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A DEMONSTRATION OF NALOXONE-PRECIPITATED OPIATE WITHDRAWAL ON SINGLE NEURONES IN THE MORPHINE-TOLERANT/DEPENDENT RAT BRAIN

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1 A comparison has been made between the effects of microelectrophoretically applied naloxone on single neurones in the frontal cerebral cortex and the striatum of naive and of morphine-tolerant/dependent rats, anaesthetized with a mixture of α-chloralose and urethane.

2 Specificity of the results obtained was evaluated by contrasting the effects of alternate applications of the (+)- and (−)-isomers of naloxone to the same neurones.

3 In naive rats naloxone had predominantly no effect, only a few cells revealing non-specific depressant responses to the drug.

4 In morphine-tolerant/dependent rats a higher proportion of neurones responded to naloxone; either with stereospecific excitatory responses, in which the activity evoked by L-glutamate or acetylcholine was increased, or with a non-specific inhibition, similar to that observed in naive animals.

5 It is suggested that these excitatory responses to microelectrophoretically applied (−)-naloxone represent opiate withdrawal responses at the single neurone level and that they reflect a latent hyperexcitability of the postsynaptic membrane in the morphine-tolerant/dependent state.

Introduction

Himmelsbach's original proposal (Himmelsbach, 1943) that opiates can induce homeostatic adjustments in the vertebrate central nervous system (CNS) has been followed by a number of attempts to explain the possible nature of such changes and their role in the closely related phenomena of opiate tolerance and dependence. These hypotheses can conveniently be divided into two groups (Collier, 1978); (1) Those that postulate an indirect induction of adaptive changes in cells that are not the primary site of opiate action; either neuronal pathways that were previously redundant (Martin, 1968) or cells that are innervated by the opiate-responsive neurones (Jaffe & Sharpless, 1968). (2) Those that postulate adaptive changes, such as altered neurotransmitter sensitivity, of the opiate-responsive neurones themselves (Collier, 1965, 1969). Hypotheses of the second type have gained increasing acceptance in recent years, with the accumulation of evidence that opiate tolerance and dependence can occur within single cells. Cultured neuroblastoma × glioma cells, for example, have been shown to develop tolerance to the specific inhibitory effects of opiates on cyclic adenosine 3′,5′-phosphate (cyclic AMP) production and exist in a state of dependence, as evinced by the higher production of cyclic AMP that occurs upon withdrawal of the opiate (Sharma, Klee & Nirenberg, 1975; Traber, Gullis & Hamprecht, 1975). Similarly, single neurones in the myenteric plexus of the guinea-pig ileum develop tolerance to the specific inhibitory effect of morphine *in vitro*, even under conditions of synaptic blockade (Hammond, Schneider & Collier, 1976) and display a hyperactivity upon the withdrawal precipitated by opiate antagonists (North & Karras, 1978).

Single neurones in the frontal cortex (Satoh, Zieglgänsberger & Herz, 1976), striatum (Zieglgänsberger & Fry, 1976) and locus coeruleus (Aghajanian, 1978) of the rat brain also appear to become tolerant to the specific inhibitory effects of microelectrophoretically applied opiates and opioid peptides during chronic treatment with systemically administered morphine. If these neurones exist in a state of dependence, then they might reasonably be expected to display a withdrawal hyperactivity upon microelectrophoretic application of the opiate antagonist, naloxone. A higher incidence of excitatory responses to microelectrophoretically applied naloxone has, indeed, been reported for neurones in the medial thalamus (Frederickson, Norris & Hewes, 1975), fron-

