding (G) proteins, and 500nM of GTPγS decreased the [^{3}H]DAMGO binding affinity. During acute withdrawal, both opioid consuming groups displayed a higher maximum efficacy (E_{max}) in basal [^{35}S]GTPγS binding (34 and 31%, each $P < 0.01$), but only the forced group showed a 58% higher net DAMGO-stimulated binding density B_{max} ($P < 0.01$) and 53% more activated G proteins per μ-opioid receptor ($P < 0.05$). In the presence of GTPγS both groups revealed a higher affinity in [^{3}H]DAMGO binding (each 25%, $P < 0.01$). The long-term drug-deprived groups displayed no differences in their binding characteristics.
© 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: μ-Opioid agonist; Etonitazene consumption; Withdrawal; Drug dependence; Guanosine-5′-*O*-([^{35}S]3-thio)triphosphate ([^{35}S]GTPγS) and [^{3}H][D–Ala^2, N–MePhe^4, Gly^5–ol]enkephalin ([^{3}H]DAMGO) binding

Animal models seem unequivocal to evaluate acute and chronic effects of drugs of abuse on the brain [5,15]. In the present study, rats consumed the highly potent and selective μ-opioid receptor agonist etonitazene [3,4], which is in vivo about 1000 times more potent than morphine as positive reinforcer and analgesic [2]. Chronic opioid intake for 8 weeks was studied to search for neurochemical effects in phases of spontaneous, acute withdrawal (2 days of abstinence) and of protracted abstinence (6 weeks). Two different paradigms of etonitazene intake (free choice and forced treatment) were studied [4] to search for effects of chronic drug consumption, but not for effects of opioid addiction [3,16]. The main hypothesis was that chronic etonitazene consumption would change brain μ-opioid receptors and their coupled signal transducers, i.e. the guanine nucleotide-binding proteins (G proteins). Therefore, we studied the in vitro binding properties of the non-hydrolyzable GTP analogue guanosine-5′-*O*-([^{35}S]3-thio)triphosphate ([^{35}S]GTPγS) to Gα protein subunits and of the selective agonist [^{3}H][D–Ala^2,N–MePhe^4,Gly^5–ol]enkephalin ([^{3}H]DAMGO) to μ-opioid receptors in brain membranes. [^{35}S]GTPγS binding was analyzed in the absence and presence of DAMGO. The μ-opioid agonist promotes the Gα protein activation resulting in an exchange of the bound GDP by GTP [14], that can be measured by an increase of high affinity [^{35}S]GTPγS binding [7,8]. To determine the density of μ-opioid receptors and the nucleotide efficiency on receptor-Gα protein uncoupling, [^{3}H]DAMGO binding was carried out in the absence and presence of GTPγS [6]. This study is the first investigating neurochemical consequences of chronic etonitazene consumption on the complex interaction of μ-opioid receptors and G proteins ([^{35}S]GTPγS ± DAMGO, [^{3}H]DAMGO ± GTPγS).

[^{3}H]DAMGO (specific activity 48 Ci/mmol) and [^{35}S]GTPγS (1100–1300 Ci/mmol) were purchased from DuPont, NEN, naloxone from RBI, GTPγS, DAMGO and GDP from Sigma. Etonitazene was a gift from Novartis. All other chemicals were of the highest grade available.

Forty-four male Wistar rats (Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin,

* Corresponding author. Tel.: +49-30-8445-8230: fax: +49-30-8445-8244.

E-mail address: drtorstenmay@hotmail.com (T. May)

0304-3940/99/$ - see front matter © 1999 Elsevier Science Ireland Ltd. All rights reserved.
PII: S0304-3940(99)00741-7

Germany) were treated as described previously [4]. Shortly, one group ($n = 16$) had a free choice between water and 0.5, 1 and 2 mg etonitazene/l (plus 0.125, 0.25 and 0.5 g/l of acetic acid), the other forced consumers ($n = 16$) received 0.5 mg etonitazene/l, each for 8 weeks. The choice-group and the forced consumers took 12.3 ± 2.5 and 56.7 ± 3.2 μg etonitazene/kg per day, respectively. The opioid solutions were withdrawn for 2 days (short-term abstinence, each $n = 8$) and 6 weeks (long-term abstinence, each $n = 8$) before sacrifice. The control group ($n = 12$) received water.

