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TESE DE LIVRE-DOCÊNCIA

ESTUDOS SOBRE A CARACTERIZAÇÃO E LOCALIZAÇÃO
DO HORMÔNIO CONCENTRADOR DE MELANINA (MCH)
E DOS PEPTÍDEOS (NEI E NGE) DERIVADOS DO MESMO
PRÉPRO-HORMÔNIO NO SISTEMA NERVOSO CENTRAL
DO RATO

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RESUMO

O Hormônio Concentrador de Melanina (MCH) vem sendo estudado há muito tempo. Entretanto, somente em 1955, Kawauchi e colaboradores isolaram e seqüenciaram o MCH da hipófise de salmão. Em 1957, Pickford & Atz mostraram que este hormônio possuía a função oposta à do Hormônio Estimulante da Melanina (MSH). O MCH tem a função, em peixes teleósteos, de agregação dos grânulos de melanina, enquanto que em répteis e anfíbios foi descrita a função de dispersão. Ainda em peixes teleósteos, o MCH inibe a secreção do Hormônio Adrenocorticotrófico (ACTH) e do α MSH induzida pelo Fator Liberador de Corticotrofina (CRF) in vitro. Alguns mapeamentos foram feitos em Sistema Nervoso Central (SNC) de rato usando-se anticorpo anti-MCH de salmão (sMCH). Desses estudos surgiu uma parte da população neuronal na Zona Incerta (ZI) e na Área Hipotalâmica Dorsolateral (AHDL) marcada, entretanto, após testes de adsorção, foram encontrados neurônios que ainda permaneciam marcados, resultando dessa maneira, na suspeita da existência de um ou mais neuropeptídeos que deveriam possuir seqüências similares de aminoácidos.

Com o intuito de determinar as características do MCH de rato (rMCH), usamos os métodos de isolamento, caracterização, seqüenciamento, clonagem, hibridização in situ, imunohistoquímica para microscopia óptica e eletrônica e de produção de anticorpos policlonais gerados em coelho.

Dessa forma, através desses métodos, caracterizamos a presença de um prépro-hormônio concentrador de melanina, do qual, derivam o MCH, e dois outros neuropeptídeos, o NEI e o NGE. Utilizando-se os anticorpos específicos anti-rMCH e anti-rNEI, obtivemos um mapeamento da distribuição de axônios e células no SNC, que contém estes peptídeos, chegando finalmente, ao estabelecimento de um "marcador" para a AHDL do rato.

**ESTUDOS SOBRE A CARACTERIZAÇÃO E LOCALIZAÇÃO DO HORMÔNIO
CONCENTRADOR DE MELANINA (MCH) E DOS PEPTÍDEOS
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NO SISTEMA NERVOSO CENTRAL DO RATO**

I - INTRODUÇÃO

A Área Hipotalâmica Lateral (LHA*), algumas vezes chamada de núcleo hipotalâmico lateral (Geeraedts et al, 1990a e b) é delimitada no homem medialmente pelo trato mamilotalâmico e pela coluna anterior do fórnix, a borda medial da cápsula interna e a região subtalâmica formam o seu limite lateral. As porções rostral e caudal são estreitas, mas a tuberal é larga. No rato, ela é definida por um plano sagital através do qual o fórnix descendente atravessa o hipotálamo separando-a das regiões mediais (Bleier et al, 1979), entretanto, os mesmos autores citados anteriormente, preferiram em trabalho posterior também em rato, não especificar os limites da LHA (Bleier & Byne, 1985), reconhecendo apenas as suas subdivisões, como consequência de suas relações topográficas adjacentes à região medial, como seguem: pré-óptica, anterior, tuberal e posterior.

(*) As abreviações usadas nesta tese são as mesmas (obedecendo a nomenclatura em inglês) dos trabalhos que vêm a seguir, para que não surjam divergências.

A LHA contém vários grupos celulares, sendo inclusive descrita como a região de maior diversidade celular em um mesmo grupo anatômico e funcional dentro do sistema nervoso central (Krieg, 1932; Nauta & Haymaker, 1969; Veening et al, 1982; Carpenter & Sutin, 1983).

A LHA tem sido implicada em funções tais como: o processamento de informações sensitivas, a modulação de respostas somatomotoras (particularmente na expressão de comportamentos associados a fome e sede, agressão e reprodução); a mediação em determinados níveis da ativação cortical e a participação no controle da pressão arterial (Stevenson, 1969; Hitt et al., 1970; Saper et al, 1979; Siegel & Edinger, 1981; Swanson, 1987; Luiten et al, 1987; Karadi et al. 1990; Allen & Cechetto, 1992). Parada e colaboradores (1990), demonstraram o envolvimento da dopamina, quando injetada na LHA, na inibição da locomoção relacionada à procura por água e alimento. Schwartz e colaboradores (1990a e b), demonstraram a participação da serotonina e do triptofano no comportamento de procura de alimento, mediado pela LHA. Em 1990, Winn e colaboradores, deixaram clara a participação da LHA no comportamento alimentar, através de lesões desta área com N-metil-D-aspartato (NMDA). Alguns desses comportamentos citados anteriormente podem aparecer após estimulação elétrica, serem atenuados ou até mesmo abolidos através de lesão eletrolítica da LHA (Swanson, 1987).

No que diz respeito à função nos processos sensitivos, está bem estabelecido que pela técnica de registro celular único, a frequência de disparo de um neurônio da LHA pode ser influenciada por uma grande variedade de estímulos originados nos sistemas olfatório, gustatório, visual, auditivo e somatosensitivo, assim como da orofaringe, estômago, intestino e fusos neuromusculares (Brooks et al, 1962; Nicolaidis, 1969; Kawamura et al, 1970; Norgren, 1970; Jeanningros, 1984; Levine et al., 1991). A participação da LHA na esfera da organização de comportamentos, pôde ser relacionada através da síndrome de "negligência sensitiva", que aparece após grandes lesões da LHA (Marshall et al., 1974). Lesões da LHA podem trazer como sinais a hipoatividade ou acinesia (Levitt & Teitelbaum, 1975), e algumas vezes até a catalepsia (Schallert et al., 1978), enquanto que a estimulação elétrica de intensidade média pode provocar locomoção (Valenstein et al., 1969; Levy & Sinnamon, 1990).

