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STIMULATION OF BRAIN-STEM PROTEIN SYNTHESIS BY MORPHINE

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Abstract—Rats were intoxicated with morphine as intraperitoneal (i.p.) single doses, or for 4 days (final dose 130 mg/kg b.w.) or for 13 days (final dose 340 mg/kg b.w.) using an ingestion method where intoxicated and control rats received the same amount of calories and fluid. The intoxicated groups showed different degrees of physical dependence, demonstrated by variously expressed abstinence symptoms after withdrawal of the drug or after administration of the opiate receptor antagonist naloxone. Soluble protein synthesis was measured *in vivo* in brain stem by double labelling with ^{3}H and ^{14}C valine and followed over time in the various rat groups after i.p. morphine injection in different doses. Protein synthesis in astroglial-enriched primary cultures from brain stem and secretion of labelled protein to the serum free incubation medium was also evaluated after morphine treatment.

There were dose- and time-dependent effects of morphine on brain stem protein synthesis with an initiai decrease and a later increase, 1–3 hr after a single dose of morphine administrration. Following a morphine single dose of 25 mg/kg b.w. the stimulation was more rapid in onset and more pronounced in rats with a higher degree of physical dependence. Specific protein fractions including one with a subunit M.W. of approx. 80,000 were identified by electrophoretic separation of labelled proteins. Some similar protein fractions increased in synthesis and were released to the serum-free incubation medium when separating astroglial primary culture proteins after morphine treatment.

It might be that the biphasic changes in protein synthesis after morphine administration underlie adaptive phenomena such as tolerance/physical dependence development and that some of the identified proteins including proteins synthesized in astroglial cells and secreted to the incubation media participate in these processes.

With regard to the mechanisms involved in development of morphine tolerance and physical dependence it has been proposed that changes in brain protein synthesis are responsible [1, 2]. Goldstein and Goldstein [3] proposed that narcotics inhibit a rate-limiting enzyme, the end-product of which is necessary for normal brain function. Low levels of this end-product in the brain lead to an increased amount of the inhibited enzyme. As a result, the enzyme will be greatly increased in amount in the tolerant/physically dependent brain, according to the proposition.

Changes in brain protein synthesis after morphine treatment have been found, but the results are controversial. Acute administration of morphine to rodents has been reported to transiently inhibit brain protein synthesis [4–8]. The chronic administration of morphine has been reported to decrease [4, 7], to increase [9] or to leave unchanged [6, 8] the rate of biosynthesis of brain proteins. Several reasons for these discrepancies exist. (1) Morphine decreases food intake which might result in a relative undernutrition of long-term morphinized rats (see 10, 11). (2) Different intoxication procedures have been used, such as daily injections for short periods [4, 7], or daily injections for longer periods [9], or pellet implantation for 2 or 3 days [6, 8]. (3) Different doses have been administered at different times preceding sacrifice (see e.g. refs 9, 12 and 13). (4) Large brain regions, in some studies the whole brain, have been examined (see e.g. refs 6 and 14). (5) Little or no account has been taken of the different cell types reacting to the opiates, such as protein synthesis in glial cells compared to neurons.

The crucial findings with respect to morphine effects are that inhibitors of protein and RNA synthesis diminish the development of tolerance and physical dependence to opiates [15, 16]. Way *et al.* [17] suggested that the proteins affected by repeated administration of morphine may be enzymes associated with the putative neurotransmitters, especially 5-hydroxytryptamine (5-HT).

In this paper dose- and time-dependent changes of protein synthesis are reported from brain stem of rats intoxicated with morphine using an ingestion method where intoxicated and control rats received and consumed the same amount of nutrients and liquid [18]. Two levels of physical dependence were evaluated. The results were compared with protein synthesis in an astroglial-enriched primary culture (see refs 19–22) and the secretion of specific proteins to the serum-free incubation medium was evaluated. Extracellular proteins were studied since they make up a rather large proportion of whole brain proteins (see ref. 23), and they might play a role in the macromolecular communication between cells (see e.g. ref. 24) and also in adaptive processes in the nervous system (see ref. 25).

MATERIALS AND METHODS

Animals. A total of 575 male Sprague–Dawley rats, with an initial body weight of approx. 150 g, were used. The animals were kept at a constant

temperature of 20° and were started on a daily diet of 90 ml of nutritionally complete fluid, as described by Zeuchner *et al.* [18]. After 2 days the animals to be morphinized received morphine in their diet with a daily increase according to the schedule 25, 55, 90, 130, 175, 225, 280 and 340 mg/kg b.w./day. 144 rats were intoxicated for 4 days (end-dose 130 mg/kg b.w.). Another 144 rats were intoxicated for 8 days up to a dose of 340 mg/kg b.w./day and maintained for a further 5 days on that dose. The rats were pair-fed the control fluid diet, thereby ensuring the same nutrient intake for both morphinized and control animals. The plasma morphine level was 3 μg/ml on a dose of 340 mg/kg b.w. Rats withdrawn from morphine for 2 days exhibited classical signs of withdrawal (see results).