The rats were sacrificed by decapitation, the 'parieto-occipital cortex' (cerebral cortex minus frontal part) was dissected, homogenized (glass-teflon, 4°C) in 10 ml of 50 mM Tris–HCl (pH 7.4) and centrifuged (40 000 × *g*, 10 min). The pellets were rehomogenized in 10 ml; 1 ml was taken for the [^{35}S]GTPγS binding assay. The 9 ml homogenates were incubated (35°C, 30 min) and centrifuged (40 000 × *g*, 10 min). The pellets were rehomogenized in 5.7 ml. The 1 ml homogenates plus 0.5 ml of 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 3 mM $MgCl_2$ were used (10 μl, 17.0 ± 0.5 μg protein) for the [^{35}S]GTPγS binding assay. The 2 ml samples contained 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 3 mM $MgCl_2$, 40 μM GDP and 56.3 ± 1.7 pM [^{35}S]GTPγS (about 300 000 cpm). The samples were incubated in the absence (total binding: about 10 000 cpm) and presence of 10 μM GTPγS (non-specific binding: 3–9% of total binding), and of 10 further concentrations of unlabelled GTPγS (0.25–100 nM), both in the absence and presence of 1 μM DAMGO, each in duplicate. All samples were incubated (30°C, 60 min) before filtration (cell harvester, GF-B filters). The radioactivity on the dried filters was determined by liquid scintillation spectrometry (counting efficiency about 95%). For the [^{3}H]DAMGO binding assay 100 μl homogenate (290 ± 9 μg protein) were used. The 3-ml samples contained 50 mM Tris–HCl (pH 7.4), 0.3 nM [^{3}H]DAMGO in the absence (total binding) and presence of 1 μM naloxone (non-specific binding; 10–20% of total binding) and of six further concentrations of unlabelled DAMGO (0.5–16 nM), each in triplicate. The samples were incubated in the absence and presence of 500 nM GTPγS. All samples were incubated (22°C, 60 min) before filtration (cell harvester, GF-B filters). The radioactivity on the dried filters was determined by liquid scintillation spectrometry (counting efficiency 50–60%). The protein content was determined in 1-ml samples with bovine serum albumin as standard [1].

The results are presented as mean ± SEM. The binding data were analyzed with non-linear, unweighted least squares procedures (GraphPadPrism, USA). The [^{3}H]DAMGO binding experiments were calculated by one-site saturation analyses to estimate B_{max} (binding density in mol/mg protein) and K_d values (inverse of binding affinity in nM). The [^{35}S]GTPγS binding data were analyzed by one-site concentration-response curves to reveal EC_{50} (effective concentration inducing 50% response, in nM), E_{max} (maximum efficacy, in fmol/mg protein) and Hill coefficient (nH) values. Furthermore, K_d and B_{max} values were calculated from the net DAMGO-stimulated [^{35}S]GTPγS binding by Scatchard analyses [7,8]. The amplification factor was calculated by division of these B_{max} values by the B_{max} values from [^{3}H]DAMGO binding [8]. Two-way analysis of variance (two-way ANOVA, treatment X GTPγS concentrations) was applied to compare the concentration-response curves between controls and drug-taking groups. Differences among the calculated parameters between the groups were assessed by means of one-way ANOVA followed by post-hoc tests using *t*-statistics ($P < 0.05$, two-tailed). The DAMGO and GTPγS effects on the corresponding [^{35}S]GTPγS and [^{3}H]DAMGO binding parameters were analyzed by two-tailed paired Student's *t*-tests ($P < 0.05$).

In the [^{35}S]GTPγS binding experiments, DAMGO induced higher E_{max} values in each group and lower EC_{50} values in all but one group (Table 1). Both in the free choice and forced group, the [^{35}S]GTPγS binding curves of 2 days abstinent rats were significantly different from the control curves (Fig. 1A,B). In comparison to the controls, the short-term opioid-deprived groups revealed higher E_{max} values both in the absence (31 and 34%) and presence of DAMGO (26 and 35%), whereas the EC_{50} values were not significantly different (Table 1). The net DAMGO-stimulated [^{35}S]GTPγS binding parameter B_{max}, describing the intrinsic agonist efficacy [7], was in previously forced etonitazene-taking, 2 days abstinent animals by 58% higher ($P < 0.01$) than in controls. The other previously etonitazene-consuming groups displayed no significant differences (Table 1). In the absence of GTPγS, [^{3}H]DAMGO binding saturation analyses revealed no significant differences in K_d and B_{max} values between opioid-administering groups and controls (Table 1). The presence of GTPγS did not change the B_{max} but increased the K_d values 3.3–4.1 times in each of the five groups ($P < 0.001$). Thereby, the K_d values in the two short-term deprived groups were significantly lower (25%; $P < 0.01$) than in controls (Table 1). The number of activated G proteins per μ-opioid receptor, the amplification factor [8], was by 53% higher in the previously forced etonitazene-taking, 2 days abstinent group vs. the controls ($P < 0.05$), the other etonitazene-groups displayed no significant differences (Table 1).

