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Immunohistochemical evidence of down-regulation of μ-opioid receptor after chronic PL-017 in rats

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Abstract

In a previous study, μ-opioid receptor binding was decreased by chronic treatment of rats with a μ-opioid receptor-selective agonist [CH_3Phe^3, D-Pro^4]morphiceptin (PL-017) [Tao, P.L., Lee, H.Y., Chang, L.R., Loh, H.H., 1990. Decrease in μ-opioid receptor binding capacity in rat brain after chronic PL-017 treatment. Brain Res. 526, 270–275]. However, there was a lack of correlation between the time course of receptor down-regulation and the loss of pharmacological effects of the drug. In the current study, we used immunohistochemistry to reinvestigate this issue. Male Sprague–Dawley rats were chronically treated with PL-017 i.c.v. for 1, 3 or 5 days, using an escalating dosage paradigm (0.75–6.0 μg), which resulted in a 1.4 to 32-fold increase in the AD_{50}. Rat brains were removed, frozen, coronally sectioned (14 μm) and processed for μ-, δ- or κ-opioid receptor immunohistochemistry by the avidin–biotin complex (ABC) method. Significant decreases in OP3 immunodensity were found in many brain regions which are enriched with OP3 after chronic treatment of PL-017. Time-dependent decreases in OP3 were detected and reached a plateau around 3 days of PL-017 treatment. No significant change in OP1 or OP2 immunodensity after chronic treatment with PL-017 was found. Our conclusion is that chronic treatment with PL-017 of rats selectively down-regulates μ-opioid receptors in the brain. This may be an important mechanism for PL-017 tolerance. © 1998 Elsevier Science B.V.

Keywords: PL-017; Antinociception; Tolerance; Opioid receptor; Down-regulation; Immunohistochemistry

1. Introduction

A compensatory decrease in the number of active receptors is one possible mechanism for the development of drug tolerance. This agonist induced down-regulation has been demonstrated in a number of hormone or neurotransmitter systems (Conn et al., 1978; Goldfine et al., 1978; Schlessinger et al., 1978; Amatruda et al., 1982; Lloyd and Ascoli, 1983). In opioid systems, we have found decreases in both μ- and δ-opioid receptor binding in rats chronically treated with etorphine (Tao et al., 1987). In a subsequent study (Tao et al., 1988), we obtained similar results after chronic administration of [D-Ala^2, D-Leu^5]enkephlin (DADLE), except that chronic DADLE treatment preferentially reduced δ-opioid receptor binding activity. Etorphine and DADLE are not highly selective opioid ligands and interact with both μ- and δ-opioid receptors (Gillan et al., 1980; Wolozin and Pasternak, 1981; James and Goldstein, 1984). Thus the observed decrease in μ-opioid receptor number could reflect activation of δ-opioid receptors only or vice versa. To clarify this point, we treated rats chronically with a selective μ-opioid receptor agonist [CH_3Phe^3, D-Pro^4]morphiceptin (PL-017) (Chang et al., 1983) for 1, 3 or 5 days and found a significant time-dependent increase in AD_{50} (median antinociceptive dose) (Tao et al., 1990). These results indicate that the animals had developed time-dependent tolerance to PL-017. Scatchard analysis of the [^{3}H]Tyr-D-Ala-Gly-MePhe-Gly-ol (DAMGO) saturation binding data revealed a decrease in B_{max} values and no change in the K_d values after 5 days' treatment with PL-017. Although the AD_{50} increased to over 10-fold of the control value after 3 days of PL-017 treatment, we did not find a significant decrease in μ-opioid receptor binding

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at that time. One possibility is that down-regulation of the opioid receptor does not cause opioid tolerance. Another possibility is that the binding experiments with brain homogenates could not detect such a correlation. To address these two possibilities, we used immunohistochemistry to detect opioid receptor levels during chronic agonist treatment in the present study. Immunohistochemical studies enabled us to monitor changes in receptor level in a specific brain nucleus, which was not feasible in our earlier binding experiments.