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tal cerebral cortex (Satoh *et al.*, 1976) and locus coeruleus (Aghajanian, 1978) of morphine-tolerant/dependent rats. However, these studies employed only (−)-naloxone which, until recently was the only form available. Although this drug is a specific opiate antagonist (Kosterlitz & Watt, 1968), it is known to induce excitatory responses of single neurones in several areas of the CNS, in both naive and morphine-tolerant/dependent animals (see Zieglgänsberger & Fry, 1978; Fry, Zieglgänsberger & Herz, 1979). We have therefore investigated the pharmacological specificity of neuronal responses to microelectrophoretically applied naloxone in the brains of naive and of morphine-tolerant/dependent rats, by comparing the actions of (−)-naloxone with those of its enantiomer (+)-naloxone, a compound lacking specific opiate antagonist activity (Iijima, Minamikawa, Jacobsen, Brossi, Rice & Klee, 1978). The microelectrophoretic technique enabled direct tests to be made of the sensitivity of these neurones to L-glutamate and acetylcholine, putative excitatory transmitter substances (see Krnjević, 1974), during the local application of naloxone.

A preliminary account of these experiments has appeared elsewhere (Fry, Zieglgänsberger & Herz, 1978).

Methods

Male Sprague–Dawley rats (250 to 350 g) were anaesthetized with a mixture of α-chloralose and urethane (90 and 300 mg/kg, respectively, i.p.), immobilized with gallamine and respired artificially with room air. Blood pressure and heart rate were monitored through a cannula in the femoral artery, while an adjacent cannula in the femoral vein allowed the continuous infusion of gallamine and the injection of plasma expander, when necessary. A heating lamp was used to maintain rectal temperature at appropriate levels.

The rats were held in a stereotaxic frame, with the upper incisor bar 5 mm above the interaural line and a burr hole drilled through the skull 2.7 to 3.7 mm anterior to bregma and 1.5 to 2.5 mm lateral of the midline. A pool of warm liquid paraffin was then placed over the exposed brain surface and small tears made in the dura- and pia-mater, to allow insertion of electrodes through the frontal cortex and down into the rostral striatum.

The glass-coated tungsten microelectrodes (Levick, 1972) for extracellular recording were glued alongside six-barrelled micropipettes (tip diameter 6 to 8 μm) beyond which they protruded by a distance of 40 to 60 μm (Zieglgänsberger & Puil, 1973). These micropipettes were constructed from filamented tubing and filled by capillarity immediately before use with a selection of the following solutions for electrophoresis: acetylcholine chloride (Fluka) 1.0 M, pH 5.0; methionine-enkephalin (Serva) 12.5 mM, pH 5.7; monosodium-L-glutamate (Merck) 0.5 M, pH 8.0; (−)-naloxone hydrochloride (Endo) and (+)-naloxone hydrochloride (Dr A.E. Jacobsen) both at 50 mM in 150 mM sodium chloride solution, pH 5.0; Pontamine Sky Blue (Gurr) 2.5% (w/v) in 0.5 M sodium acetate buffer pH 5.6. All substances, except L-glutamate, were ejected as cations and retaining currents of 20 nA used between applications. Current flowing through the barrels containing Pontamine Sky Blue solution was continually adjusted to ensure neutralisation of total current at the pipette tip. At the end of some experiments Pontamine Sky Blue was ejected by electrophoresis along the electrode track, to leave marks which would be seen upon subsequent histological examination.

Conventional techniques were used to amplify and display the unitary action potentials. These were counted automatically by a voltage gating device, the output of which was integrated over 1 s intervals and plotted on a chart recorder. Only single units excitable by L-glutamate were selected for study and this excitant was applied either continuously or, more often, with regular 10 s pulses of current, spaced at 50 s intervals. Some neurones were also excited by acetylcholine, in which case applications of this substance were alternated with those of L-glutamate, again at 50 s intervals. Effects described in the Results section involved changes of at least 50% in L-glutamate- and/or acetylcholine-evoked discharge activity. Spontaneous activity of the neurones recorded in the present experiments was usually too low to permit accurate estimation of any changes that may have taken place and is not, therefore, described in detail.

