Concepts for Biologically Active Peptides

Abba J. Kastin[*] and **Weihong Pan**
Blood-Brain Barrier Group, Pennington Biomedical Research Center, Baton Rouge, LA 70808, USA

Abstract

Here we review a unique aspect of CNS research on biologically active peptides that started against a background of prevalent dogmas but ended by exerting considerable influence on the field. During the course of refuting some doctrines, we introduced several concepts that were unconventional and paradigm-shifting at the time. We showed that (1) hypothalamic peptides can act 'up' on the brain as well as 'down' on the pituitary, (2) peripheral peptides can affect the brain, (3) peptides can cross the blood-brain barrier, (4) the actions of peptides can persist longer than their half-lives in blood, (5) perinatal administration of peptides can exert actions persisting into adulthood, (6) a single peptide can have more than one action, (7) dose-response relationships of peptides need not be linear, (8) the brain produces antiopiate as well as opiate peptides, (9) there is a selective high affinity endogenous peptide ligand for the mu-opiate receptor, (10) a peptide's name does not restrict its effects, and (11) astrocytes assume an active role in response to metabolic disturbance and hyperleptinemia. The evolving questions in our laboratories reflect the diligent effort of the neuropeptide community to identify the roles of peptides in the CNS. The next decade is expected to see greater progress in the following areas: (a) interactions of peptides with other molecules in the CNS; (b) peptide involvement in cell-cell interactions; and (c) peptides in neuropsychiatric, autoimmune, and neurodegenerative diseases. The development of peptidomics and gene silencing approaches will expedite the formation of many new concepts in a new era.

Keywords

INTRODUCTION

As in sociology, the birth and growth of a new idea require a suitably receptive environment. Young neuropeptide researchers nowadays may find it difficult to imagine that a time ever existed in which it was inconceivable that a peptide in the periphery could affect CNS function. Even today some old-timers do not acknowledge that selective peptides can cross the blood-brain barrier (BBB) in intact form. Only a few years ago when we started to elucidate the regulation and functions of astrocytic leptin receptors, there was resistance arising in part from neuron-centric training and experience. The ability and attempt to challenge existing paradigms is presumably being encouraged, but reality reveals unnecessary delay most of the time. The developmental history of neuropeptide research is part of the forty-year legacy and ongoing efforts of our laboratories. Although this

[*]Address correspondence to this author at the Pennington Biomedical Research Center, 6400 Perkins Road, Baton Rouge, LA 70808, USA; Tel: 1-225-763-0266; Fax: 1-225-763-0265; peptides@pbrc.edu.

represents only a drop in what is now a vast ocean of neuropeptide research, the reiteration of peptide concepts may be helpful in current pharmaceutical design.

CONCEPTS

1. Hypothalamic Peptides can Act "Up" as Well as "Down"

In the classical model of the hypothalamic-pituitary-target organ axis, the story begins with the production of a hypothalamic releasing or inhibiting hormone. The hypothalamic hormone is transported to the anterior pituitary *via* the hypophyseal portal circulation. There, a corresponding pituitary hormone is produced and released into the circulation. The pituitary hormone then induces the synthesis and release of a target gland hormone, which feeds back negatively to inhibit the production of the corresponding pituitary and hypothalamic hormones. This pathway, although shown by the endocrine pioneer George Sayers and others since the late 1940's, did not take into account the rest of the brain as a target for the hypothalamic hormone.

We thought that it would be more efficient for the body to use the same hypothalamic hormones to act on brain regions "higher up" than the hypothalamus than to create new ones. The hypothesis that a peptide hormone from the hypothalamus can act on the rest of the brain was first proven by studies with MIF-1 [1,2]. Later, tract tracing studies from many neuroanatomy groups confirmed the presence of complicated projection pathways that certainly involve hypothalamic peptides [3–6]. Studies after injection of hypothalamic peptides show effects not only confined to hypothalamusrelated behavior. Numerous examples of hypothalamic peptides acting on higher CNS centers are provided in the Brain Peptides Section of the *Handbook of Biologically Active Peptides* [7].

MIF-1 is a tripeptide (Pro-Leu-Gly-NH$_2$) first isolated from bovine hypothalamus. The peptide sequence is seen in the side ring of oxytocin and in Tyr-MIF-1. Its endogenous concentration is highest in the hypothalamus; however, it may or may not be a derivative of the oxytocin gene. The isolation of MSH-release-inhibiting factor-1 (MIF-1, Pro-Leu-Gly-NH$_2$, or PLG) almost four decades ago involved the 11000-fold concentration of 5000 cow hypothalami, followed by what is now considered the rather imprecise method of thin layer chromatography [8,9]. Hypothalamic extracts were more active than cerebral cortical extracts in inhibiting MSH release. Recently, we applied a sensitive and specific triple quadrupole linear ion trap mass spectrometer analyzing three multiple reaction monitoring transitions for MIF-1 that clearly shows the presence of this tripeptide in the hypothalamus, striatum, and elsewhere in the mouse brain [10]. Furthermore, MIF-1 treatment induces region-specific activation of the immediate early gene c-Fos, the highest activation being in the cingulate cortex, infralimbic cortex, nucleus accumbens, paraventricular hypothalamic nucleus, medial basal amygdaloid nucleus, fiber tract in the piriform cortex, paraventricular thalamic nucleus, and some other thalamic nuclei [11]. These are regions involved in the regulation of mood, anxiety, depression, and memory.

