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REVISIONES

Central and peripheral endogenous morphine

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ABSTRACT

Morphine was first identified in opium from *Papaver somniferum,* and is still one of the strongest known analgesic compounds used in hospital. Since the beginning of the 80s, endogenous morphine, with an identical structure to that of morphine isolated from poppies, has been characterised in numerous mammalian cells and tissues. In mammals, the biosynthesis of endogenous morphine is associated with dopamine, as demonstrated in the SH-SY5Y human neuronal catecholamine-producing cell line. More recently, morphine and morphine-6-glucuronide has been shown to be present in the human neuroblastoma SH-SY5Y cell line and that morphine is secreted from the large dense core vesicles in response to nicotine stimulation *via* a Ca^{2+}-dependent mechanism suggesting its implication in neurotransmission.

An increasing number of publications have demonstrated its presence and implication in different biological processes at the central and peripheral levels. The present review reports the major data concerning endogenous morphine presence and implication in physiological processes.

Key words: Morphine; Alkaloid; Morphine-6-Glucuronide; Analgesia; μ Opioid Receptor.

RESUMEN

Morfina endógena a nivel central y periférico

La morfina se identificó por primera vez en el opio procedente de *Papaver somniferum*, y sigue siendo uno de los analgésicos más potentes conocidos empleados en los hospitales. Desde comienzos de la década de los 80s, la morfina endógena, con una estructura idéntica a la morfina aislada de las amapolas, se caracterizado en numerosas células y tejidos de mamíferos. En mamíferos, la biosíntesis de la morfina endógena está asociada a la dopamina, como se ha demostrado en la línea celular neuronal humana productora de catecolaminas SH-SY5Y. Más recientemente, se ha demostrado la presencia de morfina y mofina-6-glucorónido en la línea celular de neuroblastoma humano SH-SY5Y y que esta morfina es secretada desde vesículas densas en respuesta a estimulación con nicotina vía un mecanismo dependiente de Ca^{2+} sugiriendo su implicación en la neurotransmisión.

Un número cada vez mayor de publicaciones han demostrado su presencia e implicación en diferentes procesos biológicos a niveles central y periférico. La presente revisión recoge los datos más importantes sobre la presencia e implicación en procesos fisiológicos de la morfina endógena.

Palabras clave: Morfina; Alcaloide; Morfina-6-Glucorónido; Analgesia; Receptor opioide μ.

1. EXOGENOUS MORPHINE

a) History

When the capsule of the poppy *Papaver somniferum* is incised, a milky fluid exudes which, after harvesting and drying, yields a brown gum-like substance known as opium. Mention is made of this substance's efficacy as a painkiller, against diarrhoea and as a narcotic, in texts dating back as far as 4000 years BC. Morphine is one of the forty alkaloids present in opium (1) and today, its

analgesic activities —as well as its dangers addiction and overdose— are common knowledge. At this time, morphine and its precursor codeine remain the gold standard in pain relief (2, 3).

Morphine is still the most commonly used analgesic in hospitals, mainly to relieve acute pain (especially following surgery) but sometimes for chronic pain which is refractory to other active compounds. After administration (orally, subcutaneous or intravenous injection, or infusion), morphine has a half-life of about three hours. There is no recommended maximum dose and the amount administered can be progressively stepped up until pain relief is obtained, as long as there are no side effects: a typical daily dosage for chronic cancer pain in adults is 30 milligrams a day (administered by infusion). The analgesic activity is due to the binding of morphine to μ opiate receptors. Morphine often has unwanted side effects, including constipation, drowsiness, nausea and vomiting; other, less common side effects are confusion, nightmares and, at excessive doses, respiratory depression (which can cause apnoea and lead to death).

b) Mu (μ) opiate receptors

At the beginning of the 1970's, the existence of specific morphine-binding receptors was hypothesised on the basis of the drug's physiological effects, and soon such opiate receptors were indeed discovered in the central nervous system (4). Most opiates (alkaloids) and opioids (peptides) preferentially bind to Mu (μ), Delta (δ) and Kappa (κ) receptors, all proteins with seven membrane-crossing segments coupled to G proteins. Morphine and its derivative morphine-6-glucuronide (M6G) preferentially bind a receptor referred to as the Mu (μ) opiate receptor (MOR) or the Mu (μ) opioid peptide receptor [MOP (5)]. Like morphine, the endogenous peptide ligands, endomorphin-1 and endomorphin-2, have a high affinity for μ receptors (6). The extracellular N-terminal portion of the receptor molecule carries the ligand binding site while the intracellular C-terminal portion is involved in signal transduction (7). These receptors are found in both the central nervous system and the periphery. In the brain, the highest densities of μ receptors are found

in the thalamus, the putamen, the black substance, the cortex, the ventral tegmental zone, the nucleus accumbens and the amygdala (8, 9). In the periphery, μ receptors are expressed on endothelial cells (10, 11) and cells of the immune system (12), among others.

Pharmacological experiments and ligand binding studies have shown that there are several different isoforms of the μ receptor:

— μ_1 is mainly expressed in the CNS and has a high affinity for morphine -it is this form that mediates the drug's analgesic activity;

— μ_2 is expressed in the CNS, the respiratory system and the gut, and mediates most of the drug's side effects (3);

— μ_3 is found on human monocytes, granulocytes and endothelial cells.