2. Materials and methods

2.1. Treatment of animals with PL-017 and pharmacological tests

Male Sprague–Dawley rats weighing 260–350 g were treated with PL-017 via a stainless-steel cannula implanted into the left lateral cerebroventricle (i.c.v.) according to the coordinates: P 1.0 mm, L 1.25 mm, V 4–5 mm, using bregma as zero. After the rats had recovered from the operation (3 days), PL-017 was administered i.c.v. to determine the AD_{50} dose (median antinociceptive dose) by the tail-flick response (D'Amour and Smith, 1941) and the up-down method (Dixon, 1965). The tail-flick response was tested at the peak effect of PL-017, which occurs about 20–30 min after PL-017 administration. In the up-down method, a series of test doses was chosen with equal spacing between each log dose of PL-017. Then a series of trials ($n = 6$) was carried out following the rule of a decrease in PL-017 dose after inhibiton of the tail-flick response and an increase in PL-017 dose after no inhibition of the tail-flick response. Each rat was tested in one trial only. The AD_{50} value was determined from the relationship $AD_{50} = X_f + k \times d$ (Dixon, 1965), where X_f was the last dose administered, k was the tabular value outlined by Dixon and d was the interval between doses. The control tail-flick latency was between 2.5 and 3.5 s. The mean and standard deviation (S.D.) of the control tail-flick latency in a group was calculated and the animal responses were quantified by defining significant inhibition of tail-flick response (antinociception) as an increase in the individual reaction time of more than 3 S.D. of the control mean reaction time. After the tests, the amount of PL-017 administered to the animals was normalized to the first dose (0.75 μg). Then the animals were chronically treated with PL-017 by daily administration of the peptide at 09.00 and 17.00 h, starting with a 0.75 μg i.c.v. dose on day 1, followed by 1.50 μg on day 2, 3.00 μg on day 3, 4.50 μg on day 4 and 6.00 μg on day 5. After chronic PL-017 treatment for 1, 3 or 5 days, the antinociceptive AD_{50} values of PL-017 were re-determined. The control rats were injected i.c.v. with saline (2 μl) instead of PL-017.

Table 1
Alteration in antinociceptive potency of PL-017 during chronic i.c.v. PL-017 treatment

Duration	PL-017 AD_{50} (μg)		Degree of tolerance
	before treatment	after treatment	
1 day	0.28 ± 0.06	0.38 ± 0.03	1.4
3 days	0.38 ± 0.03	4.82 ± 0.43	12.7
5 days	0.35 ± 0.09	11.2 ± 1.42	32.0