MIF-1 has effects on CNS activity, including learning and behavior. When tested in a 12-choice Warden maze for a palatable food reward, rats injected with MIF-1 have shorter latencies and make fewer errors than controls during learning, but not extinction [12]. After acquisition of a visual task in an extradimensional spatial shift problem, MIF-1 facilitates acquisition of brightness discrimination, presumably by increasing attention [13]. MIF-1 also increases passive avoidance retention and decreases shock-suppressed water intake [14], and it can attenuate the amnesia induced by puromycin [15] and electroconvulsive shock [16]. It also can enhance information storage received through olfactory cues in social investigatory behavior [17]. Thus, the general effects of MIF-1 on learning seem beneficial.

In addition, MIF-1 has secondary effects on body temperature. Although it does not alter basal colonic temperature or motor activity of rats at 4 or 20 °C, it increases d-amphetamine-induced hypothermia at 4 °C and decreases the hyperthermic effect of the amphetamine at 20 °C [18]. Similarly, MIF-1 blocks the hypothermic effects of chlorpromazine at both temperatures in normal and hypophysectomized animals, another example of an extra-endocrine action not mediated by the pituitary. In addition, the hypothermia induced by reserpine [19], β-endorphin, or morphine are all antagonized by MIF-1 [20].

MIF-1 shows therapeutic effects in animal models of Parkinson's disease [1,2]. It is active in tests of dopa-potentiation [1,21–23] and oxotremorine antagonism in mice [24–26], and deserpidine antagonism in monkeys as well as mice [27]. It exerts these effects even when given orally to mice and monkeys [27–29], and does not require the presence of the pituitary gland [1,24,28]. MIF-1 is also effective against the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) that induces many of the clinical symptoms of Parkinson's disease [30,31]. In rats with unilateral substantia nigra lesions induced by 6-OHDA, peripheral injection of MIF-1 (2 ng/kg) potentiates the effect of L-dopa on apomorphine-induced turning, and it decreases endorphin levels in the caudate nucleus at a dose of 20 mg/kg [32].

In clinical investigations of the efficacy of MIF-1 in Parkinson's disease, the first study tested 16 Canadian patients and found improvement in rigidity and tremor [33]. A similar pattern was then observed in 10 German patients [34]. Akinesia and rigidity were the symptoms most improved in a study with Austrian patients, and this was evident only 2 days after treatment began, reached a maximum at 1 week, and persisted for several more weeks [35]. In some later studies of parkinsonian patients, MIF-1 reduced the side-effects and prolonged the efficacy of Madopar (levodopa + benserazide) for 2 – 6 weeks after drug cessation [36–38]. The significant potentiation by MIF-1 of the effects of Madopar in reducing dyskinesia, rigidity, and tremor in these studies is consistent with that shown in earlier studies, particularly by Barbeau [39]. This expert in clinical trials of anti-parkinsonian drugs reported that "The improvement obtained in almost all (5/6) cases was of such magnitude that it far surpassed the clinical effect of any of the numerous antiparkinson drugs which we have tested in our laboratory over the past 15 years, including levodopa itself." These findings indicate that the actions of MIF-1 are not confined to the hypothalamus.

In the Austrian patients with Parkinson's disease [35], depressive symptoms also improved, as they did in a study of 12 German parkinsonian patients receiving MIF-1 in a double-blind crossover design [40]. A small group of Canadian patients with Parkinson's disease showed a 55% reduction in the classic Hamilton score of mental depression [39]. These observations of improvement of depressive as well as parkinsonian symptoms were intriguing.

In animal models of mental depression, MIF-1 was also effective. Some of the early animal models for Parkinson's disease discussed above are also considered animal models of depression [1,2]. The more selective Porsolt forced swim test of behavioral despair in rats [41] is a classical model of depression in which MIF-1 is also active [42–44]. A peripheral dose as low as 0.01 mg/kg of MIF-1 showed anti-immobility effects, and this was antagonized by blockade of brain dopamine receptors [43]. It is consistent with allosteric modulation of the dopamine receptor by MIF-1 [45]. In the Nomura water wheel modification [46] of the Porsolt test for mice, MIF-1 is also active in doses as low as 0.01 mg/kg ip [47]. In other models in which antidepressants are active, Niesink and van Ree found that MIF-1 reversed isolation-induced increase in social activity [48], and Dutch researchers also reported that MIF-1 antagonized melatonin-induced behavioral changes

[49]. MIF-1 is also effective in the labor-intensive Katz test of unpredictable chronic stress [50,51].

Clinical studies of mental depression showed the inverted-U shaped dose-response relationship noted in animal studies. In the first two small studies of oral MIF-1 in women with major depressive illness, marked improvement was seen with single daily doses of 60 or 75 mg/day by mouth, but not the large doses (Fig. 1) [52,53]. Van der Velde then studied 20 depressed patients, half of whom received 75 mg of the tricyclic antidepressant imipramine po and the other half 60 mg of MIF-1 po daily for 4 weeks. His conclusion was that MIF-1 was "as least as effective as imipramine in this study, and that its anti-depressive effect was a rapid and often dramatic one" [54]. Another 20 patients with major depression received a single subcutaneous injection of either 10 mg MIF-1 or placebo for 5 days [55]. The treatments were reversed for a second week of 5 consecutive daily injections. At the end of the first week, 8 of 9 patients receiving MIF-1 showed marked improvement on the Hamilton test as compared with only 2 of 11 patients receiving the saline placebo. Administration of MIF-1 during the second week to the patients who had received placebo during the first week resulted in a similar improvement. A graphic summary of the studies with oral MIF-1 by R.H. Ehrensing is shown in Fig. (1). Considering the rapid onset of action after MIF-1, it is hoped that the hormesis effect [56] will not prevent its development as an antidepressant.