Twenty-four previously untreated or 'naive' rats were used for this study in addition to 37 animals that had been rendered highly tolerant to and dependent on morphine by the subcutaneous implantation of pellets (Bläsig, Herz, Reinhold & Zieglgänsberger, 1973), each containing 75 mg of morphine base, according to the following schedule; one pellet on the first day, two on the fourth day and three on the seventh day of treatment. They were used for experiments on the tenth, eleventh and twelfth days after the start of this implantation schedule.

Results

Naive rats

As can be seen from Table 1, microelectrophoretic application of (−)-naloxone (10 to 80 nA/2.0 to 4.5 min) had no detectable effect on the L-glutamate (6 to 40 nA continuously or 10 to 94 nA/10 s)-evoked ac-

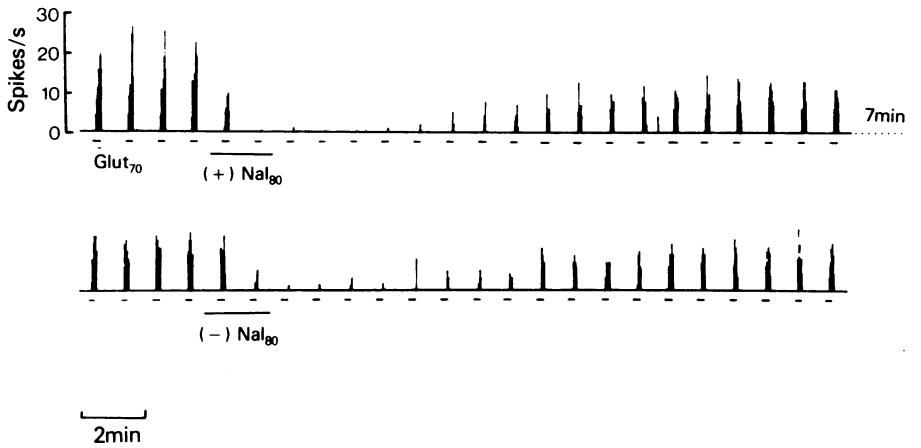

Figure 1 Ratemeter record showing depressant effects of both (+)- and (−)-naloxone (Nal) on the L-glutamate (Glut)-evoked activity of a striatal neurone in a naive rat. Electrophoretic currents are indicated in nA.

tivity of the majority of the striatal neurones recorded in naive rats. This lack of effect of (−)-naloxone could not be explained by poor electrophoretic release of the drug since it was able, on 8/10 of these otherwise unaffected cells, to antagonize the inhibitory action of subsequent applications of the opioid methionine-enkephalin (40 to 160 nA/1 min; see also Zieglgänsberger & Fry, 1976). A few striatal neurones displayed inhibitory responses to (−)-naloxone, which became apparent within 10 to 90 s of applying the drug and ended 10 to 20 s after switching off the electrophoretic current, except for one cell that remained inhibited for 12 min. These inhibitory effects occurred without any detectable depression of spike height but appeared to be non-specific, as they could be mimicked by application of (+)-naloxone to the same cells (Figure 1). Indeed (+)-naloxone (20 to 80 nA/2 min) elicited depressant responses more frequently than the (−)-isomer; 6/7 neurones being inhibited by this drug.

Of the 12 neurones in the frontal cortex tested with naloxone (20 to 80 nA/2 to 3 min), only one displayed an increase and one a decrease in L-glutamate (10 to 40 nA/10 s)-evoked activity, both of these effects being obtained by application of either the (−)- or the (+)-isomer.

