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Review

Anatomy, function and regulation of neuropeptide EI (NEI)

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ABSTRACT

This review is focused on the anatomy, role and behavior of neuropeptide-glutamic acid-isoleucine (NEI), providing a general report on the neuropeptide. In addition to hormone release, this peptide also takes part in the regulation of grooming behavior and locomotor activity. NEI is produced by cleavage of prepro-MCH that probably takes place at the Lys^{129}-Arg^{130} and Arg^{145}-Arg^{146} sites (the glycine residue on the C-terminus of NEI strongly suggests that this peptide is amidated). This same prohormone is also the precursor of MCH, widely studied in relation to food and water intake, and NGE, of which little is known. NEI and MCH are extensively colocalized throughout the central nervous system (CNS), and NEI is also present in peripheral tissues. The latter is also effective in stimulating luteinizing hormone (LH) release and, to a lesser extent, FSH from primary pituitary cell cultures. In addition to releasing LH from the medial eminence, NEI also acts directly on gonadotropes. Lastly, this neuropeptide also acts at the CNS level on gonadotropin-releasing hormone (GnRH) neurons.

Contents

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1. Introduction

Neuropeptide-glutamic acid-isoleucine (NEI) and melanin concentrating hormone (MCH) are two peptides derived from a common prepro-hormone named ppMCH. This precursor has 165 amino acids and also encodes other putative peptides such as neuropeptide-glycine-glutamic acid (NGE) [35,36,38]. These peptides, highly conserved among vertebrates, are abundant and widely distributed in the brain suggesting that they probably have important physiological functions. NEI and MCH are predominantly expressed in the lateral hypothalamic area (LHA) and incerto-hypothalamic area (formerly called rostromedial zona incerta) [6], and project broadly throughout the central nervous system [7]. Moreover, co-expression and co-secretion of NEI and MCH has been observed [36,57].

NEI can induce differential effects on brain neurons such as increased grooming behavior (EGB) and motor activity (MA) [43]. NEI injections also modify the level of noradrenalin and dopamine 35 and 60 min after administration [45]. NEI and MCH exhibit antagonistic effects on the stress-induced release of ACTH in rats [9]. It is interesting to note that NEI-induced grooming behavior is inhibited when it is simultaneously administered with MCH.

The aim of this review is to introduce the reader to NEI from an anatomical and physiological point of view.

2. NEI anatomy

Since this peptide family was first discovered in the mammalian brain (using the male rat as a model), three of its members have been found to be biologically active: MCH, NEI and NGE [35].

Few studies have been published on the role of NGE. For instance, NGE increases neurofilaments and the production of synaptophysin in rat neurons with 18 days of development [31], and also increases AMPc production in melanoma cells [28].

Immunoreactivity (MCH-/NEI-ir) and mRNA expression of both MCH and NEI have been observed in certain brain regions. Such regions include, first, those of the diencephalon: the rostromedial part of the zona incerta, later referred to as the incerto-hypothalamic area (IHy) by Sita et al. [50] the three subdivisions (anterior, tuberal and posterior) of the LHA; the area between the dorsomedial and ventromedial nuclei of the hypothalamus, which Swanson [51] designated as the internuclear area; the anterior periventricular nucleus; the dorsomedial aspect of the tuberomammillary complex. Secondly, immunoreactivity and mRNA expression has also been observed in the olfactory tubercle, located in the basal forebrain. Finally, the paramedian pontine reticular formation in the pons represents another such region [7]. It is important to note that the highest concentration of MCH-ir and NEI-ir cells is found in the IHy and the LHA. We found that nearly all the cells in these two regions were immunoreactive for both MCH and NEI (mean ± S.E.M., 96 ± 3%) (Fig. 1).

Following our description of the MCH peptide family anatomy, several groups studying the presence and function of such peptides have chosen not to use the conventional names for these regions. This has created a great deal of confusion. When describing the diencephalic region between the tip of the third ventricle and a vertical imaginary line passing through the lateral aspect of the mammillothalamic