However, whether or not these three different isoforms really exist is now controversial because all μ receptors are encoded by a single gene, *Opioid Receptor Mu 1* (*OPRM1*) in humans and *Oprm1* in mice, and the different forms are generated by alternative splicing. Currently, 28 different variants have been described in mice (13, 14), and ten different forms have been characterised in humans (15). This classification system is continually changing and it is more than likely that other variants will be identified.

Signal transduction mechanisms of the μ receptors

The activation of Gi/Go proteins inhibits cellular activity by means of three main mechanisms [reviewed in (5)]:

— by inhibiting adenylate cyclase activity leading to a reduction in cAMP generation. However, it is important to remember that repeated exposure to morphine will lead to enhanced adenylate cyclase activity, a phenomenon which seems to contribute to the addictiveness of opiates (16);

— by opening potassium channels which leads to increased K^+ flux and hyperpolarisation of the cell (17);

— by blocking voltage-dependent calcium channels and decreasing permeability to Ca^{2+} (17).

Activating potassium channels at the same time as blocking voltage-dependent calcium channels inhibits neurotransmitter release so two typical effects of binding at μ receptors are, on the one hand reduced GABA release by hippocampal interneurones (18), and on the other reduced glutamate release by neurones in the striatum (19).

In addition to these three mechanisms, it has been shown that activation of μ receptors can affect other signalling pathways specific to other cell-types (20, 21), *e.g.* in endothelial cells and some types of immune cell (notably leukocytes). Thus, the stimulation of μ receptors induces the generation and release of nitrogen oxide (NO) *via* a PKC-dependent mechanism (1, 22, 23). Morphine's immunosuppressive activity seems to be dependent on μ receptor-mediated induction of a cascade of MAP kinases in lymphocytes and polymorphonuclear cells (24).

It has also been shown that GIRK (G-protein activated Inwardly Rectifying K^+ current) channels are involved in the analgesia induced by the bolus injection of morphine, especially through its action at the Periaqueductal gray matter (PAG): the drug inhibits GABAergic interneurones thereby lifting the inhibition of PAG neurones which are involved in descending control of nociception (25-27). Other experiments have shown that opiate inhibition of neurones in the dorsal layer of the spinal cord also involves GIRK channels. This mechanism underlies the analgesic effect obtained by injecting μ receptor agonists intrathecally (28, 29). Finally, recent work has revealed the role played by GIRK channels in tolerance and addiction when morphine is administered on a long-term basis (30, 31).

c) Morphine catabolism

Enzymes

Exogenous morphine is mainly inactivated (*i.e.* detoxified) in the liver by a superfamily of enzymes referred to as the UDP-glucuronosyltransferases [UGT (32)]. Different forms of UGT are found in the gut and kidneys [reviewed in (33)] and recently, the presence of UGT2B7 was found in the brain, suggesting that

endogenous and exogenous morphine could be glucuronized inside this organ (34, 35).

These various forms of UGT have distinct but overlapping substrate specificities. To date, 28 different UGT genes have been identified and these have been divided into two families and subfamilies (UGT1, UGT2A and UGT2B) on the basis of their homology (36). Only certain of the known human UGTs appear to be catalytically active, namely UGT1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10, 2A1, 2B4, 2B7, 2B15, 2B17 and 2B28 (37, 38). In the liver, morphine is glucuronidated by a UGT at Carbon 3 or 6, to generate either morphine-3-glucuronide (M3G) or morphine-6-glucuronide (M6G; Figure 1), both highly hydrophilic compounds which are quickly excreted in the urine.

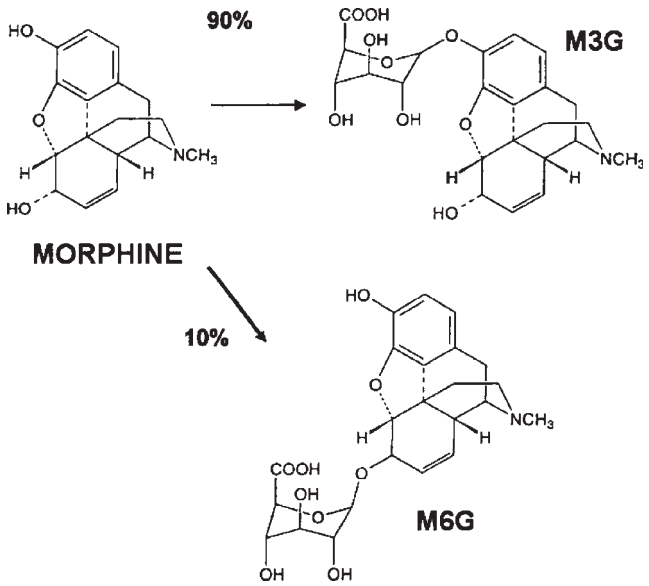

Figure 1. UGT2B7-catalysed generation of M3G and M6G from morphine in the liver.

M3G accounts for about 90% of the glucuronide products and has no analgesic activity at all (Figure 1). In contrast, M6G (the other 10% of morphine glucuronide products in the liver) is reported to be a more potent analgesic than the parent molecule [reviewed in: (32)].

In humans, most M6G and M3G formation is catalysed by UGT2B7, although UGT1A1 and 1A8 have been described as making a minor contribution to M6G (38), while UGT1A3, 1A6, 1A9 and 1A10 can only catalyse the formation of M3G (34). Most UGT enzymes are mainly expressed in the liver but some are found elsewhere, *e.g*. UGT1A6 and UGT2B7 are expressed in the brains of rats and humans (39), UGT1A6 is also expressed by rat neurones and astrocytes in primary tissue culture (40), and UGT1A6 has been detected by *in situ* hybridisation in pyramidal hippocampal neurones and Purkinje cells in the rat cerebellum (41).