The contribution of endogenous MIF-1 within the hypothalamus to actions "higher" in the brain, perhaps through axonal transport along projection pathways and molecular diffusion along fiber tracts within the CNS, has not been determined. Regardless, as shown in several animal models of Parkinson's disease and depression discussed in this section, these actions are not mediated "down" on the pituitary. Clearly, a hypothalamic peptide can act "up" to affect functions typically mediated by the cerebral cortex or the striatum.

2. Peptides in the Periphery can Act on the Brain

The prevailing view four decades ago was that peptides in the periphery could not have any effect whatsoever on the brain. This was not limited to those investigators who were sure that peptides could not cross the BBB directly, but extended to researchers who could not conceive of even indirect effects of peptides on the brain. Perhaps a contributing factor in this persistent disbelief was the failure of some early studies with adrenocorticotropin (ACTH) and vasopressin to consider the secondary effects of these pituitary hormones (e.g., on adrenal steroid release and blood pressure) or of the vehicle itself [57]. It was not realized that a peptide's actions are not completely dependent on its half-life in blood (see Section 4), so that a vehicle prolonging the half-life of the peptide is not necessary to demonstrate the CNS effects of a peripherally injected peptide.

The first unconfounded studies showing an effect of a peripherally administered peptide on the brain used the pituitary hormone melanocyte-stimulating hormone (MSH; melanocortin), rather than ACTH or vasopressin. These studies demonstrated behavioral and electrophysiological (EEG) responses in rodents as well as human beings [58–65]. Supporting studies with MSH, extending their earlier studies with ACTH and vasopressin, were then provided by the large capable Utrecht group headed by David deWied [66]. Rather than being satisfied with revealing these effects on the general process of learning and memory, we further showed in a series of what then were considered sophisticated studies that the process of attention was a key component [67–69]. Unfortunately, Organon pharmaceuticals chose for clinical studies an MSH analog whose efficacy was determined by the conditioned avoidance response in rats, rather than by tests of the native MSH itself with its broader actions. This point was emphasized in studies showing that different analogs of MSH can have differing dose-related responses in different behavioral paradigms [70,71].

A good example of a peptide injected peripherally acting on the brain and not mediated by peripheral effects was shown with a Met-enkephalin analog. In this study, naltrexone, an opiate antagonist that is able to cross the BBB, blocked the EEG effects of the Met-enkephalin analog whereas methyl naltexone, which does not readily cross the BBB, was unable to do so [72]. This is illustrated in Fig. (2).

3. Peptides can Cross the Blood-Brain Barrier

The regional distribution of c-Fos activation is different when MIF-1 is administered intravenously and when it is administered intracerebroventricularly. Although the concentration and disappearance kinetics are not identical in the two experimental regimens, intravenous MIF-1 activates more areas [11]. One possible mechanism mediating the CNS effects of MIF-1 after peripheral injection is its passage across the blood-brain barrier (BBB). The BBB provides the largest surface area for efficient exchange of information, particularly for substances coming from the periphery. The surface area of the BBB is 100–150 cm^2/g, and the microvessels composing the BBB are less than 40 μm apart [73–75]. The vast BBB contrasts with the circumventricular organs (CVOs) that provide specialized areas for rapid transmission of peripheral input to certain localized CNS regions. As for interleukin-1α, the uptake by CVOs accounts for less than 5% of total brain permeation [76]. Indeed, MIF-1 rapidly enters the brain by a saturable transport system [77]. After peripheral injection, tritiated MIF-1 is rapidly distributed to rat brain, with the highest concentration in the pituitary, followed by the striatum, hypothalamus, and midbrain [78], although occipital and frontal cortex also ranked high in an earlier study [79].

The pharmacokinetic BBB permeation of the Tyr-MIF-1 family of peptides has been quantified in mice. MIF-1 is rapidly transported into brain from blood by a partially saturable system whereas Tyr-MIF-1 only enters slowly by passive diffusion. By contrast, Tyr-MIF-1 has a saturable transport system out of the brain that is not shared by MIF-1, illustrating its selectivity structurally; but surprisingly, it is shared by a structurally dissimilar pentapeptide, Met-enkephalin that does, however, share an N-terminal tyrosine [80–82]. This can be interpreted as sharing functions, since Tyr-MIF-1 is an antiopiate and Met-enkephalin is an opiate. As shown with knockout mice lacking P-glycoprotein (Pgp) or inhibited by cyclosporin A, Pgp is not involved in the efflux of Tyr-MIF-1 or the endomorphins [83,84].

There are two principal ways by which peptides and proteins interact with the BBB: (a) transport or diffusion in intact form across the endothelial cells that are the structural backbone of the BBB [85–88] and (b) their actions on these endothelial cells which result in altered endothelial function, cytotoxicity, or cell proliferation [89–92]. We have quantified the BBB permeability of a series of bioactive peptides and polypeptides, including neurotrophic peptides, larger neurotrophins, and several classes of cytokines. Many small proteins cross the BBB (Tables 1 and 2) [93–95]. The mechanisms include saturable transport or passive diffusion based on physicochemical properties. Even for peptides and larger proteins that do not cross, they may still act on cerebral endothelial cells to change their functions. The distinctive characteristics of different peptides and proteins in their interactions with the BBB have broad therapeutic implications. For example, saturation of the transport of leptin across the BBB explains much of the phenomenon of 'leptin resistance' [96–99].