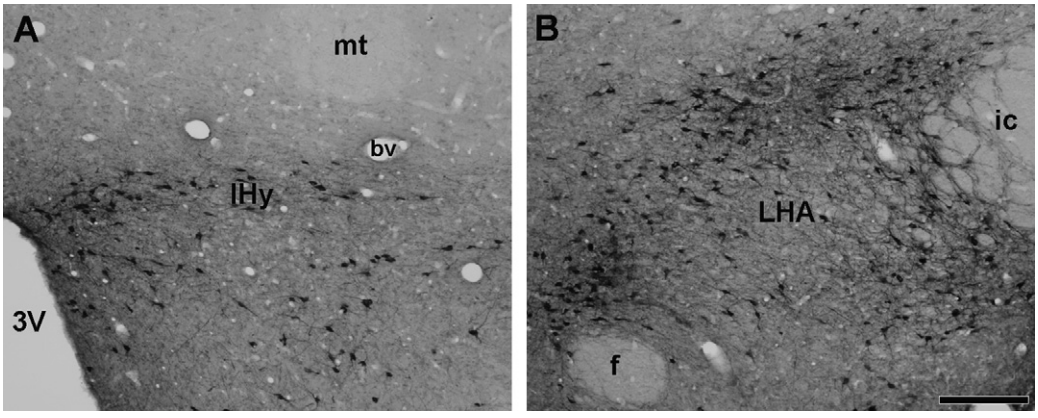

Fig. 1 – Diencephalic distribution of NEI-immunoreactivity-containing cells. Brightfield photomicrographs of immunoperoxidase material stained for NEI-ir. (A) NEI-ir-containing cells in the incerto-hypothalamic area. (B) NEI-ir-containing cells in the lateral hypothalamic area. *Abbreviations*: mt, mammilothalamic tract; bv, blood vessels; IHy, incerto hypothalamic area; 3v, third ventricle; ic, internal capsule; LHA, lateral hypothalamic area; f, fornix. Bar = 200 μm.

tract, we ourselves were at a loss for a name for the region. Due to the presence of MCH-ir and NEI-ir cells in this 'subthalamic' region, we designated it the 'rostromedial zona incerta' (rmZI) because it lies rostromedial to the classical zona incerta (ZI) and is apparently an extension of this zone. This designation further contributed to the confusion and misunderstandings regarding the anatomy and function of this peptidergic family. In order to draw clear distinctions between the areas where there is mRNA and protein expression of these peptides, we decided to study the so-called rmZI by injecting the anterograde neuronal tracer biotinylated dextran amine (BDA) into the ZI as well as into the rmZI. The results strongly suggested that indeed, a more appropriate name for the region would be the 'incerto-hypothalamic area', since the projection territories were more consistent with the anatomy of the medial hypothalamic nuclei [50].

Regarding the distribution of MCH-ir and NEI-ir fibers in the brain and hypophysis, we found that nearly every recognized cell group in the central nervous system (CNS) of the rat receives a number of these fibers. From the olfactory bulb to the spinal cord, both peptides have been identified in such terminals. However, in some areas these projections are minimal or nonexistent. Nevertheless, the distribution of MCH-ir and NEI-ir fibers is quite dense in certain regions of the rat CNS: the lateral and medial septal nuclei; the hippocampal formation; the shell part of the nucleus accumbens; the lateral part of the medial mammillary nucleus; the periaqueductal gray matter. In relation to the hypothalamic nuclei, most of the medial regions, even the paraventricular, supraoptic and ventromedial nuclei, do not receive a significant number of MCH- or NEI-ir fibers. One exception is the dorsomedial hypothalamic nucleus, which receives a moderate number of such fibers. Finally, the median eminence also receives a moderate number of MCH-ir and NEI-ir fibers in the internal lamina, although the external lamina receives significantly fewer. Consequently, the posterior lobe of the hypophysis receives a moderate number of fibers and terminals stained for either peptide. The only territory that contains MCH-ir terminals but presents few or no NEI-ir terminals is the interanterodorsal nucleus of the thalamus [7]. Some of the brain stem motor nuclei, such as the abducens nucleus, the nucleus ambiguous, the V motor and the dorsal motor nucleus of the vagus, do not receive large numbers of MCH-ir or NEI-ir fibers. To this group we can add the cerebellum, where the cortex and nuclei receive very few MCH-ir or NEI-ir fibers. We must also mention the study carried out by Rondini et al. [39], who described the presence of MCH mRNA in the caudal laterodorsal tegmental nucleus of female rats, which is independent of the female cycle. Using the anti-MCH antibody, the authors also described weak immunostaining for MCH in the same region although immunostaining with anti-NEI antibody yielded negative results.

Despite the fact that we have a great amount of data regarding the normal anatomy of MCH in the CNS, which confirms its normal distribution, the only other physiological state studied where mRNA expression and immunoreactivity for both MCH and NEI has been identified at additional sites is the nursing dam, mainly between days 15 and 21 of lactation. During this period, both peptides appear in the ventral aspect of the medial part of the medial preoptic nucleus, in the periventricular preoptic nucleus, and in the most rostral aspects of the paraventricular nucleus of the hypothalamus (PVH). However, in these lactating dams, neither peptide colocalizes with oxytocin in the PVH [32].