O entendimento de como funções tão diferentes podem ser organizadas e diferentemente controladas tem sido árduo, por ser a LHA um grupo de células atravessada por um importante feixe conector, o feixe prosencefálico medial (MFB), por isso a LHA é considerada o núcleo intersticial do MFB (Nieuwenhuys et al, 1982; Veening et al, 1982; Veening et al., 1987).

Dessa maneira, os resultados de estudos nos quais se tem procurado localizar funções particulares da LHA, através de estimulação convencional ou métodos de lesão, têm sido de difícil interpretação, pois não está claro se essas manipulações não afetariam também células e axônios não relacionados com o sistema estudado, e que apenas estariam passando pela região.

Para o estudo das aferências, deveremos levar em consideração, justamente o problema das fibras-de-passagem, pois, a este problema, devemos imputar a quase ausência de estudos das aferências oriundas do tronco encefálico, assim como dos neurotransmissores envolvidos.

As **aferências** do prosencéfalo para a LHA provêm das seguintes estruturas: núcleo olfatório anterior, área pré-límbica (Hurley et al., 1991), córtex agranular insular (Allen et al., 1991), núcleo septal lateral, núcleo intersticial da estria terminal (BST), núcleo central da amígdala, núcleo accumbens, substância inominada, subículo ventral, núcleo posterior medial da amígdala e zona medial do hipotálamo (Swanson, 1987); Jones & Moore (1977) descreveram o locus coeruleus como um dos locais de aferência da LHA, originadas no tronco encefálico. Entretanto, mais aferências do tronco encefálico ainda devem ser estudadas e seus neurotransmissores determinados.

Quanto às **eferências**, poderemos dividi-las por territórios, no intuito de facilitar a leitura. Como segue:

1 - O córtex cerebral: através de estudos combinados entre transporte retrógrado e histoquímica, ficou evidente que mais de 90% das células na LHA que projetam-se para a formação hipocampal, córtex pré-frontal e para o bulbo olfatório, contêm acetilcolinesterase e α MSH (alfa hormônio estimulante de melanina) (DeOlmos et al., 1978; Kohler et al, 1984; Kohler & Swanson, 1984). Entretanto, parece não haver camada cortical preferencialmente atingida pelos terminais originados da LHA (Kohler & Swanson, 1984), apesar de que Saper (1985), sugere que deve existir de uma maneira não muito definida a inervação preferencial de um grupo de neurônios de uma ou outra região cortical.

Evidências imunohistoquímicas demonstram que três grupos de neurônios no hipotálamo originam um número muito grande de fibras que inervam difusamente todo o córtex cerebral. Estes grupos são: **grupo 1**, neurônios α MSH e acetilcolinesterase imunorreativos (-ir), na LHA, núcleo dorsomedial e zona incerta (ZI); **grupo 2**, neurônios imunorreativos à decarboxilase do ácido glutâmico (GAD), encefalina, histamina e substância P, no núcleo tuberomamilar; **grupo 3**, neurônios imunorreativos à colecistoquinina (CCK) e peptídeo vasoativo intestinal (VIP), no núcleo supra-mamilar (Swanson, 1987). Apesar do significado funcional destas projeções não estar bem estabelecido, o hipotálamo como um todo envia o maior contingente de fibras não talâmicas ao córtex cerebral (Swanson, 1987).

2 - A região septal e amígdala: estas duas regiões telencefálicas recebem uma projeção importante da LHA, isto ficou provado por estudos de autoradiografia e por injeções de peroxidase do rábano silvestre (HRP). Entretanto, a LHA envia fibras de neurotransmissores ainda não determinados para o BST (Swanson, 1976; Saper et al, 1979), para o núcleo septal lateral (Swanson, 1976) e para o complexo núcleo septal medial/núcleo da banda diagonal de Broca (Swanson, 1976; Saper et al, 1979; Berk & Finkelstein, 1982). Embora a função destas projeções permaneça incerta, a existência de uma maciça aferência para o hipotálamo, sugere que a relativa esparsa projeção na direção oposta, atue na capacidade de modulação do fluxo de informação descendente através da região septal. Quanto a amígdala, três subdivisões dela recebem impulsos da LHA: o núcleo cortical anterior (para modulação do fluxo de informações olfatórias); o núcleo central (para modulação das projeções deste núcleo aos núcleos autônomos do tronco encefálico) e o núcleo basolateral da amígdala (o qual tem conexões bidirecionais com várias partes do córtex cerebral) (Swanson, 1976; Ottersen, 1980).

3 - Os núcleos da base: as projeções das áreas pré-óptica lateral e hipotalâmica lateral (Swanson, 1976; Saper et al, 1979), para os núcleos da base, parecem estar restritas à substância inominada (SI), esta região também é chamada de "pallidum ventral" por Heimer & Wilson (1975), e consiste de pelo menos dois componentes: um grupo de neurônios colinérgicos que se projetam para o córtex cerebral e corresponde à porção compacta do núcleo em primatas (Jones et al, 1976; Mesulam et al, 1983), e outro grupo de neurônios de projeções descendentes, os quais são similares àqueles do "pallidum dorsal", para os núcleos medianos do tálamo (Swanson et al, 1984; Young et al, 1984), para a ZI (Mogenson et al, 1985) e para o núcleo pedunculopontino (Swanson et al, 1984). As projeções descendentes da SI têm recebido atenção, porque ela recebe uma maciça e topograficamente organizada aferência do núcleo accumbens (Swanson et al, 1984). Existe uma substancial evidência implicando as vias do núcleo accumbens para a SI e para a área pré-óptica lateral adjacente, e daí para a ZI caudal e núcleo pedunculopontino, na modulação da atividade motora, particularmente no que diz respeito ao comportamento exploratório (Mogenson et al, 1985; Brudzynski & Mogenson, 1985).