In humans, M3G —the major glucuronide product of exogenous morphine— has zero affinity for opiate receptors and no analgesic activity (32). M6G —which accounts for 10% of the morphine breakdown products in the liver— can bind the μ receptor and has been reported as being 1-600 times more active as an analgesic than morphine itself, the variability depending on the model studied (notably species) and the route of administration [intraventricular, intravenous, etc.; reviewed in (2, 32 42)]. As well as these two glucuronides, smaller amounts of other metabolites are also formed and found in the urine, including morphine 3,6-diglucuronide, normorphine, normorphine-6-glucuronide and morphine-3-sulphate, with no or little analgesic activity (43, 44).

Before morphine or M6G can have any effect in the central nervous system, they have to cross the blood-brain barrier (BBB). M6G has been reported as crossing this barrier 32-57 less efficiently than morphine (45) and its analgesic effects are mainly obtained by intrathecal and intraventricular injection. Studies on the efficacy of M6G in humans have yielded inconsistent results. One study suggested that intrathecal M6G is 4-5 times more effective against postoperative pain (46) while a number of studies have indicated that very high doses of intravenous M6G are required for effective analgesia [reviewed in (3, 32)]. Because of its strongly hydrophilic nature, M6G does not cross the BBB efficiently and very high intravenous doses (of the order of 0.3 mg/kg) are necessary to reach a high enough M6G concentration in the brain for effective analgesia. In contrast to morphine, M6G analgesia seems to last longer [6-8 hours compared with just 2-4 hours (47)] and it induces fewer side effects, notably less vomiting and respiratory depression [reviewed

in (47)]. The longer-lasting action of M6G coupled with its lower incidence of adverse reactions make it a very promising analgesic. A number of clinical studies are currently underway to compare the analgesic activities of M6G and morphine in the management of postoperative pain (48).

2. ENDOGENOUS MORPHINE

a) History

In 1903, a French scientist, Dr. Mavrojannis, observed that, when he injected morphine into rats, they presented symptoms similar to those of a cataleptic attack (49). He hypothesised that an endogenous substance similar to morphine was present in the rat brain. However, it was necessary to wait for the improved detection methods of the 1970's —notably radioimmunoassay (RIA)— before a compound related to morphine could be detected in the brains of mice, rabbits and cats (50). This substance was not sensitive to proteases and had the same pharmacological receptor-binding profile as morphine. It was first called Morphine-Like Compound (MLC) (50) and was isolated from human cerebrospinal fluid (CSF), urine and brain extracts [prepared from patients who had never been given morphine (51)]. Subsequently, large quantities of MLC were purified from rabbit, rat and toad (*Bufo marinus*) epidermal tissue (52, 53). In 1985, Goldstein *et al.* purified a morphine-related compound from an extract of bovine hypothalamus and adrenal glands (54). Using chromatography and nuclear magnetic resonance (NMR), they showed that the substance was indeed morphine, with a structure identical to that of the plant molecule (54).

Subsequent experiments showed that this alkaloid could be found in the neural and immune cells of invertebrates (55). Endogenous alkaloids also seem to be produced by certain parasites, morphine having been detected in both *Schistosoma mansoni* (56) and *Ascaris suum* (57). Some scientists remained sceptical and believed that this morphine could have come from food: morphine had been detected in many plants (including hay and lettuce) as well as in cow's and human breast milk, at concentrations of between 200 and 500 ng/L (58). Experiments in livestock reared in strictly morphine-free

conditions, and in tissue cultures similarly depleted of any trace of morphine, showed that *de novo* endogenous morphine synthesis is a real phenomenon.

b) Synthesis of endogenous morphine

Definitive proof of the existence of endogenous morphine required demonstration of the *de novo* synthesis of the compound in mammalian cells. To do this, various intermediates in the synthesis of morphine were sought (Figure 2). Morphine and codeine were the first to be characterised (by mass spectrometry) in bovine hypothalamus, and then in rat brain extracts (59). Moreover, the injection of salutaridine, thebaine and codeine (three known intermediates in the plant synthetic pathway) led to rises in morphine levels in the rat brain (60). The conversion of reticulin to salutaridine by a cytochrome P-450 (an essential step in formation of the morphine skeleton) was also detected in rat liver (61) and then in microsome extracts from porcine liver tissue. It is interesting to note that higher levels of morphine and codeine can be detected in the urine of patients with Parkinson's disease on L-DOPA (62); in the brain, L-DOPA is decarboxlated to generate dopamine, an intermediate in the morphine synthetic pathway (Figure 2). Moreover, it was shown *in vivo* that mice whose tyrosine hydroxylase gene had been knocked out (tyrosine hydroxylase being necessary for dopamine formation) did not produce any endogenous morphine (63): morphine synthesis appears to depend on the presence of dopamine.

Incontrovertible proof that mammals synthesise morphine was provided by Zenk *et al.* in 2004 and 2005: when the cell line SH-SY5Y (derived from a human neuroblastoma) was cultured in the presence of $^{18}O_2$, the group were able to isolate a series of radiolabelled intermediates in the morphine synthetic pathway, as well as radiolabelled morphine [Figure 2 (64-66)]. Experiments with different radiolabelled precursors made it possible to define the entire pathway.