Several post-translational mechanisms are associated with the processing of precursors to final peptides. Cleavages, amino acid derivatization (glycosylation, phosphorylation, acetylation and myristoylation) or even spatial conformation changes can occur [23]. Many of these post-translational processes are tissue-specific and controlled by certain enzymes located in a determined region [60]. In the processing of ppMCH, MCH cleavage is followed by the cleavage of NEI (Fig. 2). These cleavages are conducted by the action of prohormone convertases (PCs). Nevertheless, although any PC is capable of cleaving MCH from the precursor, only PC2 is able to cleave the site between Lys^{129} and Arg^{130}, thus liberating NEI. This finding is supported by the fact that, in mice lacking PC2, there is a dramatic decrease in NEI and an equally dramatic increase in MCH [57]. Another post-translational mechanism is also involved in the processing of ppMCH, in which the cleavage of NEI from ppMCH occurs as a result of the activity of a carboxypeptidase E. In fact, in the free form of NEI, there are three additional amino acid residues at the carboxy-terminal end that are removed by the activity of carboxypeptidase E. In addition, the absence of this enzyme in *fat/fat* rats produces a decrease in mature NEI and a compensatory increase in ppMCH, resulting in an increase in MCH [41].

These two post-translational mechanisms are involved in the possibility of NEI nonproduction. Therefore, PC2 and carboxypeptidase E might be inhibited or absent in cells projecting to specific sites with MCH-ir but not NEI-ir terminals. Although there is a considerable amount of available data regarding this peptidergic system, we still know very little about the specific and important aspects of it.

Fig. 2 – Deduced structure of rat prepro-MCH. The relative positions of the amino (NH_2) terminal signal peptide (SP), and the putative MCH, NEI and NGE sequences at the carboxy (COOH) terminus are indicated. The amino acids sequence of NEI is expanded below it. The putative proteolitic processing sites are marked with arrowheads.

For instance, most of the studies investigating the MCH system have only used anti-MCH antibodies. Therefore, we do not know whether those cells are also immunoreactive to anti-NEI. On the other hand, some studies have only examined mRNA expression. Once again, we do not know which protein was translated, and the cells expressing MCH mRNA may contain MCH plus NEI, only MCH or just NEI.

In conclusion, considering the anatomical data regarding this peptidergic system together with the functional aspects of both peptides reported to date in the literature, we must think not only in terms of immunoreactivity and mRNA expression but also in terms of the output of the two main regions where we have at least two peptides, i.e. MCH and NEI. Thus, recent results suggest that both territories are responsible for controlling different behaviors and functions. The LHA, which seems to be an integrative center, appears to be responsible for the arousal of motivated behaviors, such as feeding. In addition, the hodological results for the IHy [50] indicate that it does not participate in the regulation of feeding behavior based on the projections from the arcuate nucleus to the MCH/NEI neurons in the LHA [10,17,18,49]. Alternative reagents or better methodological tools must be developed to improve our understanding of where, how and why ppMCH is cleaved into MCH or NEI, and thus increase our knowledge of the anatomy and functional aspects of this family of peptides.

3. Function and regulation of NEI

As previously mentioned, neuroanatomical mapping of MCH/NEI distribution and neuronal pathways show that there is a strict colocalization of the two peptides within the LHA and ZI, with extensive neural projections to the hippocampus, brainstem, posterior pituitary gland and cerebral cortex [7]. Due to this distribution, Parkes and Vale [36] examined the onset of MCH/NEI secretion in cultured neonatal rat hypothalamic cells (7- or 8-day-old rats) and found that basal levels of NEI are initially low during the first week of birth. These then increase and finally, after 20 days of culture, lower values are once again restored; eventually, after 25 days of culture, no NEI secretion is observed. The cAMP analogue, 8 Br-cGMP, produces a strong dose-dependent increase in NEI secretion, although the content of NEI remains either unchanged or slightly reduced compared to control values. This suggests that cAMP might be acting at the level of protein synthesis, maintaining the same stored amount of peptide within the cell. 8 Br-cGMP also increases NEI secretion from cultured hypothalamic cells. Parkes and Vale [36] were also the first to observe that at least two pro-MCH derived peptides, MCH and NEI, are secreted by a mammalian cell culture system and that this system can be regulated by second messengers.