4 - O tálamo: a zona lateral do hipotálamo inerva partes específicas do epitálamo, tálamo dorsal e subtálamo, embora o neurotransmissor e a distribuição precisa das células que dão origem a estas projeções não tenham ainda sido estabelecidos. É conhecido, entretanto, que neurônios da parte rostral da LHA projetam-se para o núcleo habenular lateral (Herkenkam & Nauta, 1977; Berk & Finkelstein, 1982), para o núcleo paraventricular do tálamo, para o núcleo reuniens e para a ZI (Swanson, 1976; Saper et al, 1979), assim como para os núcleos intralaminares (Berk & Finkelstein, 1982). O significado funcional destas projeções para o tálamo só poderá ser discutido de forma generalizada. O núcleo habenular lateral tem preferencialmente uma projeção difusa que trafega no seu início através ou perto do trato habenulo-interpeduncular e inerva estruturas medianas desde o núcleo dorsal da rafe caudalmente, até a zona lateral do hipotálamo rostralmente (Herkenkam & Nauta, 1979).

A estria medular, que envia impulsos para a habenula, e o trato habenulo-interpeduncular têm freqüentemente sido vistos como o componente dorsal do MFB, que se origina dos núcleos posteriores da região septal (Swanson & Cowan, 1979) e dos 2/3 rostrais da zona lateral hipotalâmica.

O significado funcional da conexão da LHA com os núcleos reuniens, paraventricular do tálamo e paratenial, são ainda mais problemáticos. Entretanto, estes núcleos da linha mediana do tálamo são conhecidos por projetarem-se para o córtex pré-frontal, formação hipocampal, amígdala, região septal e núcleo accumbens (Jones & Leavitt, 1974; Swanson & Cowan, 1975; Beckstead, 1978; Wyss et al, 1979; Luiten et al, 1982; Kelley & Stinus, 1984). Isto sugere, portanto, que estes núcleos constituem grupos celulares talâmicos que projetam-se para porções límbicas do córtex cerebral e núcleos da base, que são conhecidos por não receberem impulsos dos grupos talâmicos anteriores e do núcleo mediodorsal do tálamo. Impulsos para esses três núcleos não estão certamente restritos à região lateral do hipotálamo, embora outras fontes adicionais de aferências estejam todas associadas ao sistema do MFB ou fórnix. Estas outras fontes incluem o subículo ventral (Krettek & Price, 1978), o complexo núcleo septal medial/núcleo diagonal da Banda de Broca, o BST (Swanson, 1976) e a substância inominada (Mogenson et al, 1985) no telencéfalo; no hipotálamo, o núcleo arqueado (Krieger et al, 1979), o núcleo supraquiasmático (Berk & Finkelstein, 1981), o núcleo ventromedial (Saper et al, 1976) e o núcleo anterior do hipotálamo (Saper et al, 1978); o núcleo parabraqial (Saper & Loewy, 1980), o núcleo do trato solitário (NTS) (Ricardo & Koh, 1978), e a região A1 (McKellar & Loewy, 1982) no tronco encefálico.

Em neurônios do núcleo supraquiasmático, apenas dois neurotransmissores, a vasopressina e o VIP (Hoorneman & Buijs, 1982; Watts & Swanson, 1987), parece projetarem-se para o núcleo paraventricular do tálamo, enquanto fibras noradrenérgicas na LHA (Swanson & Hartman, 1975), provavelmente têm origem na região A1 e no NTS, o chamado grupo A2.

5 - O hipotálamo: os resultados de estudos de autoradiografia (Swanson, 1976; Saper et al, 1979; Berk & Finkelstein, 1982) sugerem que virtualmente todas as partes da região medial e periventricular recebem impulsos da região lateral. Entretanto, a organização topográfica e a especificidade de neurotransmissores dessas projeções são atualmente desconhecidas, e parece que muitos grupos celulares nas porções mediais do hipotálamo recebem preferencialmente eferências da região lateral.

Após algumas injeções de traçador retrógrado fluorescente no núcleo paraventricular do hipotálamo foram encontradas células marcadas na LHA, sem ter sido notada, entretanto, uma distribuição topográfica (Sawchenko & Swanson, 1983).

6 - O tronco encefálico: os estudos de transporte axonal indicam que a zona lateral do hipotálamo inerva mais de vinte regiões do tronco encefálico, a maioria das quais envia fibras ascendentes através e talvez de volta, para a zona lateral, e é provável que fibras descendentes dêem origem a botões terminais ou sinapses "en passant" ao longo de seu curso para a medula espinhal. De modo que, essas fibras tomam dois trajetos: um através da área hipotalâmica posterior e substância cinzenta periaqueductal até o núcleo parabraquial, e outro pela parte mais ventral da formação reticular.

Para descrever as eferências para o tronco encefálico, seria conveniente dividi-las em 4 grupos :

- **1º grupo**, constituído de núcleos associados aos núcleos de nervos cranianos, o glossofaríngeo e o vago. Estes núcleos incluem o NTS (Saper et al, 1979; Hosoya & Matsushita, 1981), a região A1 (Hosoya & Matsushita, 1981) e o núcleo parabraquial (Saper et al, 1979; Touzani et al., 1990). Todos esses núcleos servem de estações "relé" de informação sensitiva visceral para o prosencéfalo, assim como o núcleo motor dorsal do nervo vago (Hosoya & Matsushita, 1981), que primeiramente inerva as vísceras abdominais. Seria bom mencionar que células da LHA também inervam os núcleos salivatórios (Hosoya et al, 1983), e que juntamente com o núcleo motor dorsal do nervo vago, parecem constituir a única e direta eferência para os núcleos de nervos cranianos, estabelecida pela LHA.