Tolerant/dependent rats

In contrast to the results obtained in naive rats, a higher proportion of the striatal neurones recorded in morphine-tolerant/dependent animals responded to microelectrophoretic application of (−)-naloxone (see Table 1). Enhancement of L-glutamate-evoked activity, when observed, almost always occurred during application of (−)-naloxone with low currents of 2 to 20 nA/2 min, while the passage of higher electrophoretic currents (up to 80 nA) resulted in a greater incidence of inhibitory responses. In order to discover which, if any, of these responses could be regarded as specific, an additional 32 L-glutamate excitable striatal neurones were tested with both (+)- and (−)-naloxone, applied alternately with the same electrophoretic currents (starting at 0 to 10 nA/2 to 4 min and increasing, through successive doubling, to 40 to 80 nA/2 to 4 min). Results of these experiments are summarised in Table 2 and show the increase in

Table 1 Effects of microelectrophoretically applied (−)-naloxone on the L-glutamate-evoked activity of striatal neurones in naive and in morphine-tolerant/dependent rats

	Enhanced	*No clear effect*	*Depressed*	*Totals*
Naive rats	1	31	7	39
Morphine-tolerant/ dependent rats	24	10	18	52

Figures refer to the number of neurones tested.

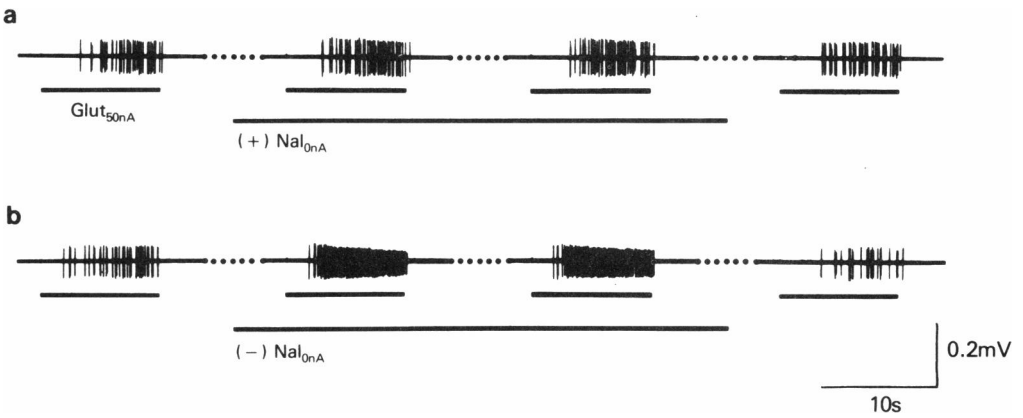

Figure 2 Retouched oscilloscope records of the discharge activity recorded extracellularly during application of L-glutamate (Glut, 50 nA/10 s; 50 s intervals) to a striatal neurone in a morphine-tolerant/dependent rat. (a) Removal of the retaining current for (+)-naloxone (Nal, 0 nA/2 min) starting 20 s before the first application of L-glutamate had no detectable effect on this L-glutamate-evoked activity. (b) Six minutes later a similar leakage of (−)-naloxone from the micropipette enhanced the excitatory action of L-glutamate.

L-glutamate-evoked activity during application of naloxone to be a stereospecific effect; occurring during application of the (−)- rather than the (+)-isomer (see also Figure 2). As in naive rats, the depressant effects of naloxone on L-glutamate-evoked activity did not appear to be mediated by specific opiate receptors, since they could be observed during the application of either (+)- or (−)-naloxone.

During the course of the above experiments 6/34 striatal neurones were found which, in addition to L-glutamate, also gave reproducible excitatory responses to acetylcholine (6 to 80 nA/10 s). On three such neurones, (−)-naloxone elicited increases in both L-glutamate- and acetylcholine-evoked activity (Figures 3, 4) the remaining 3 cells being apparently unaffected. Applied to the same six neurones, (+)-naloxone failed to enhance responses to either excitant and had predominantly depressant effects (4/6 cells), which were always greatest against acetylcholine rather than L-glutamate-induced activity (Figure 5).

