as this metabolite. It is known that aliphatic alcohols serve as substrates for sulphate conjugation [3]. The hydroxyethyl chain of tiaramide can be considered amenable, therefore, to sulphate conjugation. Lyon and Jakoby [4] have recently shown the identity of alcohol sulphotransferases with hydroxy steroid sulphotransferases. These enzyme activities have been shown to be sex-related and preferentially developed in female rats [5]. This to our knowledge is the first example of such a sex difference for sulphate conjugation being reported for a pharmaceutical agent.

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Adaptive modulations of brain membrane lipid fluidity in drug addiction and denervation supersensitivity

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The fluidity (reciprocal of microviscosity) of cell membrane lipids is implicated in many membranal functions, including transport [1], enyzmatic activity [2], receptor binding [3, 4], interaction of receptors with adenylate cyclase [5] and protein phosphorylation [6]. Under various conditions of stress which affect lipid fluidity (e.g. temperature changes), "homeoviscous adaptation" [7] is triggered so that lipid composition is altered to restore fluidity and proper membrane functions. Addictives, such as alcohol and barbiturates, have been found to have a fluidizing effect on membrane lipids [8]. In alcohol-addicted animals, tolerance is accompanied by restoration of membrane lipid fluidity by increased cholesterol level [9, 10]. Similarly, morphine has been shown, both *in vivo* and *in vitro*, to increase the lipid fluidity of synaptic membranes in a dose-dependent manner which can be blocked by naloxone [11], and to alter lipid metabolism [12].

In this study, we have investigated whether tolerance to, and dependence on, morphine may also involve a process of homeoviscous adaptation in an animal model. In parallel, we have studied the effect of a related phenomenon, denervation supersensitivity, on changes in the lipid microviscosity of brain membranes.

Morphine (HCl) was obtained from Assia (Petach Tikvah, Israel), naloxone from Endo Laboratories (Garden City, NY), 1,6-diphenyl-1,3,5-hexatriene (DPH) from Koch-Light Laboratories (Colnbrook, England), and Tris free base ("trizma") from the Sigma Chemical Co. (St. Louis, MO). Animals (Sabra rats, 2- to 3-months-old) were injected intraperitoneally with morphine (HCl) or with naloxone in doses described below. Alternatively, the animals were implanted subcutaneously with 75 mg morphine (free base) pellets according to Way *et al.* [13]. The times from treatment to decapitation are given below.

Fornix lesions were produced unilaterally, and the location of the lesion was verified histologically. The animals were decapitated 30 days after the lesions were made, and the brains were removed and dissected into regions. Nigrostriatal lesions were produced unilaterally according to Ungerstedt [14], and only animals that showed intensive circling towards the intact side when challenged with 0.5 mg/kg apomorphine were tested. The animals were decapitated 30 days after the lesions were made. The brains were removed and dissected into regions. The tissues from the various brain regions were gently ground with a Teflon pestle and vortexed. The heavy particles were allowed to settle, and the upper liquid was transferred to another set of tubes and centrifuged at 1500 rpm for 10 min. Microscopic examination showed that the pellet contained a mixture of dissociated cells and cell clusters with some cell debris. This preparation was used in most membrane fluidity measurements. Although the results only partially represent plasma membrane microviscosity, the measurements proved to be much more reproducible with this preparation than with partially purified membrane preparations (see below).

Membrane lipid microviscosity ($\bar{\eta}$—the reciprocal of fluidity) was determined by fluorescence depolarization using the lipid probe, DPH (for a review, see Ref. 15). A stock solution of 2 mM DPH in tetrahydrofuran was diluted 1:1000 in vigorously stirred phosphate-buffered saline (pH 7.2). A sample (0.5 to 1 mg) of dissociated cells was sus-

Table 1. *In vivo* effects of acute and chronic morphine and naloxone treatments on rat brain lipid microviscosity*

Treatment (N)	Time to decapitation	Hippocampus		Caudate	
		$\bar{\eta}$, 25° (poise)	C/PL	$\bar{\eta}$, 25° (poise)	C/PL
Control† (20)		6.6 ± 0.1	0.52 ± 0.04	6.8 ± 0.1	0.54 ± 0.05
Acute					
Morphine injection, 25 mg/kg, s.c. (8)	30 min	6.1 ± 0.1‡		6.3 ± 0.1‡	
Morphine pellet implantation (8)	36 hr	6.3 ± 0.1‡		6.3 ± 0.1‡	
Naloxone injection, 2 mg/kg, i.p. (4)	10 min	6.9 ± 0.2‡		6.9 ± 0.1 (NS)§	
Naloxone injection, 10 mg/kg, i.p. (4)	10 min	6.4 ± 0.1‖		6.4 ± 0.1‖	
Chronic					
Morphine pellet implantation¶ (4)	11 days	6.8 ± 1‖		6.9 ± 0.1 (NS)	
Morphine pellet implantation** (8)	11 days	6.9 ± 0.2‡	0.69 ± 0.06‖	7.1 ± 0.1‡	0.73 ± 0.10‖
As above + naloxone injection, 2 mg/kg, i.p.†† (7)	10 min	7.1 ± 0.1‡		7.3 ± 0.1‡	

* Values represent mean ± S.E.M. where (N) is the number of animals tested. Determinations of microviscosity were done in duplicate.