- **2º grupo**, consiste de regiões da LHA que são usualmente consideradas como desempenhando um importante papel no controle somatomotor, e que enviam fibras para a "pars compacta" da substância negra, e para a área tegmental ventral (Swanson, 1976; Saper et al, 1979), para a "pars reticulata" da substância negra (Swanson, 1976; Saper et al, 1979), para as camadas profundas do colículo superior (Tortelly & Reinoso-Suarez, 1980; Berk & Finkelstein, 1982; Mogenson et al, 1985), para o colículo inferior através de neurônios CRF-ir (Sawchenko & Swanson, 1989), para o núcleo pedunculopontino (Swanson, 1976; Saper et al, 1979, Mogenson et al, 1985) e para o cerebelo (Dietrichs, 1984).
- **3º grupo**, não é tão conhecido do ponto de vista funcional, todavia, recebe uma substancial eferência da LHA como um todo, este grupo consiste do hipotálamo posterior e da substância cinzenta periaqueductal, assim como de alguns pequenos grupos celulares do mesencéfalo e da formação reticular pontina (Swanson, 1976; Saper et al, 1979; Hosoya & Matsushita, 1981).

Pouco pode ser dito sobre essas vias, exceto por especulação, que elas podem influenciar a atividade do Sistema Ativador Reticular Ascendente, nas projeções reticulares bulbares ou retículo-espinhal.

- **4o grupo**, o grupo de estruturas do tronco encefálico que recebe eferências da LHA, consiste do locus coeruleus (Swanson, 1976; Saper et al, 1979) e dos núcleos da rafe (dorsal, mediano, obscuro, magno e pálido) (Swanson, 1976; Saper et al, 1979; Hosoya & Matsushita, 1981). Não existem evidências até o momento, que nos levem a conclusões funcionais.

7 - **A medula espinhal**: Kuypers & Maisky (1975) foram os primeiros a mostrar inequivocamente que as células da LHA projetam-se diretamente para a medula espinhal (Sp.Cd.) no gato, e isto tem sido confirmado em uma grande variedade de espécies, incluindo o rato. As fibras estendem-se por todo comprimento da Sp.Cd., até mesmo no filamento terminal (Swanson & McKellar, 1979). As fibras que se destinam a Sp.Cd., chegam a inervar a coluna intermédio-lateral e a substância gelatinosa (Hosoya & Matsushita, 1983) e também as fibras α MSH-ir da LHA estabelecem um difuso campo terminal por toda substância cinzenta (Kohler et al, 1984). Cechetto e Saper (1988), descreveram 4 pequenos grupos de neurônios imunorreativos a diferentes peptídeos, localizados na LHA ou em regiões muito próximas, que originam a inervação da Sp.Cd., que são: neurônios α MSH, localizados na área hipotalâmica lateral porção tuberal e na área retroquiasmática; no núcleo perifornical neurônios ANF-ir (Fator Natriureico Atrial) e DYN-ir (dinorfina); neurônios TH-ir (tirosina-hidroxilase) na área hipotalâmica dorsal.

Um fato interessante é que alguns desses neurônios α MSH-ir enviam colaterais para a Sp.Cd. e para o córtex cerebral, aonde eles estabelecem também uma difusa, embora pobre, terminação laminar (Kholer et al., 1984). Dessa forma, as evidências anatômicas, até agora, sugerem que projeções diretas da LHA podem influenciar uma variedade de mecanismos espinhais, incluindo a informação sensitiva, a aferência de motoneurônios somáticos e de neurônios pré-ganglionares.

Lind & Ganten (1990) citando os seus dados e os de outros autores, descreveram a presença de vários pequenos grupamentos de neurônios na LHA imunorreativos a: angiotensina, α MSH, dinorfina, somatostatina, encefalina, neurotensina, substância P, CRF e fator natriurético atrial. Entretanto, em nenhum desses grupos houve uma marcação extensiva da LHA, a ponto de podermos chamar algum desses peptídeos de "marcador" da LHA.

Como ficou evidenciado por tudo o que foi descrito anteriormente, a LHA foi bem estudada hodologicamente, entretanto, com métodos compatíveis para as épocas em que foram realizados, e sem possuir a vantagem, ainda, até o início de nossos estudos, de um "marcador" específico, ou seja, não havia sido descrita até aquela época nenhuma substância neuroativa que marcasse preferencialmente aquela região do sistema nervoso central do rato, ao contrário, por exemplo, do sistema magnocelular hipotalâmico (principalmente os núcleos paraventricular e supra-óptico) que são identificados pela imunolocalização de vasopressina e oxitocina.

Em 1957, Pickford & Atz mostraram claramente a existência do Hormônio Concentrador de Melanina (MCH) que funcionalmente era antagônico ao Hormônio Estimulante da Melanina (MSH). Desde então, a literatura vem mostrando uma crescente preocupação na determinação exata das características bioquímicas do MCH, através do isolamento, caracterização, seqüenciamento e clonagem do gene do prépro-hormônio do MCH, assim como de candidatos a peptídeos (Vaughan et al., 1989; Risold et al., 1992) derivados deste prépro-hormônio com a função de neurotransmissores ou neuromoduladores.

Os primeiros a purificar e seqüenciar o MCH foram Kawauchi e colaboradores em 1983, usando hipófise de salmão, demonstrando ser ele um heptadecapeptídeo. Anticorpos contra o MCH de salmão (sMCH) foram então usados e mostraram neurônios secretores no hipotálamo basal de salmão (Naito et al., 1985).

Em um dos estudos realizados com anticorpo anti-sMCH encontraram-se células marcadas na LHA e ZI do rato (Fellmann et al., 1987), que posteriormente foram identificadas como imunorreativas também ao α MSH, ao CRF (fator liberador de corticotropina) e ao fator liberador de hormônio de crescimento humano (hGRF) (Kawano et al., 1988). Watson e Akil (1979), usando anticorpo anti- α MSH, demonstraram a presença de neurônios α MSH-ir na LHA e Zi e previram a existência de um peptídeo desconhecido, ao qual deram o nome de antígeno a-2, que teria o C-terminal similar ao α MSH.