Most steps in the mammalian morphine synthetic pathway are the same as those in plants. The pathway begins with the

condensation of dopamine and 4-hydroxyphenylacetaldehyde (DOPAL), both of which are derivatives of tyrosine (64). This reaction proceeds spontaneously in an aqueous environment without catalysis. It is worth noting that the enzyme CYP2D6 is important because it is known to be involved in the formation of dopamine from tyramine, and the conversion of codeine to morphine (67). At this time, experimental results suggest that this enzyme is present in the liver, kidneys and immune cells (human white blood cells) (68). It seems that, as in plants, mammals have two ways of synthesising morphine from thebaine (Figure 2) although only a few of the enzymes actually involved in this pathway have been characterised and much information has been extrapolated from data about enzymes discovered in plants.

It is also worth noting that dopamine can be synthesised from tyramine through the action of DOPA decarboxylase/L-aromatic amino acid decarboxylase so the possibility that cells deficient in tyrosine hydroxylase (TH) could generate dopamine and therefore morphine cannot be ruled out. This is all the more likely given that TH-deficient neurones expressing DOPA decarboxylase (and therefore capable of producing morphine) have been described in different parts of the brain (69-73).

Recently, Bianchi *et al.* conducted experiments on conditional tyrosine hydroxylase knockout mice [i.e. in which expression of the gene is only abolished in central dopaminergic neurons (63)]. In the brains of conditional TH-/- mice, morphine was below the detection limit of the assay. However, the detection limit was relatively high and it cannot be excluded that some morphine was present; unfortunately, no immunohistochemical analysis was performed to complement these findings. In practice, non-dopaminergic/catecholaminergic cells such as those of the DAN-G line (pancreas) and HepG2 (hepatocytes) (66) are capable of producing morphine de novo, and it cannot be ruled out that morphine may also be synthesised by other cells in the central nervous system in a pathway dependent on DOPA decarboxylase or on an unknown enzyme rather than TH. In addition, it can also not be excluded that some particular cells are able to uptake morphine precursors (i.e. dopamine) to form morphine.

By means of immunohistochemical analysis, high-performance liquid chromatography (HPLC) and mass spectrometry, it has been

possible to characterise the presence of endogenous morphine unambiguously in various mammalian tissues, both central and peripheral.

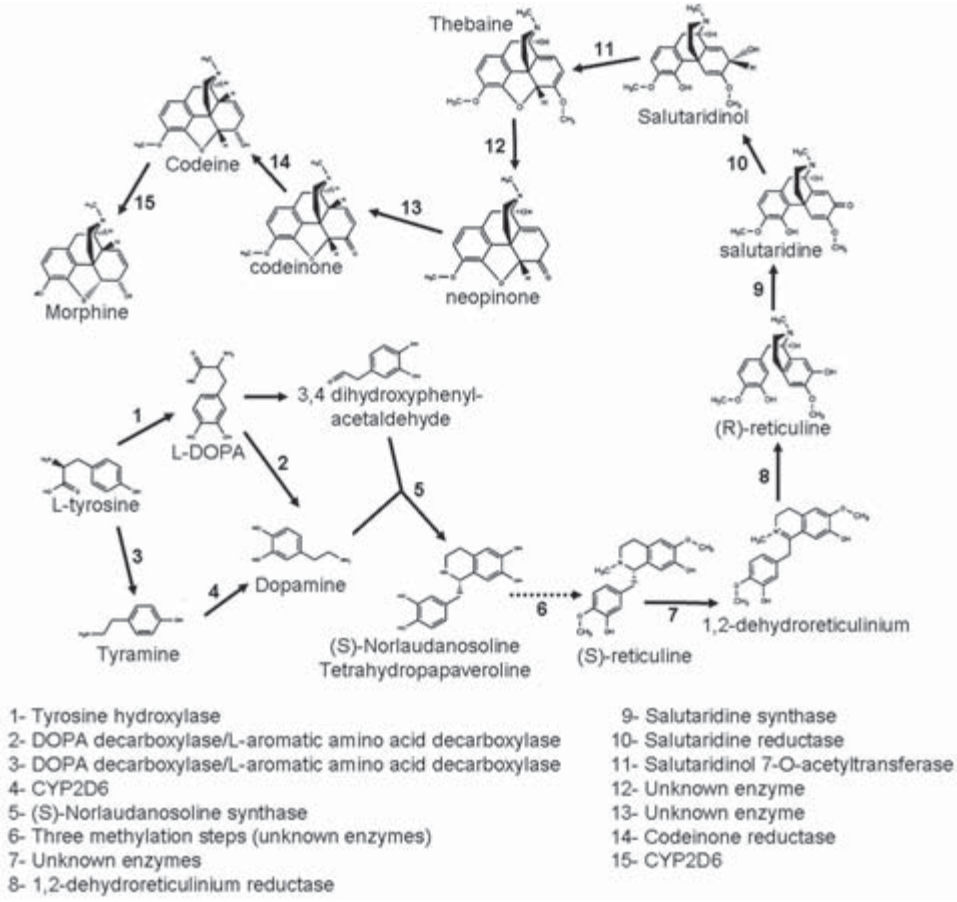

Figure 2. Intermediates on the morphine synthetic pathway in mammals: enzymes that may be involved. This synthetic pathway can take place in particular cells, but might also involve uptake of intermediates (*e.g.* dopamine) by other cells that will finish the morphine synthesis.