The etonitazene-consuming rats did not become addicted [4], i.e. they did not show an irreversible loss of control over drug intake [3,16]. However, 8 weeks of etonitazene intake induce physical dependence. Both forced and free choice etonitazene-consuming rats are more sensitive to painful stimuli for the first 3 days of spontaneous withdrawal [3]. Therefore, 2 days (but not 6 weeks) drug-deprived rats should have symptoms of an acute withdrawal due to a previous physical dependence. The experimental design produced a stringent coupling between the consumption procedure (forced or free choice) and the etonitazene doses (high or low). Thus it is speculative to differentiate

Table 1
[^{35}S]GTPγS and [^{3}H]DAMGO binding to the parieto-occipital cortex[a]

Group	Controls	2 days abstinent		6 weeks abstinent	
	Drug-naive	Choice	Forced	Choice	Forced
[^{35}S]GTPγS binding					
Without DAMGO					
EC_{50} (nM)	5.18 ± 0.27	4.60 ± 0.43	4.49 ± 0.15	5.71 ± 0.43	5.83 ± 0.35
E_{max} (fmol/mg)	217 ± 13	284 ± 23**	291 ± 23**	183 ± 16	204 ± 9
nH	0.80 ± 0.02	0.77 ± 0.04	0.79 ± 0.03	0.79 ± 0.03	0.83 ± 0.03
With DAMGO					
EC_{50} (nM)	4.02 ± 0.17^{++}	3.95 ± 0.37	3.61 ± 0.13^{++}	4.04 ± 0.25^{+}	4.29 ± 0.37***
E_{max} (fmol/mg)	262 ± 14^{++}	329 ± 25*, $^{+}$	353 ± 29**, $^{++}$	228 ± 18^{++}	260 ± 13^{++}
nH	0.83 ± 0.02	0.83 ± 0.03	0.85 ± 0.02	0.78 ± 0.02	0.78 ± 0.02
Net DAMGO					
K_d (nM)	1.97 ± 0.22	1.44 ± 0.17	2.17 ± 0.24	1.34 ± 0.19	1.95 ± 0.33
B_{max} (pmol/mg)	1.48 ± 0.10	1.49 ± 0.19	2.34 ± 0.28**	1.09 ± 0.20	1.60 ± 0.22
[^{3}H]DAMGO binding					
Without GTPγS					
K_d (nM)	1.08 ± 0.08	0.91 ± 0.08	1.00 ± 0.14	1.16 ± 0.19	0.92 ± 0.11
B_{max} (fmol/mg)	184 ± 16	227 ± 30	189 ± 20	187 ± 33	173 ± 12
With GTPγS					
K_d (nM)	4.42 ± 0.21^{++}	3.35 ± 0.35**, $^{++}$	3.31 ± 0.23**, $^{++}$	4.76 ± 0.26^{++}	3.65 ± 0.41^{++}
B_{max} (fmol/mg)	185 ± 15	229 ± 34	180 ± 18	189 ± 27	166 ± 16
Amplification factor	8.8 ± 1.0	6.7 ± 0.8	13.4 ± 2.2*	7.8 ± 2.2	9.8 ± 1.3

[a] [^{35}S]GTPγS binding was carried out in the absence and presence of 1 μM DAMGO. Nonspecific binding was determined in the presence of 10 μM GTPγS. [^{3}H]DAMGO binding experiments were performed in the absence and presence of 500 nM GTPγS. Nonspecific binding was determined in the presence of 1 μM naloxone. The data represent the means ± SEM ($n = 8$–12). The K_d values are shown in nM and the B_{max} values in mol per mg of protein. The binding parameters were calculated by one-site concentration-response and saturation curves, and by Scatchard analyses of the net DAMGO-stimulated [^{35}S]GTPγS binding data. The amplification factor was calculated by division of the net DAMGO-stimulated [^{35}S]GTPγS binding B_{max} by the [^{3}H]DAMGO binding B_{max} values. Parieto-occipital cortex membranes were used, from etonitazene consuming rats (free choice intake and forced consumption for 8 weeks) and controls. The animals were sacrificed during acute withdrawal (after 2 days of drug deprivation) and after protracted abstinence (after 6 weeks of drug deprivation), respectively. *$P < 0.05$, **$P < 0.01$ vs. the controls (ANOVA followed by post-hoc tests using t-statistics). ***$P < 0.05$, $^{+}P < 0.01$, $^{++}P < 0.001$ vs. the corresponding data in the absence of DAMGO ([^{35}S]GTPγS binding) and GTPγS ([^{3}H]DAMGO binding), respectively (paired Student's t-tests).