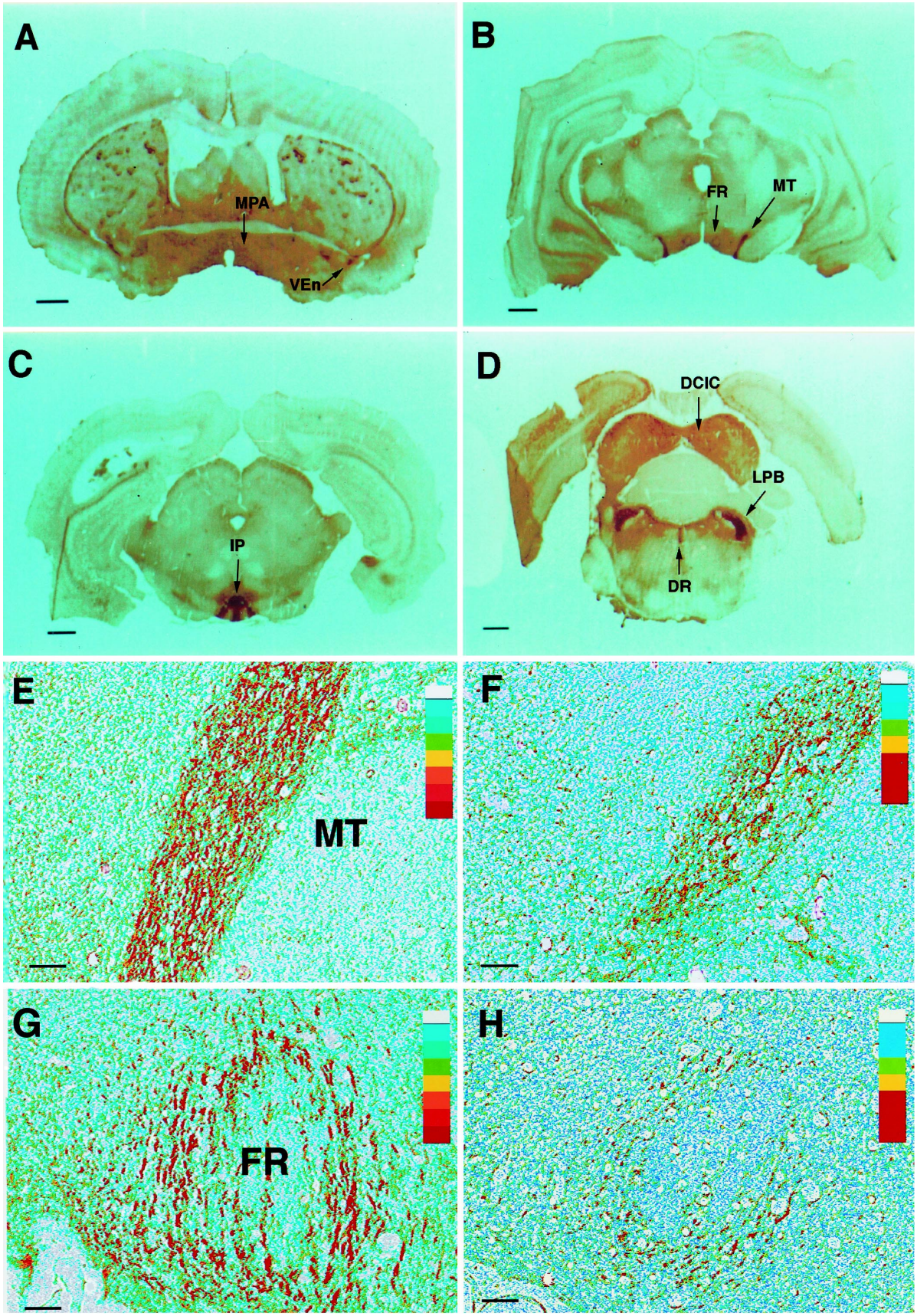
Values are means ± S.E.M.. Each group contained at least 6 rats. The degree of tolerance was calculated from the ratio of (AD_{50} after treatment)/(AD_{50} before treatment).

2.2. Fixation and tissue preparation

After chronic treatment with saline or PL-017, rats were anesthetized with chloral hydrate (350 mg/kg, i.p.) and fixed by transcardial perfusion of 200 ml Ca^{2+}-free Tyrode's solution, followed by 1000 ml of 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 6.9). Then each rat was perfused with 500 ml 10% sucrose in 0.1 M phosphate buffer (pH 7.2). After fixation, the brains were dissected and transferred to 10% sucrose buffer solution containing 0.01% NaN_3 and 0.02% bacitracin for at least 4 h before sectioning (Arvidsson et al., 1995a,b). Slide-mounted coronal cryostat sections (14 μm) were processed for immunohistochemistry.

