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The effect of morphine dependence on the vesicular content of adrenergic nerves in relation to arteriolar smooth muscle in the pancreas of the rat

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1. Sibling male Wistar rats were kept in groups of three under identical conditions. Two groups were made morphine dependent in 28 days and the third served as control. The state of dependence was established by recording the development of tolerance to the analgesic action of the drug and the effect of acute withdrawal of morphine and injection of nalorphine on one test group.

2. Animals in the other test group and the control group were killed and specimens of pancreas collected and prepared for examination by electron microscopy. The adrenergic innervation of pancreatic arterioles was located and photographed and the effect of morphinization on the percentage of granular and agranular small vesicles in the axons determined.

3. Morphinization produces a significant reduction in the granular or dense core vesicle population of these adrenergic axons.

The syndrome which follows acute withdrawal of narcotic from a heroin-dependent or morphine-dependent patient includes manifestations which could be attributed to an uncontrolled discharge of transmitter substances from peripheral autonomic nerves. It has been postulated that administration of morphine-type drugs may lead to a partial damming up of acetylcholine in the cholinergic axon which will be released in excess when the opiate is withdrawn (Paton, 1963). The literature on the pharmacological effects of morphine is voluminous and is confusing because of the different effects exerted by the drug according to the amount given and the duration of dosing (Maynert & Klingman, 1962) in different species (Vogt, 1964), and according to whether the central or peripheral nervous system has been under examination (Cairnie, Kosterlitz & Taylor, 1961 ; Klingman & Maynert, 1962, Laverty & Sharman, 1965). It is reviewed by Gunne (1963).

Crawford & Law (1958) showed that a very small dose of morphine (20 μg/kg subcutaneously) repeated four times increased the urinary output of catecholamines in the rat, and Maynert & Klingman (1962) and Klingman & Maynert (1962) that chronic dosing of rats with up to 200 mg/kg intraperitoneally increased the noradrenaline extractable from the brainstem (while 150 mg/kg had little effect) and reduced that extractable from heart, gut and spleen. Akera & Brody (1968) found an increase in whole brain noradrenaline in rats treated with morphine 30 mg/kg or

120 mg/kg for several weeks, and Sloan, Brooks, Eisenman & Martin (1963) an increase of catecholamine in the brain and decrease in heart and spleen. Morphine in single injections of 60 mg/kg causes an increase in noradrenaline in rat brain after 2 hr but not after 4 hr (Sloan, Brooks, Eisenman & Martin, 1962) and none in heart muscle. It depletes the adrenal medulla (Outschoorn, 1952).

It was thought proper to examine by another technique the question of whether chronic morphinization in the rat with the development of tolerance and the establishment of a pseudo-dependent state has a demonstrable effect on peripheral adrenergic nerve by examining the organelle population of such nerves by electron microscopy.

Methods

Induction of morphine dependence in rats

Sibling male Wistar rats were taken at approximately 100 g weight each and maintained in groups of three (*A*, *B* and *C*) in identical circumstances as to cage, food (standard diet *ad lib.*), ambient temperature (24° C), light and handling (injected subcutaneously at 09.00 hr and 17.00 hr daily for 28 days). Controls (*A*) were injected with normal saline and given 0·01% quinine sulphate in water as sole drink. Test animals (*B* and *C*) were injected at the same hour with morphine HCl (M) in 0·45% saline on a rising dose scale from 1–120 mg/kg according to the schedule in Table 1. During the first 7 days they were offered 1% solution of M and the solution of quinine *ad lib.* on choice, for the following 3 weeks M only, and the volumes consumed were recorded; the weights of the animals and the faecal output (as naturally dry faeces passed during 24 hr, collected and weighed 1 day in every 7 days) were also recorded.

The total morphine HCl intake of groups *B* and *C* by injection was approximately 2·284 g/kg and by ingestion 0·624 g/kg (group *B*) and 0·609 g/kg (group *C*).

Tolerance

On day 1 a standard noxious stimulus was applied to each rat—namely, a rubber-covered light artery forceps clipped on twice to each toe of each foot in succession. If the rat responded to the second application by vocalization and withdrawal a positive score was recorded. This test was applied three times at intervals of 30 min and the score of the last test recorded. Morphine HCl 3·5 mg/kg was injected intraperitoneally and the test repeated after 40 min. The effect was recorded as a percentage of the maximum score attained before M. This test was repeated on day 21.

TABLE 1. *Morphine HCl intake in 24 hr*

By injection				By ingestion		
Groups B and C				Group B		Group C
Day	mg/kg	Day	mg/kg	Day	mg/kg	mg/kg
1–3	2	16–17	60	1–7	7·2	7
4–6	4	18–20	80	8–14	20	17
7–9	8	21–22	160	15–21	31	32
10–11	16	23–28	240	22–28	31	31
12–14	30					

Withdrawal and slaughter

On day 28 control rats (*A*) and one group (*B*) of morphinized rats were killed and tissues harvested as described below. The third group (*C*) were injected with nalorphine HCl 10 mg/kg repeated after 12 hr and observed for evidence of acute withdrawal syndrome.