between biochemical effects evoked by these two factors. No neurochemical effect was found solely in the free choice groups. In both short-term abstinent groups, the GTPγS effect on [^{3}H]DAMGO affinity and the basal [^{35}S]GTPγS binding were lower and higher, respectively, than in controls. The nucleotides GTPγS and guanyl-5′-yl′-imodiphosphate activate G proteins and decrease the [^{3}H]DAMGO affinity [6]. A lessened nucleotide efficiency has been described in the cerebral cortex and midbrain of morphine-treated guinea-pigs and rats [10,13]. Herein, the reduced GTPγS effect on μ-opioid receptor-G protein uncoupling was accompanied by an increased basal [^{35}S]GTPγS binding activity. The G_o proteins are the most abundant G proteins in the mammalian brain and [^{35}S]GTPγS preferentially labels the α subunits of $G_{i/o}$ proteins [14]. Chronic administration of μ- but not δ- or κ-opioid agonists selectively induced higher $G\alpha_i/G\alpha_o$ immunoreactivities in the rat cerebral cortex [11]. Thus, the present results may be consequences of a larger G protein pool. On the other hand, the net DAMGO-stimulated [^{35}S]GTPγS binding and the amplification factor were increased solely in the previously forced etonitazene-taking rats vs. the controls. This short-lasting stimulatory effect of forced etonitazene intake on the μ-opioid agonist efficacy may be induced by an increased phosphorylation of the μ-opioid receptors described previously for morphine-dependent mice [12]. We did not find significant effects of etonitazene intake on the rat brain μ-opioid receptor density. Our results suggest an increased sensitiveness of the μ-opioidergic neurotransmission, in particular after forced etonitazene consumption. Challenge tests with an opioid might be useful to characterize behavioral consequences but such experiments could not be performed, since they inaccessibly affect neurotransmission parameters.

Long-lasting etonitazene-effects induced by a protracted withdrawal syndrome were not found in the signal transmission of μ-opioid receptors in the parieto-occipital cortex, but have been described previously for dopamine receptors in the limbic forebrain [4]. However, the cerebral cortex is involved in the consequences of opioid dependence and withdrawal [5,9,15]. Therefore, the short-lasting alterations in the signal transmission of μ-opioid receptors/G proteins may be signs of etonitazene dependence and withdrawal. An open question is whether such changes

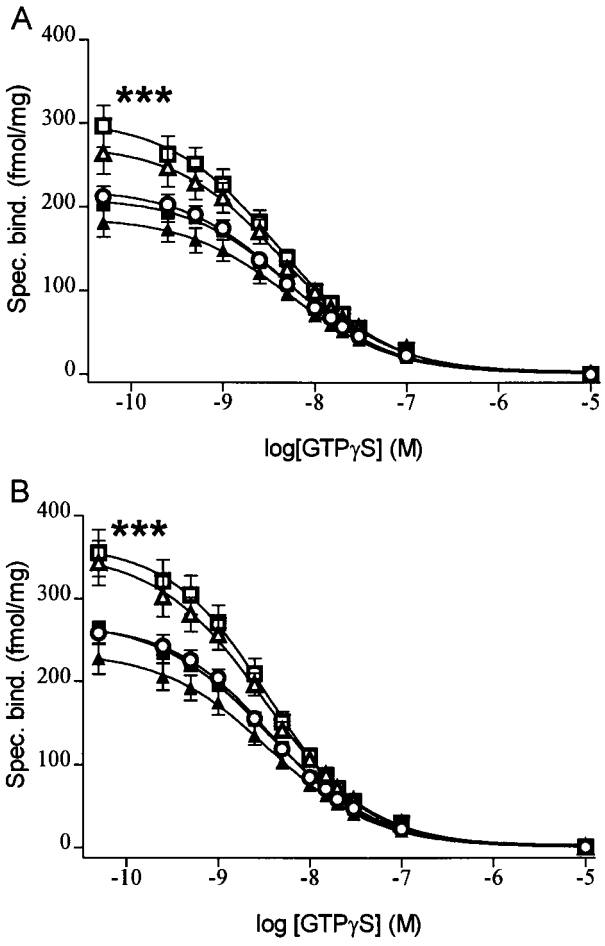

Fig. 1. [^{35}S]GTPγS binding to membranes of the parieto-occipital cortex. Each value represents the mean ± SEM (n = 8–12). The one-site concentration-response curves display the specific binding (in fmol/mg protein; ordinates) vs. the concentration of GTPγS (in M; abscissae), each in the absence (A) and presence of 1 μM DAMGO (B). The corresponding binding parameters are presented in Table 1. $P < 0.001$ (two-way ANOVA) data of each group in (B) vs. the corresponding data in (A). ***$P < 0.001$ (two-way ANOVA) data of each 2 days abstinent group vs. the controls. ○, controls; △, free choice, 2 days abstinent; □, forced, 2 days abstinent; ▲, free choice, 6 weeks abstinent; ■, forced, 6 weeks abstinent.