Novamente Cechetto e Saper em 1988 suspeitaram da presença de um outro peptídeo na LHA, quando afirmaram "...é provável, entretanto, que eles (neurônios) contenham uma reação cruzada com um peptídeo- α MSH-semelhante, ao invés de um autêntico α MSH".

A obtenção de anticorpos mais específicos também era motivo de disputa (Skofitsch et al., 1985; Naito et al., 1988), pois alguns mapeamentos realizados no Sistema Nervoso Central (SNC) do rato foram feitos com anticorpo anti-sMCH gerado em coelho (Naito et al., 1985). Deste modo, havia dúvidas quanto aos estudos que descreviam a distribuição das células e terminais nervosos MCH-imunorreativos no SNC de roedores e que utilizavam anticorpos gerados contra sMCH.

II - OBJETIVO

Ao iniciarmos nossos estudos em 1988 e trabalhando com dois grupos (liderados por Paul E. Sawchenko e Wylie W. Vale) do "Salk Institute for Biological Studies", em La Jolla, Califórnia - EUA, nosso objetivo era tentar isolar, caracterizar, seqüenciar e clonar o prépro-MCH do rato (rMCH), estudar a possibilidade de existência de outros possíveis peptídeos codificados pelo mesmo prépro-hormônio, assim como obter os respectivos anticorpos anti-rMCH e anti-rNEI gerados em coelho. Uma vez cumprida esta etapa, nossa meta, no Brasil, passou a ser o estabelecimento de uma linha de pesquisa que poderia se enquadrar sob o título de "ORGANIZAÇÃO ANATÔMICA DE UM NOVO SISTEMA PEPTIDÉRGICO NO HIPOTÁLAMO", onde o objetivo principal é o estudo neuroanatômico (hodológico e bioquímico) deste sistema.

III - MATERIAL E MÉTODOS

O material e métodos usados nos três trabalhos aqui apresentados serão a seguir brevemente comentados. Embora em cada um dos trabalhos a metodologia esteja descrita detalhadamente, achamos por bem comentar alguns pontos relevantes.

1 - Animais

Foram usados ratos albinos machos adultos jovens (Sprague-Dawley - pesando entre 125 e 300 g), e ratos pigmentados machos adultos jovens (Long-Evans - pesando entre 125 e 150 g). Usamos estes últimos animais pensando em uma diferença de imunorreatividade entre ratos albinos e pigmentados, pois já que estávamos lidando com o MCH, poderíamos encontrar divergências entre as respectivas linhagens. Todos os animais foram sempre mantidos em duplas nas gaiolas e em biotério, com água e comida (ração) ad libitum, e em ciclos de claro e escuro de 12/12 h.

2 - Isolamento e Seqüenciamento do rMCH

Para proceder o isolamento e seqüenciamento do rMCH foram utilizados 60.000 fragmentos liofilizados de hipotálamos de rato. A extração do conteúdo peptídico dos hipotálamos deu-se por homogeneização em meio ácido a quente (com esse procedimento as enzimas responsáveis pela degradação dos hormônios peptídicos são inativadas).

A seguir, esse homogenato foi resfriado e centrifugado, e da fração sobrenadante foram extraídos os compostos lipossolúveis com múltiplas lavagens com éter e éter de petróleo. Seguindo, a fração aquosa foi utilizada para o isolamento do rMCH.

Como primeiro passo cromatográfico de purificação utilizamos a filtração em gel em larga escala. Este procedimento cromatográfico fundamenta-se na separação pelas diferenças de peso molecular (podemos dizer que esta separação se dá pelas diferenças de raio que os diferentes peptídeos ou proteínas possuem). Para tal, foi empregada uma coluna (20 x 106 cm) empacotada com 85 cm de Sephadex G-50 e com 5 cm de Sephadex G-10 no topo (volume total 33 litros). Esse último procedimento (a colocação de Sephadex G-10 no topo da coluna) mostrou-se eficiente para separar melhor o rMCH de outros peptídeos que não nos interessavam. Todo o seguimento do rMCH foi realizado por radioimunoensaio (RIA), utilizando um anticorpo anti-sMCH produzido em coelhos. Depois de agrupar as frações contendo rMCH provenientes da filtração em Sephadex G-50, prosseguimos com a purificação utilizando uma coluna de imunoafinidade. Resumidamente, a coluna foi preparada utilizando proteína-A Sepharose CL-4B e imunoglobulinas de coelho anti-sMCH. O anticorpo foi covalentemente ligado à proteína-A usando-se dimetilpimelimidato dihidroclorato (Schneider et al, 1982).

Prosseguimos lavando a matriz anticorpo-proteína-A duas vezes com ácido acético 1N e equilibrando com 50 mM de NaHEPES, pH 7,5 e 150 mM NaCl. Misturamos então, a fração contendo rMCH proveniente da filtração em gel e a matriz da coluna de afinidade (anticorpo-proteína-A-Sepharose CL-4B), e deixamos sob agitação durante uma noite.

No dia seguinte empacotamos e lavamos extensivamente para remover ligações inespecíficas com o tampão de equilíbrio (50 mM de NaHEPES, pH 7,5 e 150 mM de NaCl). O material especificamente absorvido foi então eluído com ácido acético 1N. A seguir o material proveniente da cromatografia de imunoafinidade foi submetido à cromatografia líquida (FPLC, do inglês "fast protein liquid chromatography"), usando duas colunas de Superose 12B em série. Este procedimento permite separar peptídeos ou proteínas em função das diferenças no peso molecular (mesmo princípio da filtração em gel em larga escala), entretanto com uma eficiência e reprodutibilidade extremamente superiores.