There are also peptide-peptide interactions at BBB transport. Urocortin is a 4 kD peptide that binds to corticotropin releasing hormone receptors (CRHR) and has multiple effects on CNS functions, including enhancing concentration, memory, inhibiting food intake, and providing neurotrophic support. Although of relatively low molecular weight, urocortin does not show significant penetration across the BBB in the basal state. However, the addition of

leptin, or glucose, can greatly enhance the permeation of urocortin across the BBB, apparently activating the transport system from blood to brain. Once activated, urocortin transport is saturable [100]. This unusual facilitation is also seen in cultured cerebral microvessel endothelial cells, where urocortin undergoes rapid trafficking after internalization [101].

The interactions between urocortin and leptin occur not only at the level of transport but also at the level of intracellular signaling. Both CRH receptors can mediate the saturable transport of urocortin [102]. Urocortin binding activates adenylate cyclase and induces cAMP production. While leptin enhances the endocytosis of urocortin, urocortin potentiates leptin-induced transcriptional activation of signal transducer and activator of transcription (STAT) -3. The interactions are not reciprocal, since leptin fails to further increase urocortin-mediated cAMP production [103].

Melanocortin and leptin also show signaling interactions at the BBB. In the cerebral microvessels that compose the BBB, receptors for both melanocortin and leptin are present. For the central effects of feeding, ObRb, which is not a G-protein coupled receptor, is the main signaling receptor for leptin while MC3R and MC4R, G-protein coupled receptors, are the main signaling receptors for αMSH (melanocortin). In cellular models with overexpression of murine ObRb, MC3R, or MC4R, leptin induces activation of Stat3 whereas αMSH stimulates the production of cAMP. After costimulation, αMSH potentiates the STAT3 activation induced by leptin, while leptin has only a minor effect in increasing αMSH-initiated cAMP production downstream to MC3R [104]. This novel potentiation in cellular signaling may have considerable biological implications.

4. The Actions of Peptides can Persist Longer That Their Half-Lives in Blood

Among the many possible ways by which peripheral peptides can exert CNS effects [105], the most direct route is to cross the BBB. Skepticism about the ability of peptides to cross the BBB was exemplified by a report from a "blue-ribbon" advisory panel to NIH that stated "Since the half-life of (peptides) is short, it is unlikely that significant entry occurs into brain" [106].

As mentioned in Section 2 of this review, some vehicles for the administration of peptides designed to prolong their actions can exert that action themselves [57]. But a short half-life in blood does not mean that a peptide cannot exert long-acting effects without the presence of a vehicle to prolong its actions. Even though the biological action of a substance frequently correlates with its half-life in blood, this is less applicable to peptides. Peptides in blood usually remain intact for only a few minutes, whereas the central actions mentioned above for MIF-1 and MSH can persist for several hours after peripheral administration. This was clearly demonstrated many years ago for luteinizing hormone-releasing hormone [107,108]. An exception to the rule of short disappearance times for small peptides is MIF-1. Its half-life in human plasma is 5 days although in rat plasma it lasts only a few minutes, like other small peptides including the related Tyr-MIF-1 and endomorphins [109–112]. Moreover, unlike most peptides, many studies have shown that MIF-1 is active orally in rodents [27–29,113] as well as human beings [33,52–54,114]. It is yet to be determined whether MIF-1 is a substrate for oligopeptide transporter (PEPT)-1 in the intestine and PEPT-2 in the choroid plexus, or both in the kidney. Overall, signaling cascades and secondary effects of a peptide can last longer than the half-life of the intact peptide.

5. Perinatal Administration of Peptides Exerts Long-Lasting Effects

In comparison with other peptide concepts that we introduced, the first demonstration that perinatal administration of peptides can exert long-lasting effects did not encounter the same

skepticism or derision. It was presented after it had been well established that perinatal administration of sex steroids could affect adult rodents, and provided further evidence supporting the view that a peptide's half-life in blood does not necessarily correlate with its biological actions (Concept 4 above). The ability of perinatally administered peptides to affect adult behavior was first shown with MSH [115,116] and TRH [117], and then with endorphin [118,119], Metenkephalin [120], MIF-1 [121], and Tyr-MIF-1 [122,123]. Later, perinatal administration of Tyr-MIF-1 was also found to alter its own transport across the BBB [124], and similar confirmation of this principle continues to be shown with other peptides.

6. One Peptide can Exert more than One Action

Although it is now evident that a peptide can bind to different receptor subtypes, recruit different signaling elements, and show a variety of effects in different tissues, its name tended to confine its perception at one time. Two articles in *Nature* [125,126] and one in *Lancet* [60] were the first to use the term "extra-pigmentary" to describe the effects of MSH in mammals. Persistence of the presence of MSH in rodents without much of a pigmentary function suggested to us that it may have acquired new functions consistent with the principle of peptide conservation. Since melanocytes, the target organ of MSH in lower vertebrates, are of neural crest origin, the brain seemed to us a logical target for MSH in mammals. This concept of one peptide exerting more than one action was so novel at the time it was introduced that it was inscribed in the 1982 William S. Middleton Award, the major research award of the Veteran Affairs Administration, to one of us (AJK).

In addition to different behavioral and EEG effects in rats and human beings shown for both MSH (Concept 2) and MIF-1 (Concept 1), these peptides can also exert antiopiate effects, as discussed below (Section 8). Moreover, LHRH is now frequently called gonadotropin-releasing hormone (GnRH) because of its ability to release both LH and FSH [127,128]. Whereas MIF-1 exerts antiopiate but not opiate activity, addition of a single amino acid to the N-terminus of this tripeptide yields Tyr-MIF-1 (Tyr-Pro-Leu-Gly-amide) that can exert both opiate and antiopiate actions [129]. An additional amino acid substitution results in Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-amide) [130,131] which also has dual opiate and antiopiate effects. More recent examples of many peptides exerting multiple actions have buried the "one hormone-one action theory".