Experiments. Morphine chloride (Apoteksbolaget, Sweden; 5, 10, 25 or 40 mg/kg b.w.) was injected i.p. into previously untreated or morphinized rats. In some animals 5 or 10 mg naloxone hydrochloride/kg b.w. (Apoteksbolaget, Sweden) was injected i.p. 10 min prior to the morphine. During halothane anesthesia, 30 μCi ^{3}H-valine (L-[3,4-^{3}H] valine, 56.8 Ci/mmol, NEN, Boston, MA) or 30 μCi ^{14}C-valine (D-L valine, 1-^{14}C, 37.5 mCi/mmole) was injected in experimental or control rats, respectively, into the IVth ventricle, 1.1 mm posterior to the lambda. Control rats were also treated with ^{3}H or ^{14}C valine to obtain control (^{3}H) versus control (^{14}C) values. The injections were performed using a syringe with a fixed needle stop. The radioactive amino acid was administered immediately after the drug injections, or after 30, 60 or 120 min. The animals were killed by decapitation 30, 60 or 120 min after the administration of the radioactive amino acid. It has earlier been shown that incorporation is linear at least within 60 min (see ref. 26). The brain stems were quickly dissected and the samples were frozen at −80°.

Tissue culture. Primary cultures from newborn rat brain stem were cultivated according to Hansson *et al.* [27]. The cells reached confluence after 6–7 days of cultivation. The 14-day-old cultures contained 60–70% astroglial-like cells [27]. Twenty hours prior to the experiments, fetal calf-serum was removed by repeated changes of the culture medium.

Drug exposure of the cultures. Morphine chloride (10^{-8}–10^{-5} M) or a combination of 10^{-6} M naloxone and morphine in different concentrations were administered to the cell culture media.

Five microcuries of ^{3}H-valine and 5 μCi ^{14}C-valine were added to experimental and control cultures, respectively, or to control/control cultures, respectively, to obtain control (^{3}H) versus control (^{14}C) data. The radioactive amino acid was incorporated for 30, 60 or in some experiments for 120 min. In the latter experiments the culture media were carefully removed. Media from 5 Petri dishes were pooled and passed through Millipore filter papers (mesh diameter 0.45 μm). The samples were concentrated by dialysis (keeping molecules with M.W. >5000) through polyethyleneglycol.

Extraction of protein. Experimental (^{3}H) and control (^{14}C) samples were mixed (equal protein amounts). The material was then homogenized (1:5 w/v) in 1.0 mM Tris–HCl buffer containing 0.03 M NaCl and 0.05 M sucrose at pH 7.2. Homogenates were centrifuged for 60 min at 85,000 *g* (+4°). The supernatants were used for determining incorporation of the radioactive amino acids into TCA-precipitable material. Pellet material was used in some experiments. Protein content was determined according to Lowry *et al.* [28]. The cell cultures were rinsed three times in ice-cold Tris–HCl buffer and the cells were scraped off into 500 μl of buffer and frozen at −20°. Later, homogenizations and centrifugations were performed as for the brain tissues.

Measurement of TCA-precipitable material. One hundred microlitres of supernatants or solubilized pellets were pipetted onto small Whatman papers which were placed in ice-cold 10% TCA overnight, principally according to Mans and Novelli [29]. They were boiled twice in 5% TCA and rinsed in between. Papers were then rinsed twice in absolute ethanol and placed in ether for at least 10 min. The papers were dried and counted in a Liquid Scintillation Counter (1215 Rack beta, Wallac, LKB) in a Permablend toluene cocktail. Total ^{3}H and ^{14}C activities were measured by leaving 10 μl supernatant to dry on Whatman papers, dissolving in Permablend-toluene cocktail, and counting as above.

Electrophoretic system. Gel electrophoresis in 15% polyacrylamide gels (1 cm i.d.) in sodium dodecylsulphate (SDS) after reduction with 2-mercaptoethanol was performed according to Shapiro *et al.* [30]. The gels were stained for 24 hr with a 2% solution of Coomassie Brilliant Blue in a solvent of acetic acid, methanol and water (1:5:10) and then destained for 72 hr in the solvent alone. Control brain stem samples or cultures were treated in a similar way after incubation with ^{3}H or ^{14}C valine, respectively, to obtain control (^{3}H) versus control (^{14}C) values.

Slicing of gels. After photographing the gels were sectioned into 32 slices. Each slice was dissolved in Soluene (Packard Instruments) until destained (proteins dissolved). Fifteen millilitres of scintillation fluid, consisting of 11 g Permablend (Packard Instruments) in 2 l. toluene, was added to each slice and scintillations were counted. The results were plotted as the ratio of dpm ^{3}H (experimental) over dpm ^{14}C (control) as a function of the migration distance on the gel corresponding to the M.W. of the separated proteins. The ^{3}H incorporation was generally higher by a factor of 3–5 as compared to the ^{14}C data. In control versus control data such an analysis gave a constant ^{3}H/^{14}C ratio. This ratio was used to normalize the data to a value of one, to give a relative labelling ratio. It was thus established that the ^{3}H/^{14}C (control/control) data could be represented by a line along the gel, not different from 1.0, indicating satisfactory experimental conditions [25, 31]. ^{3}H/^{14}C (experimental/control) ratio values for each gel slice were then compared to the corresponding ^{3}H/^{14}C (control/control) gel slices. In the statistical evaluation, Student's *t*-test was performed. As small groups are tested, the significances were confirmed with the Wilcoxon's signed rank test.