A região ativa proveniente deste último passo (FPLC), foi revelada por RIA, concentrada por "speed-vac", e submetida a uma outra cromatografia líquida, a HPLC (do inglês, "High Performance Liquid Chromatography"), onde o princípio de separação é baseado na hidrofobicidade dos peptídeos, ou seja, nas diferenças de interação dos peptídeos com a coluna utilizada e as diferentes misturas de solventes polar/apolar.

Para isso foi usada uma coluna C18, fluxo de 0,25 ml/min , usando como solventes, 0,05% de ácido trifluoroacético (TFA) em H₂O (solvente A), 0,05% de TFA em 90% de acetonitrila e 10% H₂O (solvente B). A amostra foi injetada a 0% do solvente B em eluição isocrática por 5 minutos, seguido de um gradiente linear de 0 a 50% do solvente B em 40 min e 5 min de eluição isocrática com 50% do solvente B. Neste caso, as frações foram coletadas manualmente em função da absorbância a 210 nm. Estas alíquotas foram analisadas por RIA e as regiões de imunorreatividade agrupadas.

Após a concentração as amostras foram reaplicadas ao HPLC numa coluna C18 de fase reversa e separadas com fluxo de 0,125 ml/min usando os mesmos solventes descritos anteriormente num gradiente linear de 0 a 36% do solvente B em 96 min. Novamente as frações foram coletadas manualmente baseadas na absorbância 210 nm e as alíquotas ensaiadas por RIA.

As regiões de mais alta imunorreatividade foram seqüenciadas pelo método da degradação de Edman, e a estrutura primária determinada : **Asp-Phe-Asp-Met-Leu-Arg-Cys-Met-Leu-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Gln-Val**.

Os hormônios peptídicos, rMCH e sMCH, foram sintetizados pelo método de síntese em fase sólida e purificados por HPLC de fase reversa (pureza estimada maior que 95%).

3 - Síntese e Purificação de rMCH e sMCH

Na caracterização dos peptídeos foram utilizadas as técnicas de rotação óptica, sendo $-24,2^{\circ}$ para o rMCH, e $-10,6^{\circ}$ para o sMCH, a análise da composição de aminoácidos mostrou-se de acordo com o esperado, na espectrometria de massa o íon molecular do rMCH apresentou 2386.1 u.m.a. (unidade de massa atômica), e por HPLC analítico de fase reversa, em condição isocrática, foi observado que o rMCH é mais hidrofóbico que o sMCH, como indicado pelos tempos de retenção que é explicado pelas seqüências dos peptídeos.

4 - Imunohistoquímica

4.1 - Imunohistoquímica

Para o estudo imunohistoquímico foram usados animais intactos e também aqueles que foram submetidos previamente (48 a 72 h antes da perfusão) ao tratamento com colchicina; visto que a colchicina é uma droga que causa a ruptura de microtúbulos, portanto, paralisando o fluxo axoplasmático, ela foi usada como um método coadjuvante para concentrar o máximo possível do peptídeo no citoplasma e dendritos dos neurônios, realçando assim não só a reação imunohistoquímica, como também evidenciando neurônios imunorreativos que sem a colchicina não apareceriam.

Outros dois artifícios foram utilizados visando escolher a melhor maneira de visualizarmos todos os aspectos desse novo sistema. O primeiro deles foi a escolha do fixador (pois sabemos que para peptídeos diferentes devem ser usados fixadores diferentes), os melhores resultados foram obtidos com o fixador a 4% de paraformaldeído em tampão borato 0.1 M e o fixador de Zamboni; o segundo foi o tempo de pós-fixação, onde encontramos excelentes resultados com tempos variando entre 0 e 2 h, pois tempos maiores mostraram-se prejudiciais à boa visualização principalmente de fibras imunorreativas ao MCH/NEI. Foram usados os métodos Complexo Avidina-Biotina (ABC) para localização de pericários e terminais nervosos e de imunofluorescência para os estudos de co-localização.

4.2 - Imunohistoquímica seqüencial dupla

Este método, chamado de eluição, foi usado para estabelecermos em que extensão os neurônios que expressavam um determinado peptídeo, também expressavam outro peptídeo. Para esta finalidade usamos o método de Tramu et al. (1978) modificado, que permitiu colocarmos os cortes em contato com um primeiro anticorpo primário (por exemplo, anti-rMCH), em seguida o anticorpo secundário e logo após, foram feitas fotografias dos campos em que eram visualizadas células imunorreativas, a seguir estes anticorpos eram retirados e os mesmos cortes submetidos à incubação de um segundo anticorpo primário (por exemplo, anti-rNEI), novamente

fosfato 0,1 M de pH 7,4, seguido de outro fixador a 2% de paraformaldeído no mesmo tampão. O tempo usado para esta perfusão era de 25 min e a perfusão prévia com fixador de acroleína mostrou-se como a melhor para microscopia eletrônica.

4.5 - Anticorpos e controles

Os anticorpos anti-rMCH foram gerados em coelhos (três para cada peptídeo). Estes animais foram imunizados com peptídeos sintéticos conjugados a α -globulina humana e glutaraldeído. Três outros animais foram imunizados contra Tyr-Gly-rNEI conjugados à mesma proteína e benzidina-bis-diazotizada. A conjugação do peptídeo a uma proteína de maior peso molecular é necessária para tornar o peptídeo antigênico. Assim, anticorpos reconhecendo o complexo proteína-peptídeo são gerados e, a posteriori, pode-se separar os anticorpos gerados exclusivamente para a região do peptídeo. Para isso, todos os antisoros foram pré-adsorvidos com α -globulina humana antecedendo o uso. Apesar de todos os anticorpos terem marcado neurônios na LHA, apenas os anticorpos PBL-234 (MCH) e PBL-237 (NEI) mostraram-se os mais efetivos desde o início e durante todo o trabalho.