c) Endogenous morphine: localisation in the central nervous system and physiological functions

Maps of the brains of the dog and rat generated using various methods (HPLC and RIA) have shown the presence of morphine and/or its derivatives in neurones and nerve fibres (74, 75). In 1999, Meijerink *et al.* detected and quantified morphine in the thalamus, cortex, hypothalamus and cerebellum [Figure 3 (76)]. However, these experiments were conducted after 24 hours of fasting and fasting has been reported to increase the concentration of endogenous morphine in the brain (77). More recently, our group (78) was able to complete this work, characterizing morphine amounts present in the normal mouse brain (Figure 3). Moreover, morphine has also been detected in human cerebrospinal fluid (79). At the intracellular level, morphine has been detected in the cell bodies, axons and terminals of neurones in the putamen, hippocampus, hypothalamus, brain stem, cerebellum and spinal cord (74). Bianchi et al. have also shown that these neurones can accumulate tritiated morphine after intra-cerebroventricular infusion (74, 80), suggesting that these neurones have a system to uptake morphine.

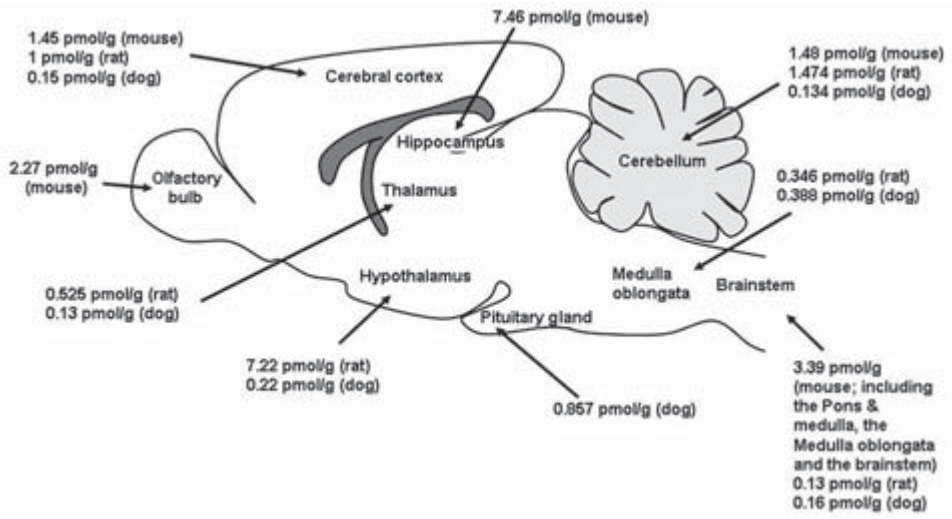

Figure 3. Cerebral distribution of endogenous morphine. Amounts of morphine present in the mouse (78), and in the fasting rat and dog (76).

Our recent studies have focused on the functional roles of endogenous morphine in the central nervous system (78). In experiments on the human neurone line SH-SY5Y [which is used to study neuronal secretion (81)], immunohistochemical analysis showed morphine/M6G/M3G (Figure 4) colocalizing with chromogranin A (CGA, a granular marker) in vesicle-like organelles with a dense core observed in certain neurons.

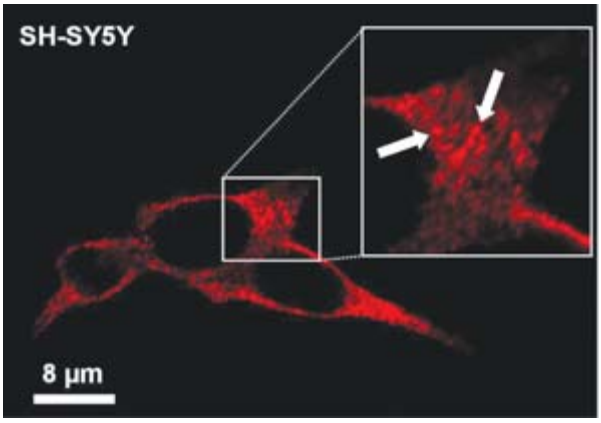

Figure 4. Confocal laser micrograph showing SH-SY5Y cells immunostained for morphine. Colocalisation highlighted in yelow.

Morphine and M6G were purified from SH-SY5Y cells and analysed by mass spectrometry. The identification of M6G pointed to the presence of UGT2B7 which is the main enzyme known to be able to convert morphine into this metabolite, and experiments based on RT-PCR and Western blotting showed, for the first time, that UGT2B7 was being expressed in these cells. Our experiments showed that morphine secretion by SH-SY5Y cells following nicotine-induced depolarisation was Ca^{2+}-dependent. Patch-clamping experiments showed that morphine and M6G could both induce naloxone-dependent electrophysiological responses at low concentrations (10^{-10}-10^{-9}M).

In order to extend the observations made in the *in vitro* model, we looked for endogenous morphine in various parts of the brain of mice [which are reported not to produce M6G (82)] using microscopy

and a morphine-specific ELISA. This showed the presence of endogenous morphine in various places, including the hippocampus and the cerebellum (Figure 3 and Figure 5).