The ability of (−)-naloxone to enhance the L-glutamate- and/or acetylcholine-evoked activity of striatal neurones in morphine-tolerant/dependent rats became apparent within 1 min of applying the drug (see Figures 2, 3, 4, 5) and on 13 cells outlasted the period of application by 1 to 13 min (Figure 5). Another seven cells differed in that their excitatory responses to (−)-naloxone gave way to a depression of chemically-evoked firing, which persisted for 5 to 9 min. In all cases, however, these excitatory actions of (−)-naloxone were found to become attenuated during repeated applications of the drug (spaced at regular intervals of 4 to 22 min; see Figures 4, 5); only 4/13 cells showing increases in L-glutamate- and/or acetylcholine-evoked activity that were reproducible during more than two successive applications of (−)-naloxone. When this loss of responsiveness to the

Table 2 Comparison of the effects of microelectrophoretically applied (+)- and (−)-naloxone on the L-glutamate-evoked activity of the same striatal neurones in morphine-tolerant/dependent rats

		(−)-*Naloxone*			
		Enhanced	*No clear effect*	*Depressed*	*Totals*
(+)-*Naloxone*	Enhanced	1	0	0	1
	No clear effect	8	9	0	17
	Depressed	2	4	8	14
	Totals	11	13	8	32

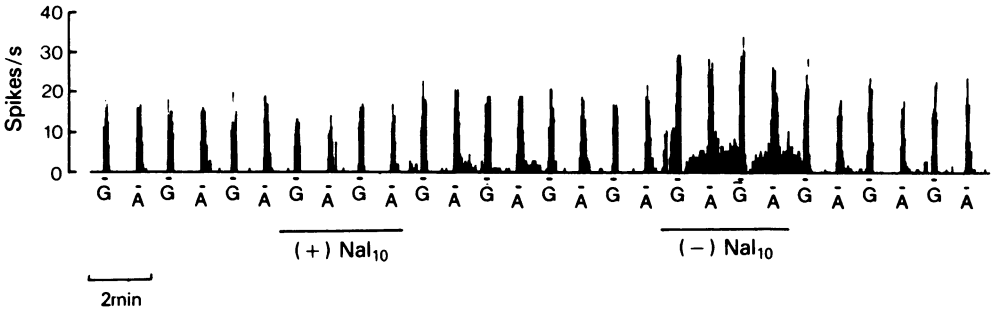

Figure 3 Effects of (+)- and (−)-naloxone (Nal) on a striatal neurone in a morphine-tolerant/dependent rat. The neurone was induced to fire by alternate applications of L-glutamate (G, 30 nA/10 s) and acetylcholine (A, 20 nA/10 s). During application of (−)-naloxone the spontaneous activity of the neurone increased, an effect associated with increased responsiveness to the excitatory effects of both L-glutamate and acetylcholine. No such changes were observed during the previous application of (+)-naloxone.

excitatory effects of (−)-naloxone occurred, it could not be overcome by increasing the electrophoretic current. In fact, as mentioned above, such increases in the amount of (−)-naloxone applied normally resulted in a higher incidence of inhibitory responses.

Inhibitory effects of (+)- or (−)-naloxone also became apparent within 10 to 90 s of application and usually dissipated 10 to 20 s after switching off the electrophoretic current (Figures 4, 5), although on eight neurones these inhibitory responses were more prolonged and outlasted the application by 1 to 12 min. They were not associated with any detectable decrease in spike height.

While inserting electrodes down into the striatum, some recordings were also made of neurones in the overlying frontal cortex. Of 17 such neurones, six dis-