4.6 - Teste de adsorção

Uma vez que a LHA e a ZI exibem grupos de neurônios que expressam α MSH, rCRF e hGRF, os quais possuem seqüências de aminoácidos similares aos peptídeos MCH, NEI e NGE, codificados pelo prépro-MCH, usamos o teste de adsorção para determinar a especificidade do anticorpo. Esse teste foi conduzido, colocando-se juntos os peptídeos e anticorpos homólogos e heterólogos em uma solução e em concentrações crescentes em contato com os cortes histológicos.

5 - Hibridização in situ

Esta técnica, atualmente uma das mais modernas e aplicadas em Biologia Molecular (aliás, a base dela), ajuda no campo da Neurociência a responder importantes perguntas, no que diz respeito à determinação com exatidão, de qual substância neuroativa é expressa nos neurônios sob determinada investigação. Experimentos de hibridização permitem aos pesquisadores identificarem fragmentos de DNA, moléculas recombinantes de DNA e RNAs com seqüências específicas de bases. Tal identificação depende do uso de "probes" ou sondas derivadas da seqüência do DNA em estudo. A obtenção de sondas pode ser através de síntese de oligonucleotídeos sintéticos ou síntese de cDNA ou cRNA in vitro, utilizando-se enzimas RNA ou DNA polimerase e o próprio DNA de interesse como matriz. Além disso, oligonucleotídeos sintéticos poderão ser utilizados, embora com menor especificidade.

a - Preparação das sondas : o vetor de transcrição foi construído inserindo-se o fragmento de cDNA obtido por digestão do vetor pRMCH11 (Nahon et al., 1989 - vide trabalho 2), com as enzimas de restrição EcoRI e AccI, em Bluescript SK (Stratagene, California). Esse fragmento continha a região codificante do ppMCH (495 pb - pares por base) e as regiões 3' (192 nucleotídeos) e 5' (41 nucleotídeos). O vetor Bluescript foi escolhido por conter os promotores para T7 e T3 RNA polimerases ladeando a sequência inserida. Isto permitiu a síntese das sondas "senso", utilizando-se linearização com a endonuclease SmaI e T3 RNA polimerase, e "anti-senso", utilizando-se HINDIII e T7 RNA polimerase, ambas as sondas marcadas com ^{35}S -UTP α S (uridina tri-fosfato alfa enxofre).

b - Hibridização in situ : o tecido foi processado como já descrito para imunohistoquímica (item 4.1 , perfusão com paraformaldeído a 4%, sem tratamento prévio com colchicina). Os cortes foram montados em lâminas recobertas por poli-lisina e submetidos à hibridização durante uma noite a 56-58°C. A solução de hibridização continha as sondas a uma concentração de 10^7 cpm/ml, numa solução de 50% formamida, 0,3 M NaCl, 10 mM Tris (pH 8,0), 1 mM EDTA, 0,05% tRNA, 10 mM dithiothreitol, solução 1x Denhardt's e 10% de dextran sulfato. Após esse período os cortes foram tratados com ribonuclease A (20 $\mu\text{g/ml}$) por 30 min a 37°C para digestão de todo RNA não hibridizado, e lavados com NaCl 15 mM/citrato de sódio 1,5 mM, numa temperatura de 55-60°C, até a queda máxima possível da radiação detectável medida por contador.

Os cortes foram então tratados com xileno para remoção de lípidos, lavados em etanol absoluto, secos ao ar livre e recobertos com emulsão fotográfica KODAK MTB-2. Após exposição por tempo adequado (4 dias a 3 semanas) a 4°C e em ambiente seco, as lâminas foram reveladas com revelador Kodak D-19 (3,5 min a 14°C), lavadas rapidamente em água destilada, fixadas com fixador rápido Kodak (2 min a 14°C), e lavadas no mínimo por 1 hora em água destilada. Cortes da série adjacente foram corados por tionina, para serem usados como referência citoarquitetônica.

- OS RESULTADOS, DISCUSSÃO E CONCLUSÕES PODERÃO SER EXAMINADOS MAIS DETALHADAMENTE, NAS CÓPIAS DOS TRÊS TRABALHOS PUBLICADOS QUE VÊM A SEGUIR, REFERIDOS COMO ANEXOS A, B e C.

1 - Nahon, J-L.; Presse, F.; Vaughan, J.; Fischer, W.; **Bittencourt, J.C.**; Hoeger, C.; Schoepfer, R.; Rivier, J.; Sawchenko, P.E. and Vale, W. (1989): Characterization of mammalian melanin-concentrating hormones and their precursors. In F.F. Casanueva and C. Dieguez (eds.): **Recent Advances in Basic and Clinical Neuroendocrinology**. Amsterdam: Ex. Med. Int. Congress Series, Elsevier Publishers B.V., pp 15-23.
(ANEXO A)

2 - Nahon, J-L.; Presse, F.; **Bittencourt, J.C.**; Sawchenko, P.E. and Vale, W. (1989): The rat melanin-concentrating hormone messenger ribonucleic acid encodes multiple putative neuropeptides coexpressed in the dorsolateral hypothalamus. **Endocrinology** 125, 2056-2065.
(ANEXO B)

3 - **Bittencourt, J.C.**; Presse, F.; Arias, C.; Peto, C.; Vaughan, J.; Nahon, J-L.; Vale, W. and Sawchenko, P.E. (1992): The melanin-concentrating hormone system of the rat brain: an immuno- and hybridization histochemical characterization. **J. Comp. Neurol.** 319, 218-245.
(ANEXO C)