also participate in the withdrawal syndrome of opioid-addicted rats [3,16].

We thank Steffen Lutter for technical assistance and Dr. Carsten Tober for reading this manuscript. Etonitazene was a gift from Novartis AG, Switzerland. The study was supported by a grant of the German Bundesinstitut für Arzneimittel und Medizinprodukte (FO 2.1–68503–201).

[1] Bradford, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72 (1976) 248–254.
[2] Dhawan, B.N., Cesselin, F., Rafhubir, R., Reisine, T., Bradley, P.B., Portoghese, P.S. and Hamon, M., International union of pharmacology. XII. Classification of opioid receptors. Pharmacol. Rev., 48 (1996) 567–592.
[3] Heyne, A., The development of opiate addiction in the rat. Pharmacol. Biochem. Behav., 53 (1996) 11–25.
[4] May, T., Juilfs, F. and Wolffgramm, J., Long-lasting effects of chronic μ-opioid intake on the signal transmission via dopamine D_1 receptors in the limbic forebrain of drug deprived rats. Neuropharmacology, 37 (1998) 997–1006.
[5] Nestler, E.J. and Aghajanian, G.K., Molecular and cellular basis of addiction. Science, 278 (1997) 58–63.
[6] Ofri, D., Ritter, A.M., Liu, Y., Gioannini, T.L., Hiller, J.M. and Simon, E.J., Characterization of solubilized opioid receptors: reconstitution and uncoupling of guanine nucleotide-sensitive agonist binding. J. Neurochem., 58 (1992) 628–635.
[7] Selley, D.E., Sim, L.J., Xiao, R., Liu, Q. and Childers, S.R., μ-Opioid receceptor-stimulated guanosine-5′-*O*-(γ-thio)-triphosphate binding in rat thalamus and cultured cell lines: signal transduction mechanisms underlying agonist efficacy. Mol. Pharmacol., 51 (1997) 87–96.
[8] Sim, L.J., Selley, D.E., Xiao, R. and Childers, S.R., Differences in G-protein activation by μ- and δ-opioid, and cannabinoid, receptors in rat striatum. Eur. J. Pharmacol., 307 (1996) 97–105.
[9] Simonato, M., The neurochemistry of morphine addiction in the neocortex. Trends Pharmacol. Sci., 17 (1996) 410–415.
[10] Tao, P.-L., Lee, C.-R., Law, P.-C. and Loh, H.L., The interaction of the mu-opioid receptor and G protein is altered after chronic morphine treatment in rats. Naunyn-Schmiedeberg's Arch. Pharmacol., 348 (1993) 504–508.
[11] Ventayol, P., Busquets, X. and Garcia-Sevilla, J.A., Modulation of immunoreactive protein kinase C-α and β isoforms and G proteins by acute and chronic treatments with morphine and other opiate drugs in rat brain. Naunyn-Schmiedeberg's Arch. Pharmacol., 355 (1997) 491–500.
[12] Wang, Z., Bilsky, E.J., Porreca, F. and Sadee, W., Constitutive μ opioid receptor activation as regulatory mechanism underlying narcotic tolerance and dependence. Life Sci., 54 (1994) 339–350.
[13] Werling, L.L., McMahon, P.N. and Cox, B.M., Selective changes in μ opioid receptor properties induced by chronic morphine exposure. Proc. Natl. Acad. Sci. USA, 86 (1989) 6393–6397.
[14] Wieland, T. and Jakobs, K.H., Measurement of receptor-stimulated guanosine 5′-*O*-(γ-thio)triphosphate binding by G proteins. Methods Enzymol., 237 (1994) 3–13.
[15] Wise, R.A., Neurobiology of addiction. Curr. Opin. Neurobiol., 6 (1996) 243–251.
[16] Wolffgramm, J. and Heyne, A., From controlled drug intake to loss of control: the irreversible development of drug addiction in the rat. Behav. Brain. Res., 70 (1995) 77–94.