† Control samples represent values obtained from either untreated, saline-injected, or sham-implanted animals. The results were similar and, therefore, merged.

‡ $P < 0.01$, compared to control (Student's *t*-test).

§ Not significant.

‖ $P < 0.005$, compared to control (Student's *t*-test).

¶ Rats were implanted with one pellet and decapitated 11 days later.

** Rats were implanted with one pellet, another one on day 4, and decapitated 11 days after the first.

†† Same as (**) but injected with naloxone 10 min before decapitation.

pended in 2 ml of the DPH dispersion and incubated for 30 min at 25°. The degree of fluorescence polarization, P, was then recorded at 25° as described [13, 15]. The lipid microviscosity was determined by the approximate empirical relation—$\bar{\eta} = 2P/(0.46 - P)$—which, on a relative scale, should be close to linear [15]. Cholesterol (C) [16] and phospholipids (PL) [17] were determined on homogenates from the various brain regions after extraction with chloroform–isopropanol (5:3, v/v). The results are presented as C/PL mole ratios.

The effects of acute and chronic treatments with morphine on the lipid fluidity of brain membranes were tested in partially purified membrane preparations (e.g. P_2M), in purified synaptic plasma membranes (SPM), and in dissociated cell preparations. Significant and reproducible changes in the lipid microviscosity could be detected only in the dissociated cell preparations. It seems that this preparation conserves the membrane integrity and, therefore, resembles the *in situ* state more closely. The observed changes in the cell preparation relate to all organelles and, therefore, only partially represent the changes at the cell plasma membranes. The results of the various *in vivo* morphine experiments are summarized in Table 1. Acute morphine treatment, either by intraperitoneal injection or by pellet implantation, produced a significant decrease in $\bar{\eta}$, both in caudate and hippocampus. In other brain regions, the effects on $\bar{\eta}$ were smaller or insignificant (data not shown). Chronic morphine treatment, however, resulted in a reversal of the fluidizing effect which, in fact, surpassed the control value in both brain regions and was accompanied by an increase in C/PL (see Table 1). An injection of the opiate antagonist naloxone (2 mg/kg, i.p.), which induces withdrawal symptoms [18] in rats chronically treated with morphine, caused a small further increase in $\bar{\eta}$. When the same dose of naloxone was injected into naive rats, it also produced a small increase in $\bar{\eta}$ as compared to rats injected with saline. Higher doses of naloxone (10 mg/kg) caused a small decrease in $\bar{\eta}$.

Parallel *in vitro* experiments, in which samples of brain tissue prepared as above were treated with opiates, are summarized in Table 2. Morphine at a low concentration (10^{-8} M) caused a significant decrease in $\bar{\eta}$, which could be further decreased with 10^{-5} M. Naloxone at a low concentration (10^{-9} M) seemed to partially reverse the fluidizing effect of morphine. Naloxone itself at a low concentration (10^{-9} M) caused an increase in $\bar{\eta}$, whilst at a lower concentration it reduced $\bar{\eta}$. These effects of naloxone may explain some of its agonist-like effects at high doses.

Steady-state fluorescence depolarization of DPH in biological membranes is a sensitive and reproducible tool for monitoring lipid dynamics [15]. The results can be presented in qualitative units of the determined degree of fluorescence polarization (P) or in the operational quantitative units of microviscosity [15]. Analysis of the latter has shown that it is a complex combination of the true viscosity of lipids and their degree of order [19]. Nevertheless, this term was found to correlate well with the lipid free volume [20, 21] and with membranal activities [20], and it is of direct physiological relevance [21].

The microviscosity of the hippocampus after fornix lesions was found to increase markedly on the denervated side ($\bar{\eta} = 7.9 \pm 0.1$) compared to the control side ($\bar{\eta} = 6.6 \pm 0.1$) ($P < 0.01$ tested with nine animals, from three separate experiments). No significant change was found in caudate. On the other hand, nigrostriatal lesion resulted in a significant increase of $\bar{\eta}$ in caudate on the denervated side ($\bar{\eta} = 7.3 \pm 0.1$) compared to the control side ($\bar{\eta} = 6.8 \pm 0.1$) ($P < 0.01$ tested with nine animals, from three separate experiments), with no significant change of $\bar{\eta}$ in hippocampus. These changes in lipid microviscosity were in a heterogeneous population of cells, which has the disadvantage of not representing the more relevant synaptic plasma membranes. The latter, as well as other membrane preparations, did not display significant and reproducible effects of morphine treatments. It is plausible that, during the homogenization steps in the preparation of the membranes, exchange of lipids between the various membranes and lipid pools took place which abolished some of the effects.