Studying the expression of pro-MCH mRNA, Hervieu and Nahon [25] found that MCH and NEI were present in adult rat peripheral tissues using a polymerase chain reaction (PCR) of reverse-transcribed RNA. Subsequently, the expression of MCH mRNA and pro-MCH derived peptides was analyzed in rat peripheral tissues and low levels of MCH transcribed gene were evidenced in the testicles, stomach and intestines of Sprague-Dawley and Wistar rats. However, a much larger degree of expression is found in the hypothalamus. Additionally, strong evidence indicates the presence of both mature and immature RNAs in the spleen, stomach, testis and intestines of Wistar rats. A weak hybridization signal at 720 NT was also observed in the heart and lung of both Wistar and Sprague-Dawley rats. Furthermore, rat MCH gene products were detected in ovaries, thymus, pancreas and adrenal gland, although no expression was found in kidneys and liver. The length of MCH RNAs is clearly different between the hypothalamus (about 1.0 kb) and peripheral tissues (around 0.8 and 1.6 kb RNA species in Sprague-Dawley rats, and 0.8 kb in Wistar rats). Hervieu and Nahon observed genuine local expression of endogenous transcripts as positive *in situ* hybridization signals were found inside the tissue and could be suppressed by previous RNA treatments.

In testis, MCH mRNA and immunoreactive MCH/NEI are found in Sertoli cells but are absent or below the threshold of detection in other testicular cells types including Leydig cells. However, in a later study these components were observed in Leydig cells [26,27] and then went on to determine whether the presence of NEI and other associated peptides could be related to discrete stages of the seminiferous epithelium cycle. Thus, the authors characterized MCH transcripts from isolated Sertoli and germ cells from adult rats, determined the sites of MCH production within adult rat and mice testes, and finally examined the expression of MCH during the development of rat and mouse testes, as well as in germ-cell-deficient strains of mice and in adult men.

It is interesting to note that compared to other peptides found in testicles, the concentration of MCH/NEI (0.1–0.2 pmol/g tissue) is in the same order as values for ACTH and β-endorphin-related peptides [14,55]. These reduced levels of MCH/NEI suggest that the peptides could be acting as paracrine, juxtacrine or autocrine components rather than endocrine hormones. The authors suggest that NEI possibly potentiates hCG stimulated testosterone synthesis, but that MCH exerts no action. Also, both peptides evidence certain antagonism between them, as previously observed under the stress paradigm, ''tempting to speculate that MCH and NEI play opposite roles in testis'' as MCH inhibits the action of NEI on testosterone production under high-hCG stimulation when Leydig cells are simultaneously treated with MCH and NEI.

3.1. *The role of NEI in hormone regulation*

The first authors to study whether NEI was involved in regulating or inhibiting hormone release in the anterior pituitary gland were Bluet-Pajot et al. [9], who showed that neither MCH nor NEI modify the basal secretion of GH or PRL at any time, nor affect ether stress-induced changes on these two hormone secretions.

Exogenous MCH induces a moderate decrease in ACTH secretion under resting conditions 3 h before the onset of darkness, but not after dark. NEI showed no effect on ACTH levels under any of the conditions tested. However, acute or repeated injections of NEI with MCH (at a 1:5 or 2:3 ratio) suppressed the inhibitory effect of rat MCH on ACTH stimulation following ether stress at night, suggesting that as both MCH and NEI are concentrated in the hypothalamic perikarya, they may cooperate in controlling neural functions.

Regarding the relationship with other hormones, Kennedy et al. [30] studied the regulatory effect of NEI and MCH on TSH and TRH. MCH intracerebroventricular (icv) injections reduced plasma TSH levels and inhibited TRH release; to a similar extent, NEI inhibited the release of TRH from mediobasal hypothalamic explants. This is particularly interesting considering that NEI has been previously designated as a physiological MCH antagonist in both the hypothalamo-pituitary-adrenal axis (HPA) and the hypothalamo-pituitary-gonadal axis (HPG), and that MCH blocks the stimulatory effect of NEI and α-MSH on grooming behavior. As in this case, both MCH and NEI are capable of reducing TH, in other words the two peptides are acting in the same sense. However, the inhibitory effect of MCH on NEI, or of NEI on MCH was not evidenced in this study.

Kennedy et al. [30] also observed that starvation leads to a hypothalamic suppression of TRH, TSH and circulating thyroxine, which subsequently decreases energy consumption, and thus proves to be an important response to minimize energetic needs. A subsequent study discarded any possible regulatory role of NEI on feeding behavior [34], in accordance to a similar result previously published by Rossi et al. [40]. However, Maulon-Feraille et al. [34] observed that the effect of the pro-MCH 131–164 region codifying NEI-MCH is stronger than MCH in stimulating rat feeding behavior. These authors proposed that NEI-MCH is less susceptible to proteases than MCH, which explains its enhanced activity on feeding behavior, and suggested that ''NEI-MCH represents the first MCH natural functional superagonist so far described''.