7. Increased Peptide Doses can Result in Decreased Effects

Frequent criticisms from referees of our early papers were that we were using unfamiliar statistical techniques (analysis of variance followed by a multiple comparisons test) and that some of the effects we observed did not follow a linear dose-response relationship. Even now the mechanism for the inverted-U, bell-shaped dose-response relationship remains elusive, but it has become so generalized a phenomenon that Calabrese published 14 papers in Crit Rev Toxicol (volume 38, 2008) describing this "hormesis". These reports nicely summarize the occurrence of non-linear dose-response relationships in a variety of experimental and clinical situations, to which we added a commentary about peptides [56]. With this emphasis on hormesis, there now exists an *International Dose-Response Society* and an online *Dose-Response* journal.

Non-linear dose-response relationships may be one of the reasons that "big pharma" has not pursued the promising early results with the clinical use of MIF-1 to treat mental depression. Unlike other antidepressants, MIF-1 exerts its maximal effects in less than a week, usually within 3–5 days, and these effects can persist for months even though larger doses may not be effective [52,53,55], as also was observed in several animal studies [42,47,132]. One may consider "cellular plasticity", where a higher dose or persistent presence of a peptide

activates alternative/additional pathways, downregulates signaling elements by desensitization, or induces gene silencing. Any of the mechanistic studies will have to stem from the original observation that peptides have biphasic and pleiotropic effects.

8. The Brain Contains Antiopiate Peptides

The existence of endogenous antiopiates could be considered consistent with a type of short-loop negative feedback mentioned in Section 1. Rather than representing a negative feedback from a peripheral target organ (e.g., adrenal gland) to the pituitary/ hypothalamus, antiopiates within the brain would be more efficient to regulate the actions of brain opiates. Incidentally, we avoid use of the term opioid coined for enkephalins and endorphins because its evolving meaning is no longer confined to peptides.

MIF-1 was the first peptide recognized as showing antiopiate activity [133]. It functions as an antiopiate by itself, and reverses the effects of morphine in a large variety of experimental conditions, as well as in a clinical study [114]. The many confirmatory studies have been reviewed recently [132]. In the first paper describing the antiopiate effects of MIF-1, it was predicted that antiopiate activity would be found for other endogenous peptides [133]. This prediction has been validated for peptides that had already been described, like cholecystokinin (CCK)-8, MSH, somatostatin, angiotensin II, hemorphins, and morphiceptin, as well as for peptides such as members of the NPFF family, nociceptin/ orphanin FQ, and enterostatin that were discovered after the antiopiate concept was introduced.

9. There is a Specific Peptide Ligand for the Mu-Opiate Receptor

The mu (μ) opiate receptor is the main subtype for the analgesic actions of morphine. In the early 1990's, specific endogenous peptides were already known to selectively bind to the delta and kappa opiate receptors, but an endogenous peptidergic ligand specific for the mu opiate receptor had not been identified, as endorphin is relatively non-selective. It was anticipated for quite a while that a peptide selectively binding to mu opiate receptors would eventually be found in brain. The arduous work leading to the discovery of the mu receptor specific endomorphin-1 and endomorphin-2 by Zadina and colleagues started from painstaking extraction and structural function analyses. Although MIF-1 does not bind to opiate receptors, Tyr-MIF-1, comprising the MIF-1 tripeptide with an N-terminal tyrosine, had some affinity for the mu-opiate site, in addition to its own high affinity site. Tyr-W-MIF-1, isolated from bovine [134] and human [130] brain tissue, is identical to Tyr-MIF-1 except for substitution of leucine in the third position by tryptophan. This tetrapeptide has about 200-fold selectivity for mu over delta and kappa opiate receptors [135]. Additional single amino acid substitutions resulted in endomorphin-1 (Tyr-Pro-Trp-Phe-NH$_2$) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH$_2$), isolated from bovine [136] and human [137] brain tissue with high selectivity and affinity for the mu opiate receptor (Table 3). Their structure is quite different from that of the enkephalins, endorphins, and dynorphins. More than 500 papers with the endomorphins have substantiated their role.

10. A Peptide's Name does Not Restrict its Actions

Recognition of the idea that substances can have multiple actions seemed to have eluded early investigators in the peptide field. Once a peptide is named for the action by which it was first discovered, why would a young researcher bother to explore new functions? Before the availability of crystallography and domain analyses, the nomenclature became a hindrance for thinking outside the box. We've already mentioned that MSH does more than stimulate melanocytes in mammals and LHRH releases FSH as well as LH (Sections 2 and 6). Moreover, orexin affects sleep as well as food ingestion [138] and somatostatin inhibits more peptides than just growth hormone (somatotropin) [139,140]. Not only are peptides

remaining to be discovered, but new functions for already discovered peptides can be predicted, so there needs to be frequent reminders of this obvious concept for peptides.

11. Astrocytes are Involved in Obesity

Just as some of the concepts discussed above involve new functions for peptides with previously described actions, it is reasonable to predict that new functions will be described for anatomical constituents, such as astrocytes, known to serve other purposes.