The avidin biotinylated complex (ABC) method was used to detect receptor levels (Hsu et al., 1981). Sections were washed in 0.1 M phosphate buffered saline (PBS; 0.0016 M NaH_2PO_4, 0.0084 M Na_2HPO_4, 0.09 M NaCl), incubated for 30 min in PBS containing 3% normal goat serum and 0.4% Triton X-100 (3% GS-PBS-T) and then incubated for 24 h at room temperature with the primary antisera. Rabbit anti-OP3 (551D) diluted 1:5000 or rabbit anti-OP1 (442D) diluted 1:1000 or rabbit anti-OP2 (542G) diluted 1:200 was used as the primary antiserum for μ-, δ- or κ-opioid receptors (Arvidsson et al., 1995a,b,c). The sections were washed and incubated with goat anti-rabbit IgG (Vector Lab., CA) diluted 1:100 with primary antiserum diluent (1% GS-PBS-T) for 60 min at room temperature. The sections were rinsed and then incubated for 45–60 min with avidin biotinylated complex (1:100 in 1% GS-PBS-T, Vector Lab., CA). After a further wash step, the immunohistochemical reaction product was developed in a 0.05 M Tris buffer solution containing 0.05% 3,

Fig. 1. Representative pictures of immunoreactivity of OP3 in rat brain. OP3-immunoreactivity was abundant within patches as well as in the subcallosal streak in the striatum, medial preoptic area and ventral endopiriform nucleus (A); fasciculus retroflexus and accessory medial optic tract in the midbrain (B); interpeduncular nucleus (C); inferior colliculus, dorsal raphe nucleus and lateral parabrachial nucleus (D), etc. Representative pseudocolor high magnification of immunoreactivity of OP3 in medial terminal nucleus of the accessory optic tract of control (E) and 3 day PL-017-treated animal (F); or in fasciculus retroflexus of control (G) and 3 day PL-017-treated animal (H). Scale bars: A–D, 1000 μm; E–H, 50 μm.


A
MPA
VEn
B
FR
MT
C
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D
DCIC
LPB
DR
E
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Table 2
The effect of chronic PL-017 treatment on rat brain μ-opioid receptor immunodensity

Region	Mean O.D. (control)	Mean O.D. (% of control)		
		1 day	3 days	5 days
LPB	0.552 ± 0.024	89.8 ± 4.8	69.6 ± 3.0[a,b]	68.7 ± 3.3[a,b]
IPR	0.550 ± 0.027	79.7 ± 3.6[a]	68.4 ± 3.0[a]	69.0 ± 4.8[a]
MT	0.523 ± 0.048	82.0 ± 3.7[a]	63.7 ± 3.7[a,b]	63.6 ± 2.8[a,b]
IPC	0.516 ± 0.033	84.9 ± 1.0[a]	54.5 ± 3.7[a,b]	56.7 ± 2.2[a,b]
FR	0.507 ± 0.039	88.0 ± 2.7[a]	55.8 ± 5.1[a,b]	52.1 ± 3.4[a,b]
AVVL	0.448 ± 0.030	81.5 ± 5.5[a]	63.5 ± 4.0[a,b]	63.2 ± 3.8[a,b]
MnR	0.432 ± 0.032	84.1 ± 4.0[a]	71.0 ± 2.0[a,b]	69.7 ± 2.4[a,b]
LHbM	0.419 ± 0.031	87.1 ± 4.1	73.6 ± 5.1[a]	71.2 ± 5.2[a,b]
VEn	0.416 ± 0.026	80.6 ± 5.7[a]	64.3 ± 4.0[a,b]	67.6 ± 4.0[a,b]
DR	0.405 ± 0.039	92.4 ± 5.1	76.0 ± 3.8[a,b]	77.4 ± 3.5[a,b]
IPL	0.375 ± 0.011	80.4 ± 2.9[a]	71.4 ± 2.4[a,b]	68.8 ± 4.3[a,b]
PVA	0.313 ± 0.017	85.3 ± 2.0[a]	76.5 ± 3.5[a]	78.0 ± 3.7[a]
CA1	0.301 ± 0.037	87.0 ± 3.5[a]	79.3 ± 2.6[a,b]	77.8 ± 2.6[a,b]
CG	0.290 ± 0.022	88.9 ± 2.1[a]	74.3 ± 3.5[a,b]	75.8 ± 1.8[a,b]
MPA	0.264 ± 0.016	82.1 ± 2.5[a]	76.3 ± 2.5[a,b]	76.0 ± 2.8[a,b]
DCIC	0.262 ± 0.016	82.5 ± 1.9[a]	78.8 ± 2.0[a]	78.3 ± 1.3[a]
Cortex	0.168 ± 0.011	82.7 ± 1.8[a]	72.4 ± 1.7[a,b]	72.3 ± 1.4[a,b]
CPu	0.130 ± 0.007	81.9 ± 4.8[a]	67.3 ± 3.0[a,b]	69.4 ± 3.2[a,b]