Viale et al. [56] began studying the possible interrelationship between NEI and preovulatory luteinizing hormone (LH) surge, given those NEI immunoreactive fibers and terminals were found in hypothalamic areas related to the control of LH surge such as the medial preoptic area (MPOA) where gonadotropin-releasing hormone (GnRH) neurons are found. Also, cytoplasmic estrogen receptors were found on perikarya in an area lateral to the rostral level of the ventromedial nucleus (VMH) [8], i.e. nearby MCH producing cells, suggesting a direct action of estrogen hormones on MCH/NEI production and/or release. These authors showed that MCH is predominantly expressed in the hypothalamus of macaques (*Macaca facicularis*). The exposition of ovariectomized monkeys to estradiol benzoate (EB) caused parallel phasic variations in immunoreactivity. A transient increase of NEI-ir (associated to the estradiol peak) was observed after 30 h, with a final NEI-ir increase observed at the time of the LH surge (72 h post-E2B). These results could indicate that the peptide might be involved in the regulation of the preovulatory mid-cycle in primates.

Also regarding the regulatory effect of NEI on pituitary hormones, Celis et al. were the first to show the ''*in vivo*'' effect of NEI on LH release [1]. This study demonstrated that icv administration of NEI to male rats increased LH serum levels. Administration of NEI to chronically ovariectomized rats treated with EB and low doses of progesterone also increased LH release. However, no effects were observed on GH, FSH or PRL. In another study, the same research group analyzed whether the peptide was also effective ''*in vitro*'' using primary cultures of pituitary cells [15]. The authors demonstrated a specific and direct action of NEI on LH stimulation and to a lesser degree FSH release, without modifying the other pituitary hormones studied (PRL and GH). The peptide acts specifically on LH release with a rapid and long-lasting stimulatory effect, whereas the effect on FSH release is smaller and more ephemeral. These results evidence the fact that NEI has quite a specific effect on the release of LH and FSH, although other authors have also studied the effect of NEI on other adenohypophysis hormones. Using dispersed human fetal pituitary glands (21–22 weeks of gestation) and cultured GH-secreting adenomas, Segal-Lieberman et al. [48] found that when NEI is administered at the same dose used for MCH it is twice as potent in increasing human GH secretion. In fact, NEI induced an increase similar to the GnRH. When using pituitary adenomas, the authors found that when cultured human GH adenoma cells were incubated with either MCH (10 nM) or NEI (10 nM) for 4 h, the behavior of the peptides varied according to the adenoma; in one case GH is released by NEI and MCH has no effect, and in the other experiment GH is released by MCH and NEI has no effect. Once again, as observed in fetal tissues, there was great variability in the response of different tumors to MCH/NEI treatment.

As previously mentioned, in the studies using cultured pituitary cells obtained from rats there is no evidence suggesting an effect of NEI on GH release, either ''*in vivo*'' or ''*in vitro*''. However, this discrepancy could be due to the different tissues used in the studies.

3.1.1. *How does NEI regulate LH release?*

Based on the evidence showing that the administration of NEI increases LH and in order to identify the anatomical substrate underlying this effect, Attademo et al. [2] used combined immunohistochemical methods to analyze female rat forebrains during diestrus and proestrus, as well as the forebrains of ovariectomized female rats treated with EB, EB and progesterone, or sesame oil (control animals). The authors found very little Fos immunoreactivity in the AVPV in rats perfused in the afternoon of the diestrus day, in ovariectomized rats treated with sesame oil or with estradiol benzoate; however, in rats perfused in the afternoon of the proestrus day or in ovariectomized rats with estradiol benzoate plus progesterone an increase expression of Fos immunoreactivity into the AVPV was observed. Additionally, they observed dense to moderate NEI innervation in areas related to reproduction, including the organum vasculosum of the lamina terminalis, the anteroventral periventricular nucleus (AVPV) and the median eminence. In the same work the authors found that around 10% of the AVPV neurons expressing Fos receive NEI close appositions, also virtually all GnRH neurons in the medial septal receive a great number of NEI appositions. In the median eminence the authors found that the internal layer was very dense in terms of NEI-ir fibres and terminals. In the median eminence, NEI varicosities and terminal-like structures were in close proximity to blood vessels and GnRH fibers. These results indicate that NEI can induce LH secretion by direct release into the median eminence, by the modulation of GnRH neurons located in the preoptic area, by the modulation of GnRH terminals located in the median eminence or by neurohormones. The release of NEI can also induce LH secretion indirectly by modulating AVPV neurons.