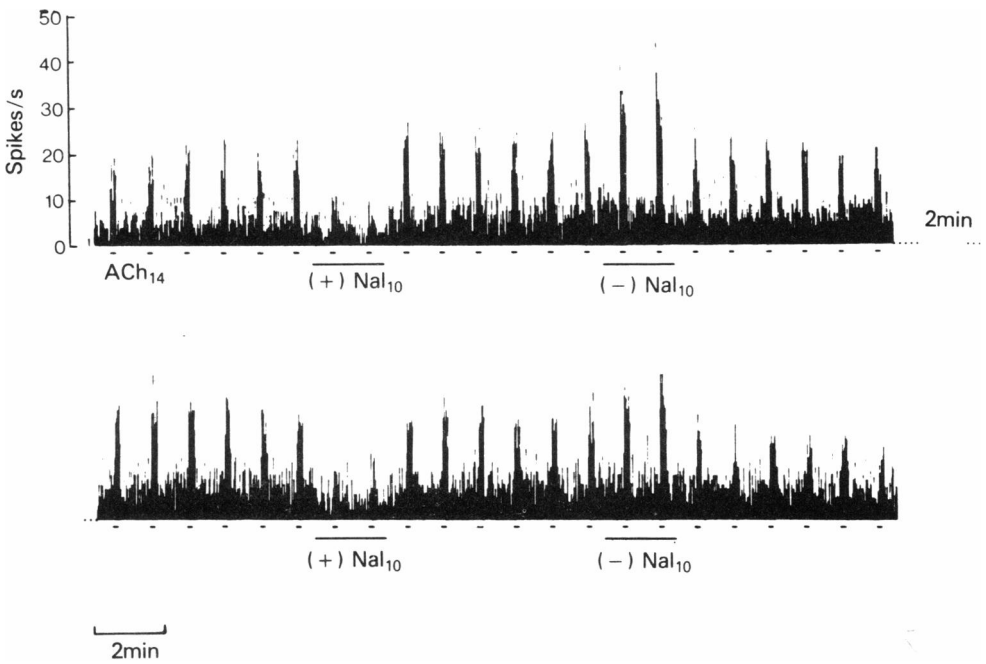

Figure 4 Application of (+)-naloxone (Nal) depresses, whereas (−)-naloxone enhances the excitatory action of acetylcholine (ACh) on a striatal neurone in a morphine-tolerant/dependent rat. The ability of (−)-naloxone to enhance acetylcholine-evoked activity was revealed without any detectable changes in the spontaneous activity of the neurone and was attenuated during the second application of this drug 22 min later.