Electron microscopic processing and analysis of results

Specimens were taken from three areas of the pancreas of each rat and fixed for 4 hr in 5% gluteraldehyde buffered with sodium cacodylate to pH 7·2, washed three times in cacodylate buffer pH 7·2, post-osmicated for 1 hr, and dehydrated and embedded in araldite. Fine sections which included the profile of an arteriole were mounted on grids by standard technique and examined under an Elmiskop 1 Electron Microscope ; photographs at 14,000 magnification were taken which displayed adrenergic nerves in close relation to arteriolar smooth muscle and enlarged $\times 3$ photographically. To ensure that terminal adrenergic axon profiles only were examined for their vesicle content, the following criteria were rigidly applied in the selection of axon profiles: the axons had to lie within the periarteriolar fibroblast sheath: axon bundles had not to contain more than eight axons: one or more axons in each bundle had to be partly or wholly denuded of Schwann cell insulation; axons of small diameter containing mostly neurotubules were excluded (considered to be pre-terminal). Axons conforming to these criteria have been shown to be exclusively of an adrenergic nature (Graham, Lever & Spriggs, 1968 ; Lever, Spriggs & Graham, 1968).

The micrographs from control and morphinized rats were shuffled to preclude identification, one author delineated acceptable axon profiles, another measured the profile areas by planimeter and counted small (mean diameter 500 Å) vesicles classified as granular (possessing an electron dense content), or agranular. Only then were the micrographs allocated to their source of origin by reference to the serial number on the reverse of the print. In this way observer bias was minimized. The vesicle counts are expressed as percentage of the total small vesicle count per unit area, as granular vesicle and agranular vesicle.

Results

Injection of morphine HCl induced a period of stillness in the rats which crouched in a corner of the cage with closed eyes. This was followed by a period of increased activity. As morphinization proceeded the initial calm interval disappeared and the whole effect was one of stimulation.

All rats grew well, group *A* (controls) from 108 to 180 g weight by day 28, group *B* from 98 to 175 g and group *C* from 103 to 160 g (means). After withdrawal of morphine and administration of nalorphine, with water *ad lib.* to drink, group *C* rats lost 8% weight in 48 hr. Fluid intake, initially approximately equal, declined in groups *B* and *C* to 82% of the control—namely, 55 increasing to 81 ml./day for group *A*, 58–67 ml./day for group *B* and 55–67 ml./day for group *C*. On withdrawal from morphine fluid intake fell in group *C* to 70% of the intake on day 28. Faecal output doubled with each group between days 1 and 28. After withdrawal

from morphine faeces became very wet and unformed but there was no watery diarrhoea. Morphinization produced tolerance to the analgesic effect of M 3·5 mg/kg on day 21, as shown in Table 2.

Withdrawal and injecting nalorphine produced marked aggression, piloerection and ptosis of the eyelids, loss of weight, loose stools, frequent attacks of the "writhing syndrome" but no "wet dog shaking" (Martin, Wikler, Eades & Pescor, 1963). It is concluded that the morphinization schedule reported in Table 1 produced morphine-dependence of moderate severity in the rats of group *B* and *C*.

The effect of morphinization on the small vesicle content of adrenergic nerve in relation to visceral arteriolar smooth muscle is shown in Table 3.

The effect of morphinization is to reduce the granular small vesicles in terminal nerve as a percentage of the total small vesicles seen per unit area of axonal profile. The agranular small vesicles increase as a percentage of the total. In absolute figures total small vesicles per unit area decline but not to a statistically significant extent.

Discussion

The morphinization adopted is similar to that advocated by Martin *et al.* (1963) for the production of morphine-dependence in rats, and gave evidence of success in producing tolerance to the analgesic action of morphine and some of the withdrawal symptoms listed by these authors. Morphinization in the rat for 28 days produces a significant reduction in the number of small granular vesicles in terminal adrenergic nerves. This is the storage site for part at least of the noradrenaline content of the

TABLE 2. *Effect of a single intraperitoneal dose of morphine HCl 3·5 mg/kg on response to standard toe pinch in groups of three rats*

Day	*A*	*B*	*C*
1	60	60	55
21	62	94	100

Groups *B* and *C* have become tolerant to the analgesic effect of morphine HCl.
Day 1 initial test; day 21 repeat test after groups *B* and *C* had been receiving chronic morphinization. The number of positive responses to the stimulus recorded after M 3·5 mg/kg is expressed as a percentage of the number recorded before.

TABLE 3. *Effect of morphinization (see Table 1 for the dose-time schedule used) on the number of small vesicles per unit area of profile of terminal adrenergic nerve in relation to pancreatic arteriolar muscle, expressed as percentages.*

	Control $N=104$	Morphine-dependent $N=227$
Total No. of small vesicles per unit area of axonal profile (means ± S.E. of mean)	100%	100%
Granular vesicles	21·5±1·8%	15±0·9%
Agranular vesicles	78·5±2·9%	85±0·7%

Morphinization decreases the percentage of granular vesicles ($P<0·001$) and increases ($P<0·02$) the percentage of agranular vesicles. Morphinization did not significantly reduce the total number of small vesicles (granular plus agranular); 221±14 for the morphinized group (*B*) compared with 254±14 for the untreated control group (*A*).
N, Number of profiles examined.

axon. Such an effect may be a consequence of reduced storage or increased discharge. The evidence of Klingman & Maynert (1962) and Sloan *et al.* (1963) is in agreement with our findings. They were able to recover less noradrenaline from peripheral tissues in the rat after chronic dosing with amounts of morphine in the same range as those used for the terminal week of the trial in this work. It does not follow that this peripheral effect (reduction of storage granules in adrenergic nerve) applies to the peripheral cholinergic nerve or that a similar effect occurs in the central nervous system or that the rat behaves similarly to man in these respects. Nevertheless, the evidence of this experiment indicates that in this species and at this site morphine dependence is accompanied by a reduction and not an increase in storage granules.

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