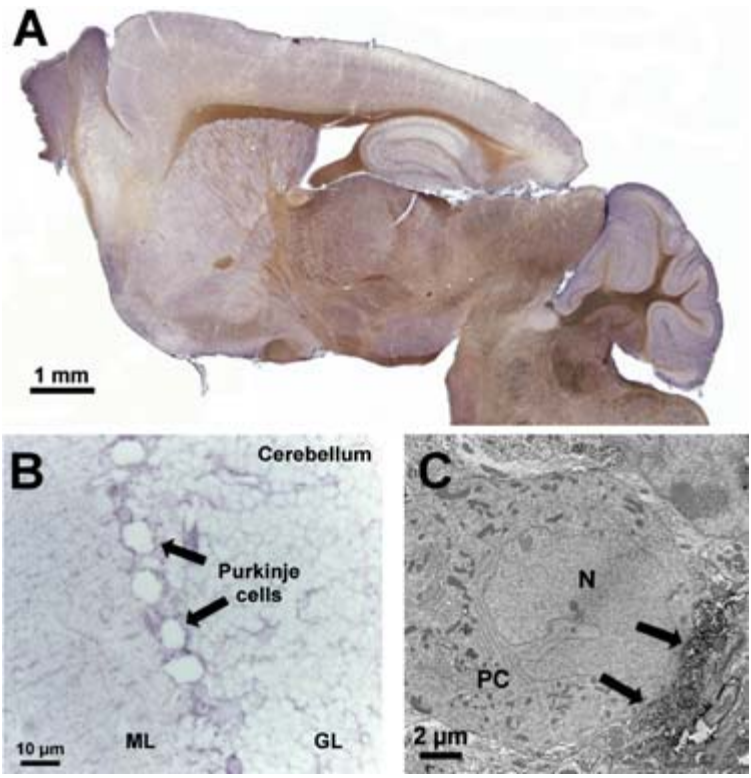

Figure 5. Localization of endogenous morphine present in the mouse brain. **A)** Immunodetection of endogenous morphine in the murine brain. The immunostaining was done with an antibody that recognises morphine and its glucuronidated derivatives. **B)** Localization of morphine label in the cerebellum using mouse monoclonal anti-morphine antibody and an HRP-conjugated secondary antibody. Morphine labelling was observed around Purkinje cells. ML, molecular layer; GL, granular layer. **C)** Electron micrograph (anti-morphine antibody) of murine cerebellum showing nerve terminals on the cell body of a Purkinje cell. The arrows indicate synaptic contacts with morphine-containing, presynaptic vesicles. PC, Purkinje cell; N, nucleus.

Focussing on the cerebellum, we showed a morphine immunohistochemical signal in basket cells (78) and their terminals synaps-

ing with the cell bodies of Purkinje cells (Figures 5B and C) which are reported as expressing μ receptors (83, 84).

Morphine in the nervous system

Various studies have pointed to the involvement of endogenous morphine in analgesia, memory, plasticity and development, as well as in the installation of addiction. A number of studies have described the effects of exogenous morphine in the CNS although, without an understanding of effective concentrations inside the synaptic cleft, it is difficult to speculate about the functions of endogenous morphine by extrapolating from results obtained with exogenous morphine. It is also likely that the level of endogenous morphine being produced and secreted varies with physiological conditions (notably stress). It is important to note that the concentrations used in most experiments (therapeutic doses) are often greater than 1 μM. Nevertheless, on the basis of the observed effects of exogenous morphine coupled with an understanding of the distribution of endogenous morphine and its receptors in the CNS, hypotheses can be formulated about the physiological roles of endogenous morphine.

Endogenous morphine and nociception

An experiment conducted in the year 2000 showed that endogenous morphine colocalises with μ receptors in various parts of the brain stem. These include the locus coeruleus, the parabrachial nucleus, the periaqueductal grey substance and the nucleus raphe, all structures known to be involved in supraspinal nociception modulation (1). On the other hand, experiments carried out by Guarna *et al.* showed that the injection of antibodies directed against morphine into murine CSF (a procedure which lowers the level of endogenous morphine in the brain) induced hypersensitivity to heat-associated pain (85). Moreover, knockout mice which do not express μ receptors are abnormally sensitive to thermal stimuli but not to mechanical stimuli (86). These findings seem to suggest that endogenous morphine mainly modulates sensitivity to thermal pain.

It should also be noted that morphine seems to have paradoxical effects according to its concentration. One study showed that the subcutaneous injection of a small quantity of morphine (1-10 μg/kg) induced hyperalgesia whereas high doses (1000-7000 μg/kg) induced analgesia (87). These results are consistent with those of Robuvitch *et al.* who showed that DAMGO, a μ receptor agonist, stimulated cAMP production in the SK-N-SH cell line (derived from a neuroblastoma) at a concentration of 10 nM, but inhibited it at 0.1 μM (88). One of this group's hypotheses is that some nociceptive neurones could be expressing a low level of G protein-coupled μ receptors with a high affinity for morphine, and these could be mediating the hyperalgesia in the presence of low morphine concentrations; these same neurones would also be expressing classic μ receptors coupled with Gi/o proteins, to mediate the analgesia observed at the higher morphine concentration (89).

Endogenous morphine and memory

An experiment in which antibodies against morphine were injected into murine CSF pointed to a link between endogenous morphine and memorisation (90): after twelve hours of fasting, control mice showed deficient memorisation whereas memorisation processes were unaffected in the mice in which the level of endogenous morphine had been artificially depressed (*i.e.* neutralized by the antibodies). Endogenous morphine could therefore inhibit memorisation at times of stress (*e.g.* during starvation).