As mentioned in Section 3, saturation of the transport system for leptin explains much of leptin resistance, in which excess circulating leptin no longer exerts its effect to reduce food intake and adiposity. In the past few years, we have taken several unique approaches in understanding the regulatory changes that direct leptin transport from blood to brain in rodents. The leptin receptor, ObR, is a member of the class I cytokine receptor family. Although the leptin receptor subtype ObRa is most abundantly expressed in cerebral microvessels and it efficiently mediates leptin endocytosis, Koletsky rats lacking ObRa because of a Tyr^{763} stop mutation in the extracellular domain [141] still transport some leptin into the brain [142,143]. Besides the ObRa isoform, we have shown that ObRb, ObRc, ObRd, and even the tailless form of ObR can endocytose leptin and release it from receptor-overexpressing cells in intact form [144]. We also found unexpected changes of ObR subtypes in cerebral microvessels and hypothalamus during development [145].

For about 15 years, CNS leptin research has focused on neuronal signaling. It was not until 2008 that astrocytic leptin receptors gained recognition from the demonstration that agouti viable yellow (A^{vy}) mice with adult-onset obesity show specific upregulation of astrocytic ObR [146]. In the BBB microvessels of A^{vy} mice, the expression of abundant ObRa is unchanged while that of the sparse ObRb is mildly upregulated. This, the shift of cellular distribution of ObR from BBB endothelia to astrocytes may represent a dynamic defensive mechanism of the CNS against metabolic disturbance. The upregulation of astrocytic ObR and concurrent reduction of neuronal ObR are also seen in B6 wildtype mice with diet-induced obesity [147]. The astrocytes respond to leptin treatment with robust cell signaling, including calcium influx [147] and induction of pSTAT3 and pERK [148]. Sadly, despite the undisputable multilevel evidence of astrocytic production of ObR, including confocal microscopic analysis of immunohistochemistry with a variety of antibodies, RT-PCR, and double-labeling in-situ hybridization [149], it took nearly two years for the neuron-centric community to accept the presence of ObR in astrocytes. Fortunately, acceptance of this concept took less time than that for some of the other concepts discussed here, undoubtedly facilitated by further demonstration of functional changes of mice after astrocytic ObR functions are modulated.

There are two aspects of the experimental proof that astrocytic ObR functions to antagonize neuronal leptin activities in pathological conditions. First, in mice pretreated with fluorocitrate, an inhibitor of astroglial metabolic activity, there is redistribution of leptin in neurons and increased pSTAT3 activation (Pan W *et al.*, unpublished observations). Second, in mice with specific knockout of astrocytic ObR that we recently generated, there is partial resistance to diet-induced obesity, and reduced susceptibility to pilocarpine-induced seizures (Pan W *et al.*, unpublished observations) and experimental autoimmune encephalomyelitis (Wu X *et al.*, unpublished observations). Overall, the improvement of behavioral outcome in these disease models indicates that the astrocytic leptin system may serve to worsen neuropathology in reactive astroglia.

It is reasonable to speculate that the astrocytic leptin system serves its normal function in the resting state. For example, it might be essential for the normal development of the hippocampus where essentially all astrocytes express ObR. Work is ongoing to determine

whether astrocytic ObR signaling serves to promote neurogenesis, synaptogenesis, and hippocampal related memory and cognitive functions during the course of development and aging.

THE CONTEXT OF PEPTIDE CONCEPTS IN THE NEUROPEPTIDE FIELD, AND FUTURE DIRECTIONS

The line of research in which we continue to engage originated at a time in which peptides were not yet considered neuromodulators or hormones. The concepts were built upon experimental evidence from somewhat unconventional approaches. They contributed to the transformation from regional to global, from single to multifocal, and from cross-sectional to longitudinal views of the peptide world. Now, neuropeptide research can take more drastic turns. The origin and fate of a peptide are better understood by use of molecular and cellular approaches, and studies of the interactions of peptides are aided by peptidomic methods. Comparative studies across species and time have been particularly valuable. Like the discovery of small interfering RNAs, it is possible that peptides will soon be deemed to have silencing functions to modulate biological activities. The broad functions of peptides are already demonstrated by the trophic, vasogenic, and immunomodulatory roles of many that are implicitly involved in neurodegenerative, oncogenic, and autoimmune diseases. Interacting with classical neurotransmitters such as monoamines, peptides play critical roles and may provide novel therapeutic targets for neuropsychiatric disorders. Peptides also serve as carriers for drug delivery, including lytic peptides and polycation peptides that penetrate cell membranes. Like amino acids and much larger extracellular matrix components, peptides can be essential for cell-cell interactions. There will also be more studies of peptides interacting with other classes of molecules. The ever expanding field is rewarding for all who have contributed a few paradigm-shifting concepts as structural backbones or building blocks.

CONCLUSIONS

As these concepts have become established, some lessons might be gleaned from the course of their progress. Richard Conniff puts it in perspective in the June 2008 issue of the *Smithsonian*: "Ideas that seem obvious in retrospect are anything but, in real life". Good advice was provided by Alexander Fleming: "Don't let your mind be cluttered up with prevailing doctrine" and by Kiekegaard: "There are two ways to be fooled: one is to believe what isn't so; the other is to refuse to believe what is so". Even earlier, Schopenhauer realized that discovery frequently undergoes three stages: ridicule, opposition, and acceptance as self-evident, with JBS Haldane adding a fourth stage: "I always said so".

Even in the 21st century, there was resistance to our last concept about astrocytic leptin, but publications in *Endocrinology, Brain,* and *Peptides* facilitated its acceptance. By summarizing this and earlier concepts in this issue of *Current Pharmaceutical Design,* we hope that it represents the beginning of successful efforts to identify novel targets for neuropsychiatric, autoimmune, and neurodegenerative diseases.