Determination of M.W. In the figures M.W.s are given according to data from control gels with M.W. markers. They are mean values from many experi-

ments with standard errors in the range ±1000 to ±1500 for the individual bands. In addition, every slice in the experimental sets covers a M.W. of approx. 2500 (in the M.W. region 12,000–70,000). The gel electrophoretic separation technique used with subsequent slicing does not allow determination of M.W. less than approx. 5000.

RESULTS

Animals

Untreated control rats, rats intoxicated for 4 days (= short-term intoxicated; final dose 130 mg morphine/kg b.w. p.o.) and long-term intoxicated rats (13 days; final dose 340 mg/kg b.w. p.o.) did not differ in their general behaviour. Naive rats intoxicated acutely with 25 mg/kg b.w. morphine showed slow reactions already after 10 min, not observed with smaller doses but very prominent already 5 min after intoxication with 40 mg/kg b.w. Short-term intoxicated rats showed a similar reaction after 40 mg/kg b.w. but less prominent symptoms at 25 mg/kg b.w. while long-term intoxicated rats showed very little effect after an acute i.p. dose of 25 mg/kg b.w. even if 40 mg/kg b.w. produced moderate effects. When morphine was excluded from the diet for one day of the treated rats, their body weights decreased by 5% and 13% in 4- and 13-day intoxicated rats, respectively. Diet intake decreased from 90 ml daily to 72 ml and 52 ml, respectively. The animals were also much more tremulous and irritable when they were handled one day after withdrawal from the higher morphine dose than after withdrawal from the lower dose. Thus there were differences in the degree of physical dependence between the two groups of rats. Injection of 5 mg naloxone/kg b.w. i.p. to the long-term intoxicated rats led to a massive tremor and irritability already 3–4 min after the injection. Two hours after the injection their body weights had decreased by 4%, demonstrating that the naloxone injection precipitated a strong withdrawal reaction, while the body weight of the short-term intoxicated rats decreased by 2% under similar conditions (see Table 1).

Incorporation of radioactive amino acid into soluble protein of brain stem

Acute morphine intoxication. Administration of 5 mg/kg b.w. morphine i.p. did not significantly affect the incorporation of $^{3}H/^{14}C$ valine into brain stem soluble protein of intoxicated rats versus controls during 3 hr. Three hours after the i.p. injection of 10 or 25 mg/kg b.w. morphine there was an increased incorporation following a slightly depressed incorporation in experimental/control ratio in the latter dose during the first hour after injection. Five or 10 mg/kg b.w. naloxone completely reversed these effects. After i.p. injection of 40 mg/kg b.w. morphine there was a depressed incorporation during the first 2 hr which was only partly reversed by naloxone in the doses used (Fig. 1).

Short-term morphine intoxication. I.p. injection of 5 mg/kg b.w. morphine to rats intoxicated for 4 days (final dose 130 mg/kg b.w.) did not affect the $^{3}H/^{14}C$ ratio during 3 hr in the system used. After injection of 10 or 25 mg/kg b.w. morphine to such animals the incorporation ratio was increased 2 hr after the administration. This increase was reversed by 10 mg/kg b.w. naloxone but not by 5 mg/kg b.w. naloxone. Injection of 40 mg/kg b.w. morphine depressed the incorporation significantly, not reversed by 5 or 10 mg/kg b.w. naloxone (Fig. 2).

Long-term morphine intoxication. I.p. injection of 5 mg/kg b.w. morphine into long-term intoxicated rats (13 days; final dose 340 mg/kg b.w.) did not affect the incorporation ratio $^{3}H/^{14}C$ in brain stem during 3 hr. There was an increase incorporation ratio $^{3}H/^{14}C$ between 2 and 3 hr after injection of 10 or 25 mg/kg b.w., the latter not completely reversed by 5 or 10 mg/kg b.w. naloxone. After injection of 40 mg/kg b.w. morphine there was a decreased incorporation ratio during the first hour after administration, not reversed by 5 or 10 mg/kg b.w. naloxone (Fig. 3).

Brain stem primary culture. Administration of 10^{-8} or 10^{-7} (final concentration) morphine did not affect the incorporation ratio $^{3}H/^{14}C$ in the experiments performed. Three hours after the administration of 10^{-6} M morphine, a slightly increased incorporation

Table 1. Characteristics of the various animal groups demonstrating degree of physical dependence

	I.p. inj. of 25 mg/kg b.w. morphine	I.p. inj. of 40 mg/kg b.w. morphine	B.w. decrease 1 day after morphine withdrawal (%)	Fluid diet intake 1 day after morphine withdrawal (ml)	B.w. decrease 2 hr§ after i.p. inj. of 5 mg/kg b.w. naloxone (%)
Untreated rats	++*	+++*			
Rats intoxicated for 4 days, final dose 130 mg/kg b.w. (= short-term intoxicated)	+(+)	++(+)	5.1 ± 0.3† (8)‡	72 ± 4† (8)‡	2.1 ± 0.1† (8)‡
Rats intoxicated for 13 days, final dose 340 mg/kg b.w. (= long-term intoxicated)	(+)	++	12.9 ± 0.5 (8)	52 ± 3 (8)	4.3 ± 0.2 (8)

* Slow reaction: +++ severely affected; ++ moderately affected; + little affected.
† SEM.
‡ Number of animals.
§ Maximal b.w. loss was seen 2 hr after the naloxone administration in the experiments.