Exogenous morphine has been observed to have various effects on hippocampal function, the hippocampus being known to be a key structure in memorisation. Morphine is known to be able to modulate neurotransmission in the hippocampus by inhibiting its GABAergic interneurones. Such inhibition would result in an increase in the discharge amplitude of pyramidal neurones in the CA1 zone (91) and modify the efficacy of glutamatergic synapses by acting on the expression of proteins important for the post-synaptic density [*i.e.* receptors (92)]. Morphine would therefore act to consolidate memories by promoting long-term potentiation (LTP). However, other experiments have shown that prenatal exposure to

morphine impairs memorisation processes and reduces LTP at hippocampal synapses (93). Moreover, another experiment in rats showed that one-off exposure to high-dose morphine (10 mg/kg administered intraperitoneally) impaired memorisation in a cross-maze model. The effects of exogenous morphine on memorisation are therefore as yet poorly understood and seem to depend on both dosage and stage of development. In the light of all these observations and because both endogenous morphine and μ receptors are known to be present in the hippocampus, it could be that endogenous morphine plays a role in controlling memorisation functions.

Morphine, neurogenesis and the growth of nerve cells

In the brain of adult mammals, there are two sites of ongoing neurogenesis, namely the sub-ventricular zone (SVZ) along the edge of the lateral ventricle, and the sub-granular zone (SGZ) of the hippocampal dentate gyrus. Many contradictory results have been published on the effects of morphine on the multiplication of neuronal progenitors in the SGZ, reflecting the complexity of the underlying mechanisms [reviewed in (94)]. In rats and mice on long-term morphine (10 mg/kg administered intraperitoneally), neurogenesis has been observed to be decreased by a factor of over 30% in the SGZ (95, 96), whereas other experiments have shown that morphine stimulated multiplication (97, 98). Moreover, in μ receptor knock-out mice, an increase of over 50% was documented in the rate of survival of newly formed neurones in the granular zone of the dentate gyrus four weeks after the injection of bromodeoxyuridine [BrdU, a marker for neurogenesis (99)]. Endogenous morphine in the hippocampus (78) could therefore regulate the production and survival of neuronal progenitors, thereby affecting synaptic plasticity in the hippocampus. These data are supported by the results of clinical studies which point to hippocampal plasticity problems in heroin addicts (100).

With respect to cell growth, *in vitro* studies have shown that morphine has dose-dependent effects on the growth of neural processes (axons and dendrites) in cell lines and primary neurones in tissue culture: at high concentrations (10 mM-10 μM), morphine

inhibited the growth of neuronal processes in primary cultures of hippocampal and cerebellar granule neurones in a naloxone-dependent fashion (101), whereas morphine concentrations of between 1 nM and 10 fM enhanced process growth in spinal cord and cortical neurones by a factor of over 20% (101), and a low concentration of morphine (10 pM) stimulated process elongation in PC-12 cells (102). These effects were not reversed by naloxone suggesting that they are mediated by special, high-affinity receptors.

In addition, a recent study showed that low doses of morphine had beneficial effects on synaptic regeneration and reconstruction at the nerve terminals of non-myelinated afferent fibres of the second lamina of the spinal cord following damage to the sciatic nerve (103).

Even more recent experiments described that low morphine concentrations (1-100 nM, 2 hours) significantly stimulated migration in rat microglial cells exposed to ATP (acting as a chemotactic agent) (104). Significant effects were observed from the concentration of 1 nM with peak activity observed at 100 nM. CTAP, a specific antagonist of μ receptors, inhibited this migration. These results suggest that endogenous morphine in the brain could modulate immune responses in the CNS.

Endogenous morphine and addiction

Experiments in two invertebrate models, *Mytulis edulis* and *Homarus americanus*, showed that exposure to addictive substances (ethanol, nicotine and cocaine) stimulates a two-fold increase in the release of endogenous morphine by the nervous system (105). A number of groups have postulated the existence of a link between endogenous morphine and addiction (106) although no experiments have yet been conducted in animals.

d) Localisation and physiological roles of endogenous morphine in the periphery

In the periphery, morphine has been detected in the liver and in a hepatocyte cell line (61, 66), and in the adrenal glands of a number

of mammalian species. The adrenal gland is one of the major organs involved in responses to stress and is composed of a cortical part (where glucocorticoids are produced) and a medullary part [mainly composed of chromaffin cells (107-109)]. Previously, morphine had also been found in PC-12 cells which are transformed rat chromaffin cells (66, 110), and in eel chromaffin cells (111). Chromaffin cells are neuroendocrine cells derived from the neural crest which are full of secretory granules containing catecholamines together with many different proteins and peptides, including chromogranins (107). In a stressful situation, chromaffin cell stimulation by the splanchnic nerve induces membrane depolarisation and degranulation which leads to emptying of catecholamines and the other granule contents into the blood stream. It is worth noting that the catecholamines (dopamine, adrenaline and noradrenaline) are all derived from dopamine and tyrosine precursors, as is morphine.