Acknowledgments

ABBREVIATIONS

BBB	Blood-brain barrier
CNS	Central nervous system
CRH	Corticotropin (ACTH) releasing hormone
CRHR	CRH receptor
MSH	Melanocyte-stimulating hormone (melanocortin)
MIF-1	MSH release inhibitory factor-1 (Pro-Lys-Gly-NH$_2$)

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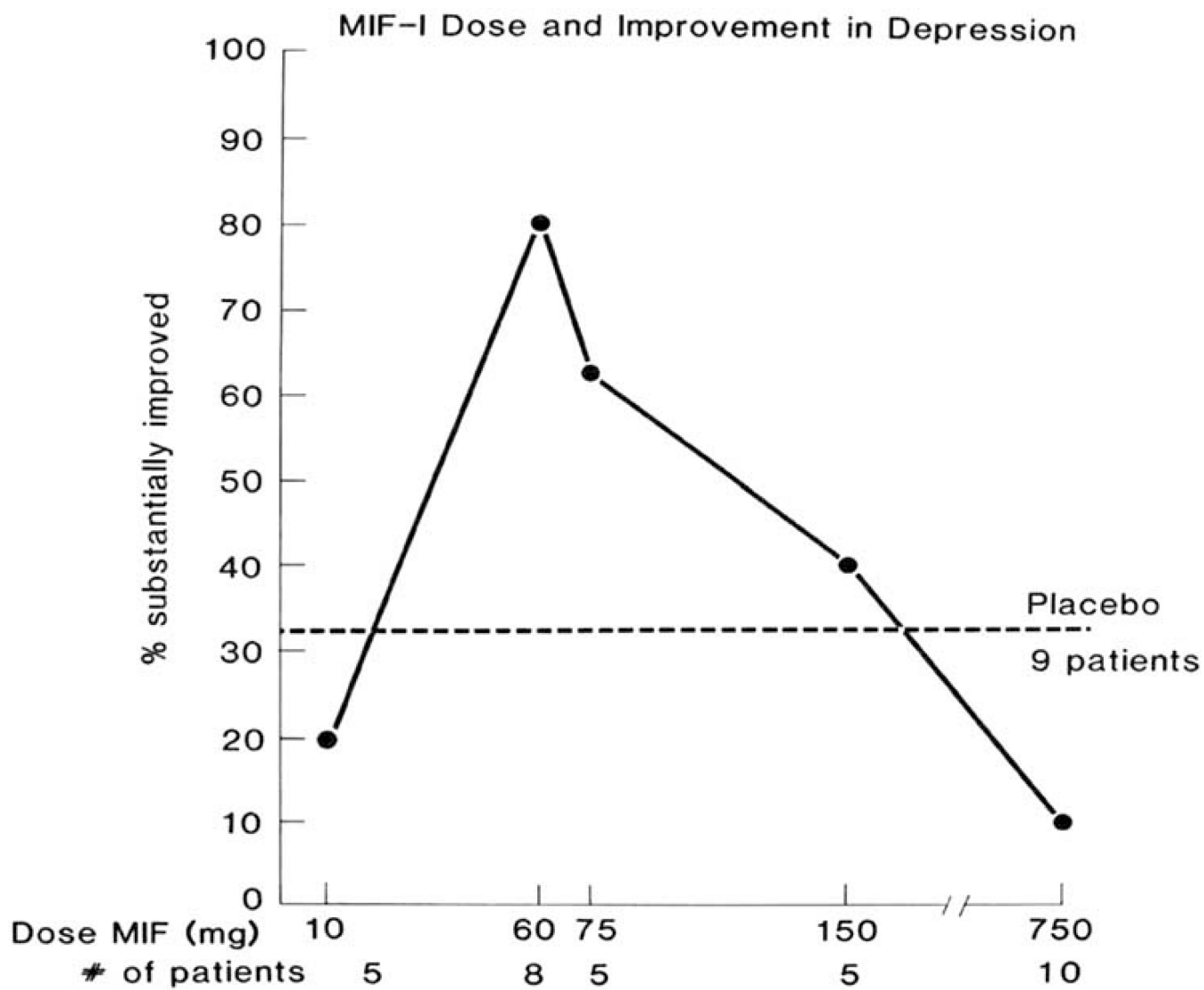
Fig. 1.
The inverted-U shaped dose-response curve seen in investigations by R.H. Ehrensing *et al.* of the effects of oral MIF-1 in patients with mental depression.
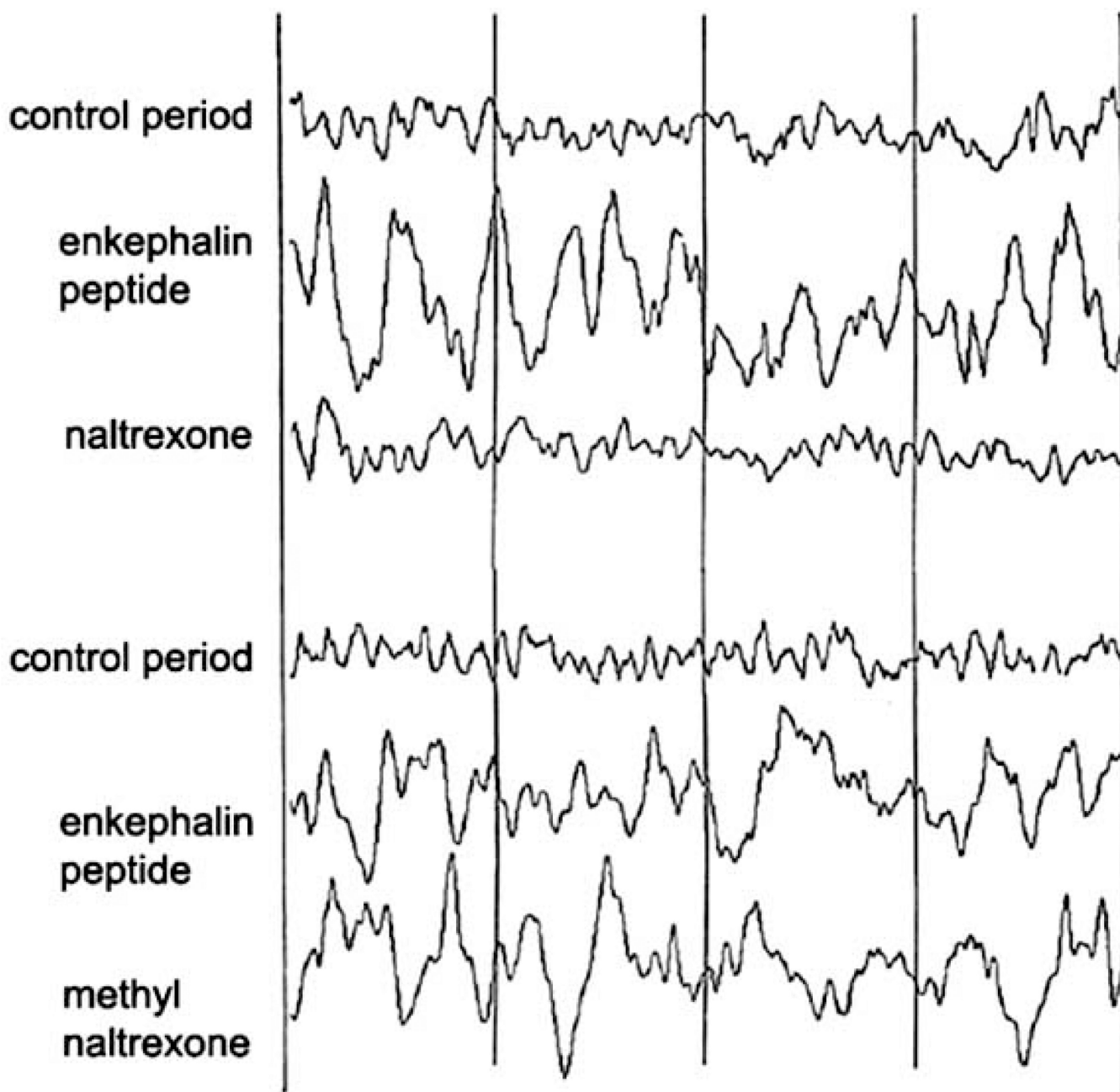
Fig. 2.
Demonstration that the electrophysiological effects of an analog of the opiate Met-enkephalin (enkephalin peptide) administered peripherally can be blocked by an opiate antagonist (naltexone) that crosses the bloodbrain barrier but not by an opiate antagonist (methyl naltrexone) that does not readily cross the blood-brain barrier (modified from Kastin *et al.* [72]).