In addition to MCH/NEI's multiple projections throughout the central nervous system, fibers with both peptides are also present in the posterior gland. This presence led Parkes and Vale [37] to study the effect of MCH/NEI on the secretion of oxytocin (OT) and arginine vasopressin (AVP) in isolated rat posterior hypophysis. The authors extracted the posterior pituitary lobe from adult male rats and incubated them in 100 nM NEI during 1–3 h. NEI treatment reduced AVP secretion by 57 ± 10% after 1 h and by 68 ± 11% after 3 h treatment. On the contrary, OT secretion increased by 245 ± 89% and 209 ± 64% after 1 and 3 h treatment, respectively. These results suggest that NEI is probably involved in the regulation of lactation, directly stimulating the release of OT in the posterior pituitary gland. The NEI-induced suppression of AVP release suggests that this peptide may be involved in maintaining fluid homeostasis, possibly inhibiting the consequential antidiuretic activity of AVP during fluid overload.

3.2. NEI's role on behavior

The different interactions between neuropeptides and neurotransmitters play an important role in the functional regulation of both motor control and behavior [21,22,52–54]. In rats, central administration of α-MSH elicits excessive grooming, locomotion, and rearing behaviors [53]. The ventral tegmental area is the target area for inducing EGB and MA by α-MSH, an effect accompanied by changes in striatal dopamine (DA) levels [52–54]. It is also a region rich in fiber terminals of both the ppMCH and α-MSH systems [7,16]. In a similar manner, icv administration of NEI can induce EGB and MA. Conversely, icv administration of MCH exerts no influence on any of these behaviors. Although MCH is ineffective when administrated alone, it antagonizes the effects induced by α-MSH and NEI [42]. As VTA is the specific site where NEI induces EGB and MA, Sanchez et al. [44,45] studied the effect of NEI on rat VTA, a structure where α-MSH, NEI and MCH are highly concentrated. In order to elucidate the biochemical mechanism of the behavioral effects of these neuropeptides, the authors determined the degree of grooming behavior along with the levels of catecholamines following neuropeptide administration. The neuropeptides were administered bilaterally in the VTA, and the rats were killed 0, 35 and 60 min later. The levels of EGB, MA, dopamine, noradrenalin (NA) and the DOPAC/DA ratio were immediately measured to determine steady state levels of catecholamines and an indirect measure of DA release and metabolism, respectively. NEI induced a significant increase in EGB, MA and total behavior scores 30 and 60 min after administration. This was correlated with an increased DOPAC/DA ratio in the nucleus caudatus, but not in the nucleus accumbens. Conversely, NEI increased the concentration of NA in the nucleus accumbens, but not in the nucleus caudatus. However, MCH injections did not generate any changes in behavior or in the level of catecholamines in the studied nucleus. In conclusion, the behavioral effect of the exogenous administration of NEI in VTA is peptide-specific and probably associated to alterations in catecholamine metabolism and release in the nucleus accumbens and caudate-putamen complex.

Berberian et al. [3] were able to demonstrate that icv injections of atropine, a general muscarinic antagonist, administrated before NEI suppresses the NEI-induced behaviors. On the contrary, previous injections of dihydro-β-erythroidine, a general nicotinic antagonist, do not affect the EGB and MA induced by the peptide. Based on this evidence, the authors suggested that NEI might be specifically acting on a cholinergic afferent to dopaminergic cells. Furthermore, the results appear to indicate that a neural target, different from the dopaminergic system, may be activated by the peptide eliciting behavioral changes such as EGB, as it was shown that NEI increases EGB and MA.

3.3. Receptors involved in cholinergic effects

Pharmacological and molecular biology studies allowed identifying different types of muscarinic receptors. Until the present, five genes encoding different receptors have been identified, named by the chronological order of their discovery: m1, m2, m3, m4 and m5 (m1 and m2 coincide with the first identified receptors, M1 and M2). On the other hand, the subtypes that have been pharmacologically characterized to this day are M1, M2, M3 and M4 [11,33].

Receptors M1, M4, M2 and M3 (in decreasing order) are expressed in the striated nucleus. M1 receptors are post-synaptic; M4 receptors are located as hetero-receptors on dopaminergic or glutamatergic afferences or as post-synaptic receptors on GABAergic neurons; M2 receptors are found as auto-receptors on cholinergic interneurons [12].