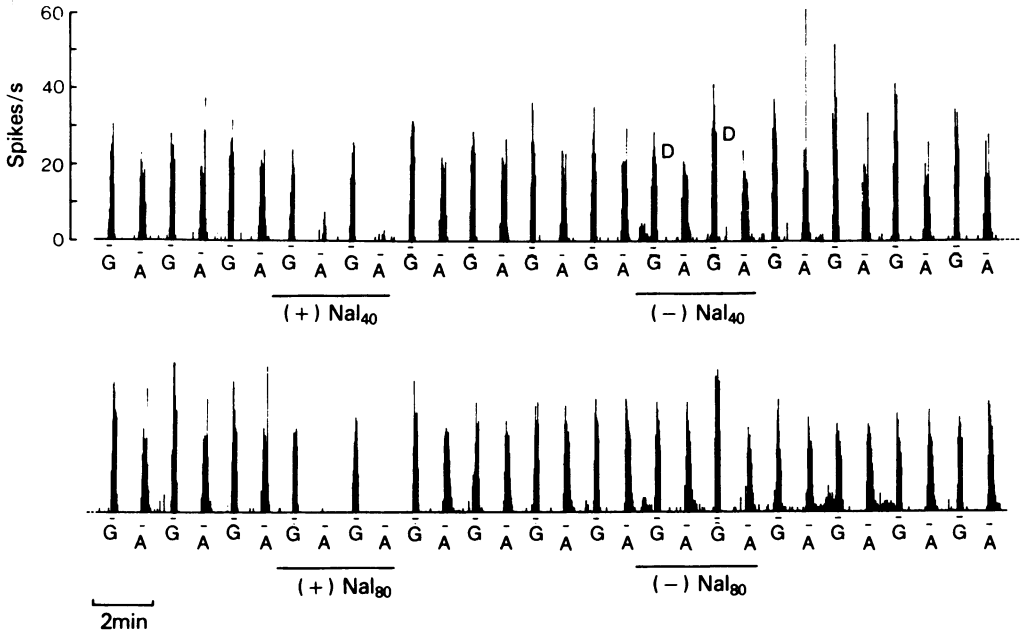

Figure 5 Continuous ratemeter record illustrating a selective inhibitory effect of (+)-naloxone (Nal) on the acetylcholine (A, 44 nA/10 s)- rather than the L-glutamate (G, 30 nA/10 s)-evoked activity of a striatal neurone in a morphine-tolerant/dependent rat. During the first application of (−)-naloxone, responses to L-glutamate were enhanced to such an extent that depolarizing block (D) of neuronal firing occurred, but these excitatory effects became less apparent upon the second application of the drug, even though the electrophoretic current had been doubled.

played increases and two decreases in L-glutamate (10 to 120 nA/10 s)-evoked activity during application of (−)-naloxone (0 to 80 nA/2 to 4 min), both changes in excitability being of a similar time course to those already described for striatal neurones. Excitatory responses to acetylcholine (10 to 100 nA/10 s) could be observed on eight of these cortical neurones and in two cases were enhanced during the application of (−)-naloxone, effects which ran parallel to increases in L-glutamate-evoked activity. However, when (+)-naloxone was applied to the same 17 neurones with identical electrophoretic currents, it failed to increase L-glutamate- or acetylcholine-evoked activity; the only effects observed (on five cells) being inhibitory.

Discussion

In morphine-tolerant/dependent rats, as compared to naive animals, a higher proportion of neurones in the frontal cerebral cortex and the striatum responded to microelectrophoretic application of naloxone, both increases and decreases of chemically-evoked activity being observed. The latter inhibitory effects appeared to be non-specific as they could be seen not only during the application of (−)-naloxone but also during the application of its (+)-isomer, an enantiomer lacking specific pharmacological activity (Iijima *et al.*, 1978). Excitatory effects of naloxone, however, appeared to be mediated by actions at stereospecific opiate receptors; occurring during application of the (−)- but not the (+)-isomer. The present results, therefore, suggest that the excitatory effects of (−)-naloxone in the brains of morphine-tolerant/dependent rats can be regarded as true opiate withdrawal responses at the single neurone level.

Our observation that the stereospecific actions of naloxone on neurones in the frontal cerebral cortex and the striatum of morphine-tolerant/dependent rats were exclusively excitatory could be interpreted in two ways. Either that the drug was acting presynaptically to increase selectively the release of excitatory rather than inhibitory transmitters, or that it was acting postsynaptically to increase the chemical excitability of the neuronal membrane. The latter explanation would appear to be more plausible, especially in view of the enhanced excitatory responses to both L-glutamate and acetylcholine during the application of (−)-naloxone. Thus, it seems that the hyperexcitability of opiate withdrawal is not due to the increased efficacy of any one particular neurotrans-

mitter substance, but to a more general increase in the effectiveness of excitatory transmitter/receptor interaction.

As observed during opiate withdrawal of isolated tissue preparations (Schulz & Herz, 1976) or whole animals (Bläsig *et al.*, 1973), the withdrawal responses of single neurones in the morphine-tolerant/dependent rat brain were opposite in effect to the acute specific inhibitory actions of opioids seen in the naive state. Applied to single frontal cortical (Frederickson & Norris, 1976; Satoh *et al.*, 1976) or striatal (Frederickson & Norris, 1976; Nicoll, Siggins, Ling, Bloom & Guillemin, 1977; Zieglgänsberger & Fry, 1976) neurones, for example, opioids typically cause a (−)-naloxone antagonisable inhibition of both L-glutamate and acetylcholine-evoked activity. In the morphine-tolerant/dependent animal these neurones become refractory to the specific inhibitory effects of the opioids (Satoh *et al.*, 1976; Zieglgänsberger & Fry, 1976) and, judging from the present results, appear to exist in a state of latent hyperexcitability; masked by the continued presence of opiate but revealed during the withdrawal precipitated by local application of (−)-naloxone.