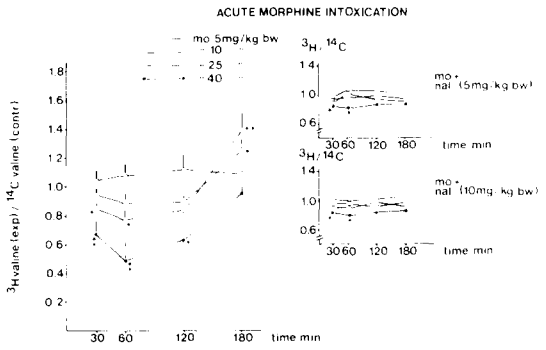

Fig. 1. *In vivo* incorporation of ^3H- and ^{14}C-valine (experimental/control) into TCA-precipitable material from 85,000 *g* (60 min) supernatant of brain stem from acute morphine intoxicated rats. The rats were injected i.p. with 5, 10, 25 or 40 mg/kg b.w. morphine and the labelled valine was administered as pulses for 30 or 60 min. The data are expressed as dpm ^3H/dpm ^{14}C ratio, normalized to 1.0 for control (^3H)/control (^{14}C) data by multiplying the dpm ^{14}C data by a factor of 3–5 (according to the text). Inset figures show results expressed in a similar way after 5 or 10 mg/kg b.w. naloxone, administered prior to the morphine. Each point represents the mean value of 3 different experimental animals and 3 controls (experiments in duplicate) ± SEM. (In the inset figures, SEM values are always less than 12%.) Statistical analysis: Student's *t*-test. * $P < 0.05$; ** $P < 0.01$.

ratio was seen. It was not obtained after administration of both 10^{-6} M morphine and 10^{-6} M naloxone. Administration of 10^{-5} M morphine decreased the incorporation ratio significantly, even after co-administration of 10^{-6} M naloxone (Fig. 4).

Incorporation of radioactive amino acid into pellet material of brain stem

The 85,000 *g* (60 min) pellets were used to follow incorporation of $^3H/^{14}C$ valine (exp./control) into membrane bound proteins in different experimental

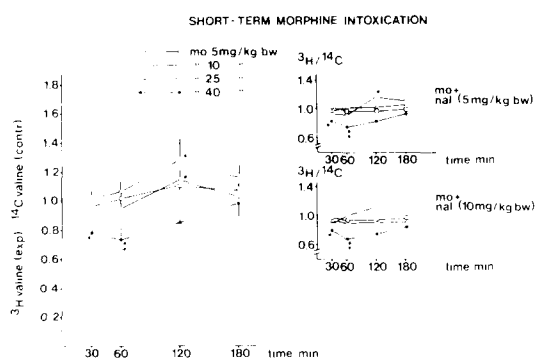

Fig. 2. Incorporation of ^3H- and ^{14}C-valine into brain stem TCA-precipitable material as in Fig. 1 of rats intoxicated with morphine for 4 days with a final dose of 130 mg/kg b.w./day. Appropriate pair-fed control animals were used. The rats were injected with 5, 10, 25 or 40 mg/kg b.w. morphine at time 0 and pulse labelled with the radioactive amino acid for 30 or 60 min. $^3H/^{14}C$ ratios were calculated as in Fig. 1, described in the text. Number of animals and statistical evaluation as in Fig. 1. * $P < 0.05$, ** $P < 0.01$.

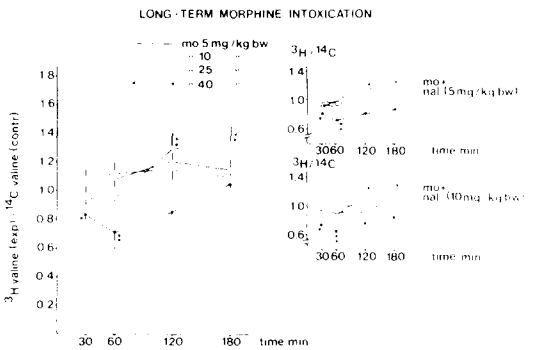

Fig. 3. Incorporation of ^3H- and ^{14}C-valine (experimental/control) into brain stem TCA-precipitable material as in Figs 1 and 2 of rats intoxicated with morphine for 13 days (final dose of 340 mg/kg b.w./day as described in the text). Number of animals and statistical evaluation as in Fig. 1. * $P < 0.05$; ** $P < 0.01$.

sets. According to Table 2 there was a slightly increased $^3H/^{14}C$ ratio 2–3 hr after i.p. morphine (25 mg/kg b.w.) treatment of control rats. Similar results were seen in short-term intoxicated rats while in long-term intoxicated rats there was an increase in the ratio already after 1 hr up to 3 hr following i.p. morphine administration.