In situ hybridization studies demonstrated that over 80% of the neurons in the striatum nucleus express M1 muscarinic receptors [4,58]. Pirenzepine, a M1 antagonist, was found to block NEI-induced EGB and MA in a dose-dependant way, suggesting that these receptors might participate in this behavior. Previous studies performed on striated nucleus slices prepared from M1-KO mice showed that the increased release of DA could not be reverted by the administration of oxotremorine (a non-selective muscarinic agonist) [61,31], and postulated that these receptors were not implicated in this pathway. However, these authors suggested that the M1 receptors modulating DA release in the striated nucleus could be located in extrastriate neurons (for example, cortical neurons) that project to the striate nucleus. On the other hand, *in vivo* microdialysis studies showed that rats with deficient M1 receptors have an elevated extracellular DA level in the striate nucleus, probably generated by an increase in DA release [19]. However, it was demonstrated that DA release in the striate nucleus is mediated by M1 receptors, in agreement with the previously described results that suggest that NEI acts via these receptors to induce EGB and MA [29,59] At the same time, the study using the M1 agonist McN-A-343 showed that EGB and MA can be stimulated in a dose-dependent way when the agonist is, injected alone, although no additional or synergic effects were observed when injected together with the peptide.

When assessing the participation of M2 and M4 receptors on NEI-stimulated EGB and MA, it was observed that the highest doses of metoctramine and tropicamide, M2 and M4 antagonists, respectively, increased the studied behavior when injected without the peptide and were not able to completely block the NEI-induced behavior (Sánchez, Celis, unpublished results). Both M4 and M2 muscarinic receptors

are coupled to protein G, of the G_o/G_i family and hence can reduce neuronal activity. Furthermore, there is considerable evidence indicating that M2 receptors are expressed as presynaptic receptors in the striate nucleus [5,24] and it has been demonstrated that rats deficient in M4 receptors exhibit a basal MA increase produced by the loss of inhibition mediated by these receptors [20]. Zhang et al. [61] observed that ACh release is mainly mediated by M4 receptors of the striated nucleus, therefore inhibiting or blocking these receptors would generate an increase in MA. All the aforementioned interactions evidence the complexity of the pathways involved in the studied behavior.

As Sanchez et al. [44,45] also observed that the peptide increases the noradrenalin content in the nucleus accumbens, the group decided to establish the role of the noradrenergic system on NEI-regulated EGB and MA. The authors observed that adrenoceptor antagonists "*per se*" have no effect on EGB or MA. icv administration of propranolol, a general β-adrenoceptor antagonist, prior to NEI inhibited the induced EGB in a dose-dependent manner and metoprolol, a β1-adrenoceptor antagonist, also blocked this behavior. However, icv injections of phentolamine, an α-adrenoceptor antagonist, and ((±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl) amino]-2-butanol), a β2-adrenoceptor antagonist, had no effect on the NEI-induced behaviors at any of the tested doses. On the other hand, isoproterenol, a general β-adrenoceptor agonist and dobutamine, a β1-adrenoceptor agonist, both elicited similar behaviors to those induced by NEI. These results support the hypothesis of a relationship between NEI and β-adrenoceptors, more specifically the β1-adrenoceptor, as observed with other similar endogenous peptides such as neurotensin, substance P and α-MSH. Hence, NEI could probably be exerting a neuromodulating effect on the CNS [47].

3.4. *Effect of NEI on binding*

As high doses of NEI stimulate the production of cAMP in the caudate putamen, Sanchez et al. [44,45] used the activation of adenylate cyclase coupled to specific D1 receptors to find evidences of a possible interaction between the neuropeptide and D1 receptors in the mammalian nervous system. The authors found that at high doses, NEI affected both the maximum binding and apparent affinity of [*n*-methyl-3*H*](R)-(+)-8chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1*H*-3-benzazepin-7-ol hemimaleate to the dopamine D1 receptor in a concentration-dependent manner. These binding results are in accordance with previous results by the authors indicating that NEI modifies cAMP response [45].

Based on these results it is possible to hypothesize that NEI stimulates binding to D1 receptor and that this stimulation could be due to one of the two following situations: (a) NEI unmasks the binding sites by recycling or modulating receptors, but not via biosynthesis or degradation; (b) NEI interacts with membrane lipids causing a change in the lipid environment of the DA D1 receptor which activates ligand-binding to this receptor. In other words, it is clear that physicochemical studies are necessary to understand the behavior of NEI at a membrane level (these studies are currently in course).