In experiments carried out in 2006 in a bovine chromaffin cell model, we detected M6G inside these cells' secretory granules (Figure 6). M6G secretion was also observed when nicotine was used to induce depolarisation in these cells in primary tissue culture. Because these chromaffin cells degranulate in response to stress, it is likely that M6G is secreted along with the catecholamines in such responses. Once in the circulation, the M6G could bind μ receptors on diverse cell types (immune cells, endothelial cells, etc.) and trigger physiological responses. We also showed that the M6G in chromaffin granules is stored as a complex with phosphatidylethanolamine-

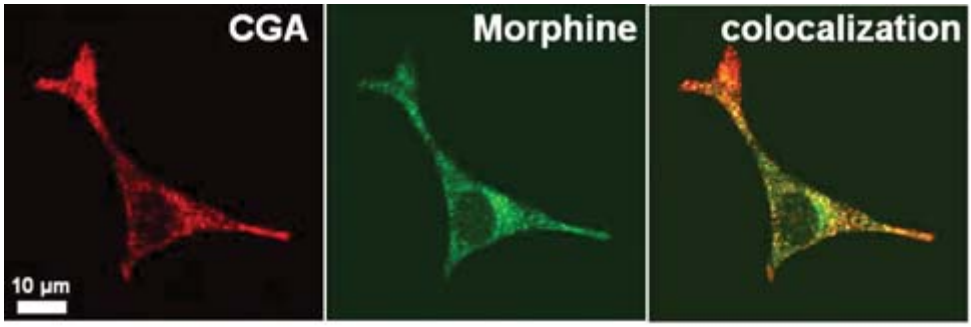

Figure 6. Confocal laser micrograph showing primary chromaffin cells in tissue cells labelled with antibodies against morphine and CGA (a granular marker). Colocalisation highlighted in yellow.

binding protein (PEBP) which would prevent it being cleared *via* the kidneys. Our findings also showed that, in the chromaffin cell model, M6G is the final product of the alkaloid synthesis pathway, and could represent a neuroendocrine effector.

A number of studies conducted on invertebrate immune cells have shown that morphine synthesis is stepped up at times of stress (55). More recently, a number of articles have reported that immune cells [notably polymorphonuclear cells and monocytes (68, 112)] synthesise morphine, pointing to a role in immune responses.

The intensive use of morphine in hospitals has spurred many studies focusing on the presence of morphine in the serum or plasma after the i.v. administration of exogenous morphine. In contrast to this body of work on exogenous morphine, there is little information about endogenous morphine in the blood although some findings suggest that endogenous morphine may play a role in stress and immune responses. Since the beginning of the 1990's, a number of studies on invertebrate immune cells had demonstrated morphine synthesis in response to stress (55). Three other studies conducted on pigs and patients who had undergone invasive surgery (*e.g.* coronary bypass surgery or laparotomy) showed elevated levels of endogenous morphine in the blood. Tonnesen *et al.* showed that coronary bypass surgery induced a rise in the amount of morphine in human serum from 0.28 nM to 3.9 nM (113-115) and, in another experiment, the same group measured a morphine concentration of about 10 nM in the blood of piglets who had undergone coronary bypass surgery while none was detectable in control animals (113). Finally, a human study suggested that the concentration of morphine in the blood was higher after open cholecystectomy than it was after laparascopy (0.2 nM *versus* 0.018 nM) (115). Surgical procedures such as bypass surgery or thoracotomy elicit massive inflammation, *e.g.* the extracorporeal circulation associated with coronary bypass surgery always causes inflammation. Taken together, these observations point to a link between rises in blood morphine and inflammation. It is interesting to note that estimated IC50's for μ receptors [of the order of 10 nM (116)] are consistent with the levels observed in the circulation, suggesting that endogenous morphine may have effects on different cell types, including immune cells and endothelial cells.

The injection of lipopolysaccharide (LPS, an integral component of the cell walls of Gram-negative bacteria) into rats is used to study sepsis. In this system, LPS is known to induce an increase in the concentration of endogenous morphine in the adrenal glands (76) and brain (77). Other types of stress (like starvation) also induce rises in morphine levels in the spinal cord (76).

Morphine is known to be able to have immunosuppressant activity *via* various distinct mechanisms (117):

(*i*) direct action on monocytes, macrophages and granulocytes: by inducing nitrogen oxide [NO (22)] and thereby inhibiting the production of pro-inflammatory cytokines as well as chemotaxis and phagocytosis (116). On the other hand, the binding of morphine to μ receptors on endothelial cells also induces NO production and inhibits the expression of certain adhesion molecules involved in the recruitment of immune cells to sites of infection and inflammation;

(*ii*) activation of the hypothalamic-pituitary-adrenal axis (HPA) and release of immunosuppressive glucocorticoids (119);

(*iii*) inhibition of the differentiation of stem cells into lymphocytes, and inhibition of T lymphocyte multiplication (120);

(*iv*) inhibition of the cytolytic activity of NK cells (119-121): this has been observed after the direct injection of morphine into the cerebrospinal fluid, suggesting that the effect is mediated in the central nervous system.

The release of morphine in the blood could represent a physiological mechanism designed to mitigate over-exuberant inflammatory reactions. Morphine is also known to induce analgesia so it could, if present at high enough levels in the blood (113, 114) or brain (77) at times of stress, modulate the activity of peripheral nociceptors.

3. CONCLUSION

In summary, numerous studies have established a role for morphine as an endogenous signalling molecule. Thus, endogenous morphine appears to be both a neurotransmitter and endocrine

mediator playing a role in physiological processes. Investigation on endogenous morphine will continue to be an important axis of research and efforts will be focus on the characterization of its implication in physiology.

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