Values are means ± S.E.M. ($n \geq 6$ animals). Significant differences from control group ([a]$P < 0.05$) or from 1 day group ([b]$P < 0.05$) were determined with one-way ANOVA and Newman-Keuls multiple comparisons test. No significant difference was found between 3 days and 5 days.
Abbreviations. AVVL: anteroventral thalamic nucleus, ventrolateral part; BSTLD: bed nucleus of stria terminalis, lateral division, dorsal part; CA1: hippocampus CA1; CG: central gray; CPu: caudate putamen; DCIC: dorsal cortex of the inferior colliculus; DM: dorsomedial hypothalamic nucleus; DR: dorsal raphe nucleus; FR: fasciculus retroflexus; IPC, IPR, IPL: interpeduncular nucleus, caudal, rostral and lateral subnucleus; LHbM: lateral habenular nucleus; LPB: lateral parabrachial nucleus; MnR: median raphe nucleus; MPA: medial preoptic area; MT: medial terminal nucleus of the accessory optic tract; PN: paranigral nucleus; PVA: paraventricular thalamic nucleus, anterior part; SNR: substantia nigra, reticular part; VEn: ventral endopiriform nucleus.

3′-diaminobenzidine hydrochloride (DAB), 0.005% H_2O_2. The sections were washed, dehydrated and coverslipped. Controls were incubated with normal rabbit serum instead of antiserum in the staining procedure.

2.3. Quantitative analysis of immunohistochemistry

The brain slices were examined through a microscope equipped with a plan-Neofluar lens (Axioskop, Zeiss, Germany). The images were captured with a CCD camera (model 4912-2010, CoHu, USA) and stored in a pentium-90-based computer equipped with an image capture interface card (IMAGRAPH, USA). The captured images were 640×480 pixels in dimension with 256 gray levels. The image analysis software we used was the Image Pro Plus for Windows version 1.3 (Media Cybernetics, USA). Spatial calibration was done with an objective micrometer. For each captured image, background correction was done. Optical density calibration for each slide was done by selecting the area without opacity as O.D. of 0 and the completely opaque area of the slide as maximum O.D. of 2.41 to fit a standard O.D. curve. The most representative area of interest on each image was manually selected with same shape and size and the average O.D. was measured.

2.4. Statistics

The results are expressed as the means ± S.E.M. One-way analysis of variance (ANOVA) was used first to analyze the data. The observed significances were then confirmed with Newman–Keuls multiple comparisons test.

2.5. Materials

The opioid peptide PL-017 was purchased from Peninsula Labs. ABC kit and biotinylated anti-rabbit IgG were supplied by Vector Lab. (Burlingame, CA). Picric acid was purchased from BDH Lab. (England). 3,3′-Diaminobenzidine tetrahydrochloride (DAB), bacitracin, gelatin, paraformaldehyde, sodium tetraborate, sucrose, Tris, Triton X-100 were supplied by Sigma Chemical Co. (St. Louis, MO). All other chemicals used were reagent grade and were supplied by Nacalai Tesque (Kyoto, Japan).

3. Results

3.1. Development of tolerance to chronic PL-017 treatment

The AD_{50} of PL-017 for eliciting an antinociceptive response in the tail-flick test was determined before and after chronic PL-017 treatment. As summarized in Table 1, after 1 day of administration of PL-017, tolerance had been produced (the AD_{50} value was increased 1.4-fold). Further chronic injection of PL-017 resulted in a time-dependent increase in the AD_{50} values of the opioid peptide. As shown in Table 1, after 3 days of chronic treatment, the AD_{50} value had increased 12.7-fold and after 5 days 32-fold, i.e. the animals had become increasingly tolerant to the effects of PL-017.