Incorporation of $^3H/^{14}C$ valine into protein bands separated by gel electrophoresis

Incorporation of ^3H- and ^{14}C-valine (experimental/control) into brain stem soluble protein was measured for 120 min between 1 and 3 hr after i.p. administration of morphine in doses of 10 or 25 mg/kg b.w. with or without a previous 5 mg/kg b.w. naloxone dose. Gel electrophoretic separation in

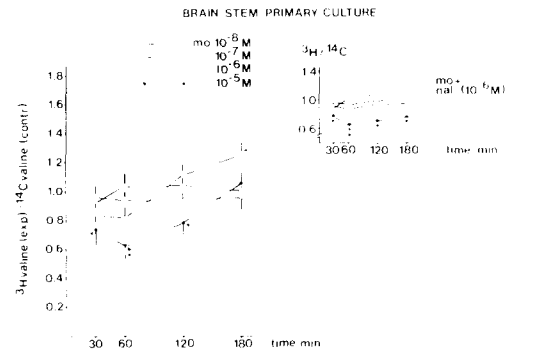

Fig. 4. Incorporation of ^3H- and ^{14}C-valine (experimental/control) into TCA-precipitable material from 85,000 *g* (60 min) supernatant of primary cultures of rat brain stem incubated with naloxone and morphine or with morphine alone, as indicated, from time 0. Pulse-labelling with the valine was performed for periods of 30 or 60 min at various times after the drug exposure. The results are expressed as dpm ^3H/dpm ^{14}C ratio normalized for control vs. control data to 1.0 as described in the text. Each point represents the mean value of 5 experimental and 5 control Petri dishes ± SEM. (When SEM are not shown, they are less than 12%.) Statistical analysis: Student's *t*-test. * $P < 0.05$; ** $P < 0.01$.

Table 2. *In vivo* incorporation of $^{3}H/^{14}C$ valine (experimental/control) pulse-labelled into TCA-precipitable material of brain stem pellet (85,000 *g* for 60 min)

Animal group	Labelling periods† 0–60 min	60–120 min	120–180 min
Acute morphine intoxication (25 mg/kg b.w. i.p.)	0.96 ± 0.13§	1.01 ± 0.19	1.31 ± 0.15‖
Short-term intoxication + 25 mg/kg b.w. morphine i.p.*	1.03 ± 0.16	1.21 ± 0.20	1.29 ± 0.14
Long-term intoxication + 25 mg/kg b.w. morphine i.p.*	1.04 ± 0.19	1.38 ± 0.17‖	1.39 ± 0.17
Control‡	1.07 ± 0.14	0.92 ± 0.18	0.98 ± 0.17

* Morphine (25 mg/kg b.w.) was administered i.p. into control rats, short-term intoxicated and long-term intoxicated rats (for treatment of rats see text).

† Labelling with ^{3}H- or ^{14}C valine (exp./contr. or contr./contr.) for 60 min various times after morphine administration (principally similar as in Figs 1–3 for soluble proteins).

‡ Control rats labelled with $^{3}H/^{14}C$ to establish control/control data (see text).

§ Values are correlated to control (^{3}H)/control (^{14}C) data according to the text. They are mean from three different animals analyzed in duplicate ± SEM. Statistical analysis: Student's *t*-test.

‖ $P < 0.05$ vs. control data.

SDS of the protein material from acute morphine intoxicated rats revealed a slight increase in the $^{3}H/^{14}C$ ratio (exp./control) in gel slice 5 (M.W. approx. 80,000) after the administration of 10 mg morphine/kg b.w. No significant changes in $^{3}H/^{14}C$ ratio along the gels were obtained when 5 mg/kg b.w. naloxone was also injected. Appropriate controls, $^{3}H/^{14}C$ incorporation from control versus control animals revealed a straight line along the gel (not significantly different from 1.0) after multiplying the ^{14}C value by a factor of 3.3–4.1 in the different experiments (Fig. 5).

When the animals had been intoxicated with morphine for 4 days (final dose 130 mg/kg b.w.) i.e. short-term intoxicated, there was an increase in $^{3}H/^{14}C$ ratio in gel slices 5 and 15 (No. 15 = M.W. approx. 40,000) between 1 and 3 hr after an injection of 10 or 25 mg/kg b.w. morphine. Even after a previous injection of 5 mg/kg b.w. naloxone there was a slight increase in the $^{3}H/^{14}C$ ratio in gel slice 5 (Fig. 6).

After long-term morphine intoxication (13 days; final dose 340 mg/kg b.w.) brain stem material separated by SDS electrophoresis showed an increased $^{3}H/^{14}C$ incorporation ratio in gel slices 4 (M.W. approx. 90,000), 5, 6 (No. 6 = M.W. approx. 70,000) 15 and 27 (No. 27 = M.W. approx. 15,000) and a decreased ratio in gel slices 3 (M.W. approx. 100,000). The stimulated $^{3}H/^{14}C$ ratio in gel slices 5 and 6 was seen also after a previous injection of 5 mg/kg b.w. naloxone while the other changes observed after morphine alone were abolished (Fig. 7).