Table 1

BBB Transport Systems of Endogenous Ingestive Peptides

Category	Representative Peptides	References
Saturable	Leptin	[96]
	Insulin	[150,151]
	TNFα	[86]
	Mahogany protein	[152]
	Pancreatic polypeptide	[153]
	Human ghrelin	[154]
	CRH	[155]
Saturable, but requires activation	Urocortin - no entry unless activated by leptin	[100]
	by TNFα	[156]
	by glucose	[157]
Passive diffusion (non-saturable)	Orexin A	[158]
	NPY	[159]
	AgRP	[160]
	CART	[161]
	α-MSH	[79,162]
	GLP-1	[163]
	Amylin	[164]
	Urocortin II	[155]
	cHis-Pro	[165]
	Nesfatin	[166,167]
	FGF21	[168]
	PYY_{3-36}	[169]
No entry	Orexin B	[158]
	MCH	[170]
	Obestatin	[171]
	Adiponectin	[171]
	Mouse ghrelin	[154,171]

Table 2

BBB Transport of Other Endogenous Peptides/Polypeptides, Mostly Cytokines and Neurotrophins

Category	Representative Peptide/Polypeptide	References
Saturable	CNTF	[172]
	EGF	[173]
	GM-CSF	[174]
	IFNγ	[175]
	IGF1	[176]
	IL1α/IL1β	[85]
	IL1RA	[177]
	LHRH	[178]
	LIF	[179]
	Neuregulin1β1	[180]
	PACAP38	[181]
Passive diffusion (non-saturable)	CINC1	[182]
	GH	[183]
	IFNα	[175]
	IL2	[184]
	IL6	[185]
	IL8	[186]
	PACAP27	[181]
	TGFα	[187]
No entry	GDNF	[188]
	IL10	[189]
	MIP1α, MIP1β	[190]
	PDGF-AA, PDGF-BB	[191]
	TGFβ	[192]

Table 3

Structures of Opiate Mediating Members of the Tyr-Mif-1 Family of Peptides

Peptides	Amino Acid Structure	References
MIF-1	Pro-Leu-Gly-NH$_2$	[8]
Tyr-MIF-1	Tyr-Pro-Leu-Gly-NH$_2$	[193]
Tyr-W-MIF-1	Tyr-Pro-Trp-Gly-NH$_2$	[130]
Endomorphin-1	Tyr-Pro-Trp-Phe-NH$_2$	[136]
Endomorphin-2	Tyr-Pro-Phe-Phe-NH$_2$	[136]