Both the *in vivo* and *in vitro* results indicate that acute morphine treatment reduced the membrane lipid microviscosity of the brain cells, and that the fluidizing effect of morphine was reversed in the chronic state. This adaptation appears to have occurred through changes in the lipid composition, as indicated by the concomitant increase in the C/PL mole ratio, in chronically treated animals. Part of the observed restoration of lipid microviscosity may also have been due to a concomitant increase in the sphingomyelin to lecithin mole ratio or a decrease in the degree of unsaturation of the phospholipid acyl chains. These parameters are probably the main determinants of membrane lipid fluidity *in vivo* [15]. It should be borne in mind that the actual fluidity changes in the regions mediated by morphine *in vivo* could be much greater than the apparent changes measured, since lipid domains which are not affected by the drug can dilute the effect. Nevertheless, even these small changes in the apparent $\bar{\eta}$, which should be considered as a lower limit, are probably sufficient to cause significant alterations in membrane function [3, 4, 6].

A distinction should be made between two effects in considering the modulation of membrane lipid fluidity: (a) the specific association of the ligand with a receptor, as in

Table 2. *In vitro* effects of morphine and naloxone on rat brain lipid microviscosity*

	$\bar{\eta}$, 25° (poise)	
	Hippocampus	Caudate
Untreated	6.58 ± 0.04	6.75 ± 0.05
Morphine (10^{-8} M)	6.25 ± 0.06†	6.41 ± 0.06†
Morphine (10^{-5} M)	5.95 ± 0.08†	6.25 ± 0.06†
Morphine (10^{-8} M) ± naloxone (10^{-9} M)	6.41 ± 0.05‡	6.41 ± 0.09 (NS)§
Naloxone (10^{-9} M)	6.92 ± 0.08†	6.92 ± 0.09‖
Naloxone (10^{-5} M)	6.25 ± 0.06†	6.58 ± 0.06‖

* The ligands were incubated with the dissociated cells in the DPH dispersion for 30 min at 25°. Concentrations indicated are final. Values represent mean ± S.E.M. from five separate experiments, three animals in each. Determinations of microviscosity were done in duplicate.

† $P < 0.001$, compared to untreated (Student's *t*-test).

‡ $P < 0.05$, compared to morphine (10^{-8}M) (Student's *t*-test).

§ Not significant.

‖ $P < 0.05$, compared to untreated (Student's *t*-test).

the case of acetylcholine [22] and β-adrenergic agonists [23], and (b) the non-specific incorporation of a drug into the lipid bilayer, as in the case of alcohol, barbiturates, and other anesthetics [8]. In the latter case, there may also be differences in the actual lipid region into which the drug is incorporated. The low concentrations of morphine required to produce a fluidizing effect on membranes and the fact that naloxone partially reverses this effect and by itself acts in the opposite direction indicate that morphine exerts its fluidizing effect, at least in part, through binding to its receptor. The occupied receptor may then undergo conformational changes which, in turn, induce structural changes in the membrane lipid domain [22, 23]. The other part of the fluidizing effect of morphine may be due to direct interaction, both specific and non-specific, with the membrane lipids. The latter possibility may occur in the hippocampus where the fluidizing effect of morphine is large, whilst this region is known to be relatively poor in opiate receptors [24]. The ensuing process of adaptation to morphine, on the other hand, may also involve direct action of morphine on lipid-metabolizing enzymes [12, 25].

One of the first suggestions as to the mechanism of tolerance to, and dependence on, narcotic drugs was that postsynaptic cells become supersensitive to their normal transmitter or its functional analogues [26]. Supersensitivity has been shown in many cases to involve an increase in the number of postsynaptic receptors [27, 28]. We have shown recently that increased microviscosity of membrane lipids is accompanied by an increase in the number of receptors to several neurotransmitters, agonists and antagonists, which become unmasked, probably by vertical displacement [3, 4, 6]. A similar process may also occur during denervation supersensitivity. This possibility is supported by our finding that denervations are accompanied by an increase in membrane microviscosity which could, in turn, result in unmasking of serotonin, β-adrenergic and opiate receptors [3, 4, 6]. The changes in membrane lipid fluidity reported here could provide a common mechanism for the phenomena of denervation supersensitivity and tolerance to addictive drugs, since in the hyperviscous membranes receptors may become overexposed, thus giving rise to the abstinence syndrome which is characterized by a general supersensitivity of the CNS to neurotransmitters [18, 29].

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