Some speculation can be made as to the possible mechanisms underlying the hyperexcitability of frontal cortical and striatal neurones during (−)-naloxone-precipitated opiate withdrawal. There could, for instance, be a partial depolarization of the postsynaptic membrane towards the threshold potential for generation of action potentials (Fleming, 1976) or, perhaps, a decrease in this threshold. Alternatively, the actual depolarizing responses to the excitatory substances themselves may be enhanced, without any changes in the electrical excitability of the membrane. This latter mechanism remains an intriguing possibility especially if, as implied earlier, opiate withdrawal responses are to be regarded as a mirror image of the acute specific inhibitory effects of these drugs which, at least in the cat spinal cord (Zieglgänsberger & Bayerl, 1976; Zieglgänsberger & Fry, 1976) appear to occur without detectable changes in resting membrane potential or conductance. On the basis of the increased contractile responses to potassium ions observed in myenteric plexus/longitudinal muscle preparations from morphine-tolerant/dependent guinea-pigs, it has been suggested (Johnson, Westfall, Howard & Fleming, 1978) that partial depolarization may contribute to the development of morphine tolerance in myenteric neurones. These preparations, however, were bathed in opiate-free solutions and probably studied while already in a state of partial withdrawal (Schulz & Herz, 1976; North & Zieglgänsberger, 1978). Resolution of the above questions must presumably await intracellular recordings in morphine-tolerant/dependent animals.

Depressant effects of naloxone on neurones in the frontal cortex and the striatum of the naive rat brain appeared to be non-specific, occurring more frequently during application of the (+)- than the (−)-isomer, as has also been observed in the rat brain stem (Gayton, Lambert & Bradley, 1978). Both (+)- and (−)-naloxone, moreover, induced a higher incidence of inhibitory effects in the brains of morphine-tolerant/dependent animals. A similar finding has been reported for the spinal cord of morphine-tolerant/dependent rats, in which (−)-naloxone became more effective as a depressant of the acetylcholine- and ventral root-evoked action potentials of Renshaw cells (Davies, 1976). Such selective inhibitory effects of naloxone on acetylcholine-induced firing of Renshaw cells in the rat (Davies, 1976) and cat (Duggan, Davies & Hall, 1976) and of neurones in the ventrobasal thalamus of the cat (Duggan *et al.*, 1976) have been interpreted as evidence for a specific interaction between opiate and acetylcholine receptor sites in the postsynaptic membrane. In the present experiments naloxone also appeared to be more potent as a depressant of acetylcholine than L-glutamate-evoked activity. Such depressant effects occurred more frequently with the (+)-isomer, however, and thus did not appear to be mediated by specific opiate receptors. Indeed, continuous occupation of specific opiate receptors in the morphine-tolerant/dependent state seems to have rendered neurones in the frontal cortex and the striatum even more susceptible to these non-specific depressant actions of naloxone, a phenomenon similar to that observed in the hippocampus (Fry, Zieglgänsberger & Herz, 1979), which is particularly predisposed to the non-specific actions of opioid agonists and antagonists.

In conclusion, the present experiments indicate that excitatory effects of naloxone on neurones in the frontal cerebral cortex and striatum of morphine-tolerant/dependent rats are stereospecific and presumably mediated by opiate receptors in the postsynaptic membrane. Further evidence is therefore provided that tolerance to the specific inhibitory effects of opioids can develop at the single neurone level in the vertebrate CNS. As first suggested by Collier (1965; 1969), these homeostatic adjustments that take place during the development of tolerance to and dependence on morphine, appear to involve an enhanced sensitivity of the postsynaptic membrane to excitatory transmitters, the mechanism of which remains to be elucidated.

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