3.5. *Effect of NEI on middle-striatum brain co-cultures*

NEI and NGE have epitopes that are recognized by antibodies against α-MSH. In the case of NEI, the peptide has a very similar sequence to α-MSH: Pro-Ile-NH_2 for NEI compared to Pro-Val-NH_2 for α-MSH. NEI neurons project to the caudate putamen and the cerebral cortex, and both areas lack pro-opiomelanocortin (POMC)-containing fibers. A study was carried out on 18-day gestation rat fetal neurons using striatal and mesencephalic-cultured cells, as the amidated form of NEI is secreted by hypothalamic neurons. Positive immunoreactivity of NEI and NGE to α-MSH antibodies suggests that these peptides might interact with cellular structures binding to α-MSH. The effect of NEI and NGE was measured by quantifying neurofilament proteins (NF), growth-associated proteins (GAP-43) and synaptophysin in the synaptosomal fraction as markers for neuritogenesis, growth cones and terminal neuronal differentiation. The results show that NEI increases neurofilaments and the production of synaptophysin: NGE is only active at the highest studied concentration, 2500 nM; however, NEI is active at 250 nM and increases NF by 22% and α-MSH by 9.5%. The α-MSH receptor is expressed in fetal neurons together with the MCH receptor. MC3-R RNAm disappears in the midbrain at approximately postnatal day 7. It is still unclear whether exogenous NEI affects NF via a melanocortin receptor or another receptor. The receptors for MCH are highly selective for MCH and are not activated by NEI, NGE or MCH-gene-overprinted-polypeptide (MGOP) [31].

3.6. *Interaction between NEI, α-MSH and MCH*

Using mouse B16 melanoma cells, Hinterman et al. [28] studied possible molecular interactions between α-MSH, MCH, NEI and NGE at the level of the pigment cell MCH receptor subtype and different melanocortin (MC) receptors. Mouse B16 melanoma cells express either MCH-R or MC1-R. Interaction with other subtypes of melanocortin receptors was studied with MC-Rs transiently expressed in COS cells. These cells express MC3-R, MC4-R and MC5-R, but neither MCH, NEI nor NGE caused a significant displacement of [^{125}I]α-MSH. However, doses between 100 and 10 nM MCH, NEI and NGE induced the formation of cAMP and melanin synthesis that could be blocked by agouti protein or by inhibiting adenylate cyclase or protein kinase A. These experiments show that mammalian MCH-precursor derived peptides can mimic MSH signaling via MC1-R activation at relatively high but still physiologically relevant doses, as observed in other autocrine/paracrine signaling mechanisms [28].

Work done by Gonzalez et al. [22] demonstrated that NEI injections in the ventromedial nucleus (VMN) stimulates exploratory activity, increases anxiety and reduces DA and DOPAC release, but has no effect when injected in the MPOA. Both behavioral effects of NEI are blocked by α-MSH. NEI stimulates sexual behavior in the MPOA but not the VM, while in this latter area it antagonizes the stimulatory action of MCH. Whereas NEI exerts its effects mainly in the VMN, also inhibiting DA release in this area, MCH acts mainly in the MPO where it has an anxiogenic effect and antagonizes the

exploratory action of α-MSH. Additionally, MCH only affects amine release in the MPOA.

Based on the interrelation that exists between α-MSH, NEI and MCH on EGB and MA, Sanchez et al. [46] sought to determine the effects of MCH, NEI and α-MSH on the levels of cAMP in brain slices from male albino rats containing caudate-putamen and accumbens nuclei including their interactions with α-MSH. The authors showed that at low doses (0.6 μM), NEI and MCH do not modify total cAMP levels compared to control values (Kreb-Ringer-bicarbonate), but that at high doses (3.6 μM) these peptides are capable of increasing the content of cAMP. As previously demonstrated by Sanchez et al. [43] both high and low doses of α-MSH (0.6 and 3.6 μM) significantly increase cAMP compared to Krebs-Ringer-bicarbonate. MCH blocks the effect of low-α-MSH doses and, to a lesser degree, the effects of higher α-MSH doses, 3.6 μM. On the other hand, the addition of NEI to the α-MSH incubation does not induce any further effects. Simultaneous addition of NEI and MCH produced a slightly additive effect in the production of cAMP [43]. This research group also studied the effect of MCH/NEI in the striatum as well as their interaction with α-MSH on IP3 content and found that at low doses (0.6 μM) none of the peptides modified the IP3 content compared to the control slices. Several combinations of the peptides were also studied at a dose of 0.6 μM, but still had no effect on the IP3 content. However, when using the combination of NEI/MCH or of α-MSH/MCH at the highest dose (3.6 μM), the IP3 increase induced by MCH alone at this concentration was blocked.

4. Perspectives for the future

NEI has already shown to be very versatile. It is gratifying to work with it because it acts at different levels and in very diverse situations. This peptide may influence LH release or TSH inhibition and therefore could be related to fertility and be important in minimizing energetic needs. As it also acts in behavior, we believe that it probably has an important role in the functional regulation of both motor control and behavior, with an interaction between the neuropeptide and neurotransmitter. Furthermore, NEI increases neurofilaments and the production of synaptophysin and is also found in peripheral tissue and posterior hypophysis. Taking all this into account, there is still a broad range of studies to be carried out on this neuropeptide.

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