Gel electrophoretic separation of tissue culture material after $^{3}H/^{14}C$ incorporation

The tissue culture material from brain stem was incubated with 10^{-6} or 10^{-5} M morphine for 3 hr and ^{3}H or ^{14}C-valine (exp./contr.) was added 60 min after the administration of morphine, and was incorporated into protein for 120 min. $^{3}H/^{14}C$ ratios were increased in gel slices 5 and 15 even after co-administration of 10^{-6} M naloxone and decreased in gel slices

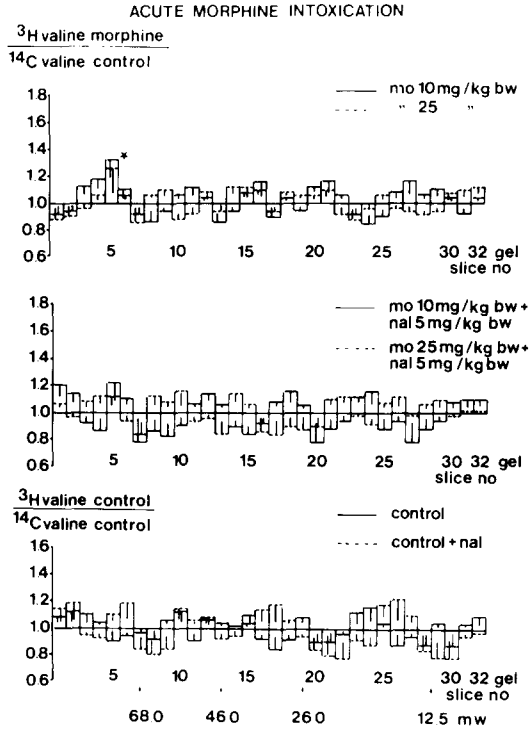

Fig. 5. Electrophoretic separation (SDS 15%–polyacrylamide; gel diameter 1 cm) of $^{3}H/^{14}C$ (experimental/control) valine incorporated *in vivo* into soluble protein from brain stem material of acutely intoxicated rats. The radioactive amino acid was administered for 120 min 1 hr after the i.p. injection of the drug in the concentration specified. Approximately 1 mg protein was applied to the gel, containing 10,000–12,000 dpm ^{3}H and 2500–3500 dpm ^{14}C activity. The gels were sectioned into 32 slices. M.W.s are indicated. Control/control ($^{3}H/^{14}C$) experiments were performed. The data for the ^{14}C controls were multiplied by a factor of 3.3 as a scale plotting factor and to normalize the ratio of the incorporation data. Each point represents the mean value of 3 experimental animals and 3 controls, run in duplicate ± SEM. Statistical analysis: Student's *t*-test. The significances indicated had also been confirmed with the Wilcoxon paired signed rank test. * $P < 0.05$; ** $P < 0.01$.

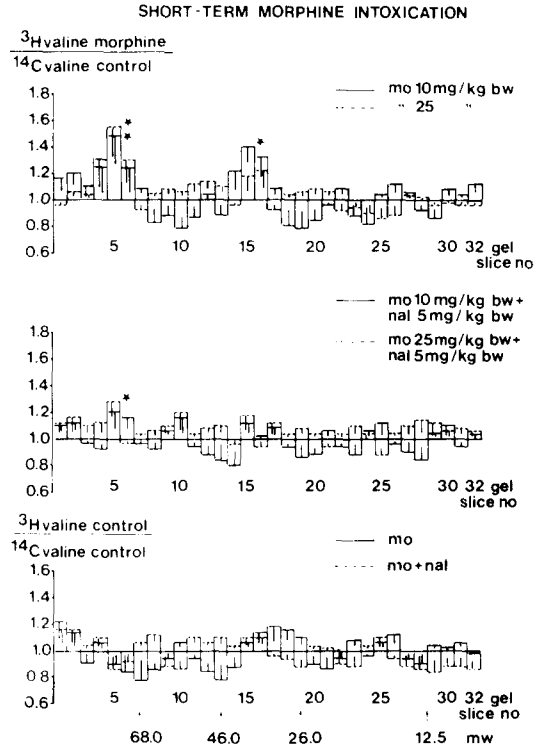

Fig. 6. Results from double labelling experiments of brain stem material of short-term morphine intoxicated rats injected i.p. with morphine and naloxone or morphine alone in the concentrations specified. The radioactive valine was administered 1 hr after the drug and was incorporated for 120 min. Control ^{14}C data were here multiplied by 4.02 (see Fig. 5). Control rats were pair-fed with the intoxicated. Number of experiments and statistical analysis were similar as in Fig. 5. * $P < 0.05$; ** $P < 0.01$.