3.2. Immunohistochemistry of opioid receptors in rat brain

OP3-immunoreactivity was found in a number of regions throughout the brain, such as the cerebral cortex,

Table 3
The effect of chronic PL-017 treatment on rat brain δ-opioid receptor immunodensity

Region	Mean O.D. (control)	Mean O.D. (% of control) 3 days
CA1	0.274 ± 0.009	94.9 ± 5.0
MPA	0.277 ± 0.016	98.2 ± 4.8
BSTLD	0.305 ± 0.018	94.8 ± 4.2
SNR	0.342 ± 0.019	104.0 ± 3.9
DM	0.371 ± 0.034	93.0 ± 5.7
IPR	0.377 ± 0.028	96.9 ± 2.8

Values are means ± S.E.M. ($n \geq 6$ animals). No significant difference was found after 3 days of PL-017 treatment.

Table 4
The effect of chronic PL-017 treatment on rat brain κ-opioid receptor immunodensity

Region	Mean O.D. (control)	Mean O.D. (% of control) 3 days
LPB	0.224 ± 0.008	104.2 ± 5.4
SNR	0.235 ± 0.011	97.0 ± 8.6
MPA	0.242 ± 0.012	101.0 ± 5.5
PN	0.275 ± 0.022	108.0 ± 8.6

Values are means ± S.E.M. ($n \geq 6$ animals). No significant difference was found after 3 days of PL-017 treatment.

caudate putamen, medial preoptic area, several thalamic nuclei, hippocampal regions, habenular nuclei, interpeduncular nuclei, several raphe nuclei, the colliculi, parabrachial nuclei, etc. Some representative pictures are shown in Fig. 1(A)–(D). The representative pseudocolor pictures of high magnification of immunoreactivity of OP3 in the medial terminal nucleus of the accessory optic tract in control and 3-day PL-017-treated animal are shown in Fig. 1(E) and (F). The other set of representative pictures of the fasciculus retroflexus (FR) are shown in Fig. 1(G) and (H). OP1-immunoreactivity was found in the interpeduncular nuclei, hippocampus, medial preoptic area, dorsomedial hypothalamic nucleus, substantia nigra, etc. OP2-immunoreactivity was found in the medial preoptic area, substantia nigra, paranigral nucleus, parabrachial nucleus, etc. (not shown).

3.3. The effect of chronic PL-017 treatment on rat brain opioid receptor immunodensity

When we used an image analysis system (Image Proplus) to quantify the optical density (O.D.) of the OP3-immunoreactive brain areas, we found that the mean O.D. of each area decreased by about 10–20% (i.e. 80–90% of control value) after 1 day of treatment with PL-017 (Table 2). After treatment of PL-017 for 3 days, the mean O.D. of OP3-immunoreactivity decreased further to about 55–75% of the control value in most areas. However it did not show decrease further after treatment with PL-017 for 5 days. No significant change on OP1-immunoreactivity or OP2-immunoreactivity was found after chronic PL-017 treatment for 3 days (Tables 3 and 4).

4. Discussion

In general, chronic exposure to an agonist usually induces two different processes at the receptor level: a rapid desensitization (reduction of the high-affinity state and uncoupling of the receptor from effector) and a late down-regulation (decrease in the density of receptors) (Taylor and Insel, 1990). With respect to μ-opioid receptors, the appearance of regulatory modifications in receptor properties after chronic opioid treatment is well documented in vitro: up-regulation of opioid receptors after chronic treatment with opioid antagonists (Tempel et al., 1985; Yoburn et al., 1989) and down-regulation after chronic opioid agonist treatment (Puttfarcken et al., 1988). Studies on the in vivo regulation of μ-opioid receptors by chronic exposure to agonists have yielded controversial results, with several groups reporting lack of changes, down-regulation and up-regulation (for review, see Cox, 1993). Several variables, including the type of drug, animal species, tissue preparation process, etc., can influence the results of such studies (Cox, 1993).

In our previous ligand binding studies (Tao et al., 1990), we demonstrated that chronic treatment of rats with a μ-selective agonist PL-017 resulted in a reduction in μ-opioid receptor binding during the course of tolerance development. However there was a more than 10-fold increase in the AD_{50} value after 3 days of chronic PL-017 treatment but no detectable down-regulation of the μ-opioid receptor in this period. Down-regulation of the μ-opioid receptor was only shown after 5 days of chronic PL-017 treatment. One possibility is that the down-regulation could have been obscured by receptors that were not down-regulated in adjacent brain regions after 1 day or 3 days of PL-017 treatment. Therefore in the present study, we used immunohistochemical methods to further investigate this issue. We used antisera against the C-terminal peptide of OP3 (Arvidsson et al., 1995a), N-terminal peptide (3–17) of OP1 (Dado et al., 1993; Arvidsson et al., 1995b) and C-terminal peptide (366–380) of OP2, (Arvidsson et al., 1995c) to determine the immunodensity of opioid receptors before and after chronic PL-017 treatment. We found there was a time-dependent increase in the AD_{50} of PL-017 (i.e. degree of tolerance) (Table 1) and a time-dependent decrease in OP3-immunoreactivity in many brain nuclei (Table 2). However, the decrease in OP3-immunoreactivity reached a maximum around 3 days of PL-017 treatment when the degree of tolerance was still increasing. Our previous binding data (Tao et al., 1990) showed that down-regulation of μ-opioid receptors occurred after 5 days of PL-017 treatment but not after 3 days of PL-017 treatment. Since the radioligands ([^{3}H]diprenorphine or [^{3}H]DAMGO) only bind to membrane opioid receptors, we think that down-regulation should have occurred to a greater extent after 5 days of PL-017 treatment. However, the immunohistochemical studies were performed with fixed brain tissue and membranes may have been made permeable by paraformaldehyde and the antibodies could have entered the cell to react with the intracellular opioid receptors. If this is the case, the OP3-immunoreactivity we detected in the present immunohistochemical study represents the total of membrane and intracellular OP3-immunoreactivity. Therefore the similarity in OP3-immunoreactivity after 5 days of PL-017 treatment and 3 days of PL-017 treatment may imply that there are more μ-opioid receptors being internalized after 5 days of PL-017 treatment than that after 3 days of PL-017 treatment, i.e. the μ-opioid receptor density on the cell surface after 5

days of PL-017 treatment is less than that after 3 days of PL-017 treatment, due to the increase in internalization of OP3 after 5 days of PL-017 treatment. Therefore, the total OP3-immunoreactivity detected after 5 days of PL-017 treatment was not significantly different from that after 3 days of PL-017 treatment. The methods used in the present study are more sensitive than the previous biochemical binding study to detect changes in opioid receptors in different brain regions after different durations of chronic treatment with opioids. Even so, we did not find any significant change in OP1 or OP2-immunoreactivity after chronic treatment with PL-017.

One may argue that opioids may interfere with the labeling of the receptors by our antibody. We chronically treated rats with morphine for 6 days to induce tolerance. However, no significant change in OP3 immunodensity was found in most brain regions we examined. This result indicates that although morphine is also a μ-opioid agonist, it does not cause down-regulation of μ-opioid receptors as PL-017 does. This result also confirms that opioids do not interfere with the labeling of the receptors by our antibody.

In conclusion, chronic treatment of rats with a μ-opioid receptor-selective agonist PL-017 selectively down-regulated only μ-opioid receptors. The down-regulation of μ-opioid receptor occurred after 1 day of PL-017 treatment and showed a time-dependent correlation with the development of tolerance. Therefore, both down-regulation of OP3 as well as uncoupling of mu-opioid receptors and G proteins (Tao et al., 1995) may be important mechanisms for PL-017 tolerance.

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