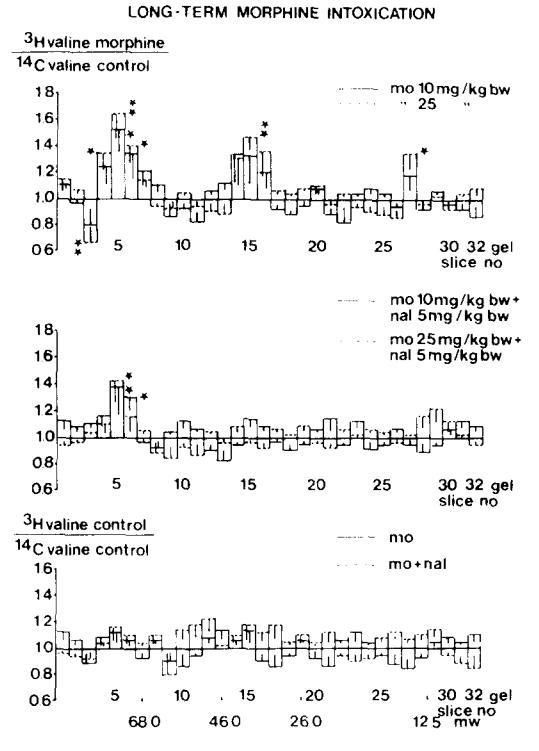

Fig. 7. SDS gel electrophoretic separation of brain stem material from long-term morphine intoxicated rats i.p. injected with naloxone and morphine or morphine alone as described in the text and in Figs 5 and 6. ^{14}C control data were multiplied by 3.6. Number of experiments and statistical analysis as in Fig. 5. * $P < 0.05$; ** $P < 0.01$.

3 and 7 (No. 7 = M.W. approx. 65,000) (Fig. 8). Even in the serum-free incubation medium the $^{3}H/^{14}C$ ratio was increased in gel slices 5, 15 and 27 and decreased in gel slices 3 and 7. Similar results were obtained after 10^{-6} M naloxone had been administered prior to morphine (Fig. 9).

DISCUSSION

The results confirm and extend previous data [32, 33] and demonstrate a phase of stimulated protein synthesis in brain stem of long-term morphine intoxicated rats. The double-labelling procedure used ensures that brain material from experimental animals is compared with identically treated control material. It also ensures that all technical features of the isolation procedure are common to both the experimental and control material. The double-labelling method removes problems connected with obtaining quantitative yields of products from the small amounts of tissue material used, since any losses during the stages of isolation and purification would be the same for both radioactive amino acids.

It should be noted that the stimulatory phase appeared earlier and was more pronounced in animals with a higher degree of physical dependence, even if it could be obtained after acute intoxication. Interestingly, the stimulation of protein synthesis occurred between 1 and 3 hr after morphine administration, which might allow time for messenger RNA molecules to move into the cytoplasm and thus become available for influencing the pattern of protein synthesis.

Separation of the soluble proteins by SDS–gel electrophoresis showed a relative increase in incorporation of a protein band with a molecular weight of approx. 80,000, not completely blocked by naloxone. A similar result was obtained from brain stem astroglial-enriched cultures [27] after exposing for 10^{-6} or 10^{-5} M morphine. This fraction was also identified in the serum-free incubation medium. Proteins synthesized by glial cells and secreted to the extracellular medium have been proposed to participate in adaptive responses in the nervous system (see e.g. ref. 25).

It is important to recognize that electrophoresis in polyacrylamide–SDS gels separate proteins according to their molecular size so that each band could represent a number of different proteins. Moreover, the principal staining component may not necessarily represent the protein(s) which exhibit(s) the largest change as a result of morphine treatment. In addition, the double-labelling method used to identify the observed protein changes cannot actually distinguish between increased synthesis and

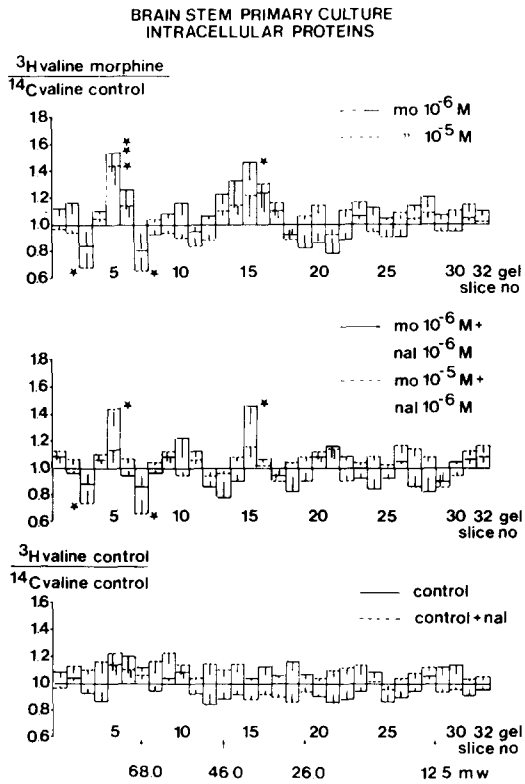

Fig. 8. Double labelling with ^{3}H- and ^{14}C-valine (experimental/control) of brain stem primary cultures after incubation for 3 hr in 10^{-6} or 10^{-5} M morphine or after a prior addition of 10^{-6} M naloxone. The labelled amino acid was added 1 hr after the drug and was incorporated for 120 min. The gels were sectioned into 32 slices. Total activity was 8,000–10,000 dpm ^{3}H and 2000–2500 dpm ^{14}C. Control experiments ($^{3}H/^{14}C$) were performed and ^{14}C values were multiplied with a factor of 3.7 (see text and Fig. 5). Each point represents the mean of 5 different gels (material from 4 Petri dishes pooled in each) ± SEM. M.W.s are indicated. Statistical analysis: Student's *t*-test comparing $^{3}H/^{14}C$ values of experimental/control with $^{3}H/^{14}C$ values of control/control slices. The significances indicated were also confirmed with the Wilcoxon paired signed rank test. * $P < 0.05$; ** $P < 0.01$.

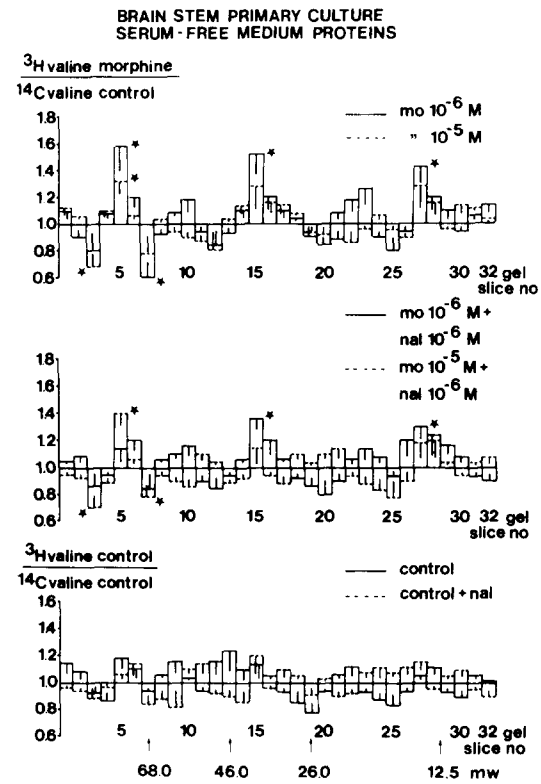

Fig. 9. Double labelling with ^{3}H- and ^{14}C-valine (experimental/control) of brain stem primary cultures as in Fig. 8 and electrophoretic separation of proteins in the incubation media. The gels were sectioned into 32 slices. Total activity was 4000–5000 dpm ^{3}H and 2500–3000 dpm ^{14}C. ^{14}C values were multiplied by 4.1 according to the text. Number of experiments and statistical evaluation as in Fig. 8. * $P < 0.05$; ** $P < 0.01$.

increased turnover rate. Thus, if all proteins in the experimental group increased or decreased in comparison to controls, then the $^{3}H/^{14}C$ ratio would be constant for all components and no specific changes would be observed. Earlier results obtained from experiments with single labelling have not demonstrated such an effect (see ref. 32). The technique used for protein synthesis does not take into account possible changes in free amino acid pool size that might occur during the various experimental conditions. Nor does it take into account differences in uptake of the radioactive amino acids. Labelled proteins were therefore separated by electrophoresis in the same experimental sets to correlate an increased synthesis with increases in the labelling of single groups of polypeptides/proteins. In addition, protein synthesis changes in a brain primary culture model system was followed over time and after exposure for different morphine concentrations. The results from the two systems showed similarities, further diminishing the risk for evaluating "artifacts". Furthermore, in the membrane-bound fraction from the *in vivo* experiments changes in protein formation showed similarities to those of the soluble protein fractions in the different experimental sets.

The results might be discussed in terms of receptor specificity. Brain stem has been shown to express opiate receptors [34]. Astroglial cells, however, do not seem to bear opiate receptors (see e.g. ref. 35) although contradictory results exist [36]. The inhibition of protein synthesis after 40 mg morphine/kg b.w. was not blocked by naloxone even in a concentration of 10 mg/kg b.w. Even the stimulation of protein synthesis was not completely blocked by the opiate antagonist naloxone in two concentrations. This might be interesting as the receptors can be blocked with the tolerance/physical dependence degree still left.

In view of the main results from the present study, namely (i) a more pronounced stimulation of protein synthesis in brain stem from rats with a higher degree of morphine physical dependence, (ii) the identification of electrophoretically separated protein fractions with an increased amino acid incorporation

even in the incubation medium of brain stem astroglial-enriched cultures, and (iii) the stimulation being not completely linked to opiate receptors, it is possible that the proteins involved participate directly or indirectly in the metabolic changes underlying adaptive phenomena such as tolerance/physical dependence to develop. Even if much more work remains to prove any connection between the stimulated protein synthesis including the 80 kD fraction and tolerance development, the results demonstrate the importance of using a well characterized system concerning intoxication, dose, time and biochemical methodology. As the intoxication system is very complicated biochemically, further studies might be performed in cell cultures where effects on different cell types and cell interactions could be measured.

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