IV - REFERÊNCIAS BIBLIOGRÁFICAS

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ANEXOS

CHARACTERIZATION OF MAMMALIAN MELANIN CONCENTRATING HORMONES AND THEIR PRECURSORS

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INTRODUCTION

In teleosts, melanin dispersion is under dual regulation by melanin stimulating hormone (MSH) and Melanin Concentrating Hormone (MCH). Although the existence of a principle opposing MSH had been proposed earlier for amphibians (1) yet not substantiated, in 1955 Enami (2) demonstrated the presence of both MSH and MCH like activities in the pituitary and hypothalamus of a teleost. Baker and colleagues (3)(4) were able to chemically separate the two activities and established that pituitary MCH was of hypothalamic origin. In 1955, Kawauchi, Baker and associates were able to isolate and determine the sequence of salmon MCH as a heptadecapeptide with a disulfide bridge forming a 10 amino acid ring. Immunohistochemical experiments with anti-salmon MCH sera revealed the presence of MCH containing perikarya in the teleost ventral hypothalamus with projections to the pituitary where fibers terminate on capillaries and in some species directly on corticotropic and melanotropic cells (5). Salmon MCH is a potent inhibitor of CRF induced ACTH and alpha MSH secretion by teleost pituitary glands incubated *in vitro* and is also reported to have a similar, albeit, weaker activity on the rat pituitary (6) (7). Addition of anti-MCH serum to incubated fish pituitary glands increases the spontaneous release of MSH. Chronic administration of MCH to the rainbow trout caused melanin concentration and paling and suppression of plasma MSH levels (7). The role of MCH as an inhibitor of ACTH secretion is also supported by findings that eels transferred from a black to a white tank, e.g. under a condition presumed to be associated

with the increased production of MCH, the release of ACTH and cortisol in response to moderate stressors is attenuated (8).

Antisera directed towards synthetic salmon MCH detected MCH like molecules in the brains of various vertebrate species including rats and human beings (9) (10) (11) (12) (13). Rat hypothalamic extracts and partially purified fractions were reported to possess MCH-like bioactivity on melanin dispersal in teleost melanocytes as well (14) (15). An extensive system of MCH positive cell bodies are located in the dorsolateral hypothalamus and zona incerta; fibers are widespread and diffuse and include aspects of the olfactory bulb, basal ganglia, neocortex, hippocampal formation, and broad expanses of the brainstem and spinal cord (Review, (16)). Coexisting within the same dorsal hypothalamic perikarya are peptides immunologically related but not identical to alpha MSH (17) (18) (19) (20), corticotropin releasing factor (CRF) (21) (22) (23) and growth hormone releasing factor (GRF) (24)(25). Despite much speculation and the efforts of several groups, the nature of the peptides sharing epitopes with alpha MSH, CRF and GRF within the dorsal lateral hypothalamus had not been identified.

With the goal of characterizing mammalian MCH's and their precursors we designed synthetic oligonucleotide probes based upon the salmon MCH sequence and screened rat and human brain libraries. Although these probes consistently hybridized to a ~0.3 kb mRNA which was the 7SL RNA component of the signal recognition particle (26), we were unable to detect mammalian MCH precursors. The MCH precursors from Chum (27) and Chinook (28) salmon were cloned and sequenced by Kawauchi's and Watson's groups. We cloned and sequenced the Coho salmon MCH precursor (26) and used partial and full length salmon probes to screen mammalian libraries but again we could not detect rat or human MCH positive clones. We, therefore, decided to determine the structure of a mammalian MCH by traditional means involving the isolation of MCH-like peptides from natural sources (29).

ISOLATION OF RAT MCH

We developed a sensitive radioimmunoassay (RIA) for MCH using rabbit anti-salmon MCH sera and iodinated salmon MCH as tracer. This assay detects immunoreactive (ir) MCH in rat hypothalamic extracts. We located zones of irMCH in fractions from our purification of CRF and GRF from 60,000 rat hypothalamic fragments (Figure 1).

Figure 1. Scheme for the purification of irMCH from rat hypothalamic extracts according to Vaughan et al.(29).

At the G-50 stage, CRF and GRF were separated from a low molecular weight zone of irMCH which was further purified by immunoaffinity chromatography using the anti-salmon MCH sera and subsequently by gel permeation on FPLC and by reversed phase HPLC and narrow bore HPLC. Several highly purified fractions of irMCH were thus obtained and subjected to Edman degradation in a gas phase protein sequencer (Applied Biosystems 470 A). Microsequencing from the amino-terminus yielded an identical amino acid sequence for all fractions with the PTH amino acid signal becoming undetectable at various cycles for each fraction. Analysis of a pool of the fractions, reduced and treated with 4-vinylpyridine and repurified on narrow bore HPLC, gave the sequence of the nonadecapeptide shown in Figure 2. The fractions may have differed in length or by oxidation of methionine⁴ or 8 or by deamidation of glutamine¹⁸; cycle 18 showed a mixture of Gln and Glu, a finding we interpreted as an indication of partial deamidation in these 8 year old samples. This deamidation was fortuitous because [Glu¹⁸]-rMCH is detected much better by the salmon RIA than is rMCH (with Gln¹⁸), thus explaining why our old fractions displaced more completely than do fresh

hypothalamic extracts or synthetic rMCH (29). The major zone of MCH activity in fresh rat hypothalamic extracts co-elutes with synthetic rMCH. Synthetic rat MCH, synthesized according to the methods of Rivier et al.(30) displaces fully as do rat hypothalamic extracts in RIA's using rat MCH as a tracer with both anti salmon and anti rat/human MCH antisera. Compared to salmon MCH, rat MCH is extended from the N-terminus by 2 amino acids and has 4 additional substitutions.

Figure 2. Comparison of primary sequences of rat (29) and salmon (31) MCH.

CLONING AND SEQUENCES OF THE RAT AND HUMAN MCH PRECURSORS

Using an oligonucleotide probe designed from the peptide sequence of purified rMCH we have screened a rat hypothalamic cDNA library and isolated and fully characterized four rMCH clones. All clones were identical except at their 3' ends where they had different lengths of poly(A). The largest cDNA clone comprises the complete coding region of the MCH precursor (495 nucleotides), 41 nucleotides of the 5' non-translated sequence, all of the 3' non-translated region (192 nucleotides) including a single putative polyadenylation sequence, followed by a poly(A) tract. The deduced prepro rMCH is 165 amino acids long with a probable signal peptide cleavage site at Gly²¹ yielding a precursor of 144 amino acids; the 19 amino acid MCH peptide is located at the carboxy terminus as found for the salmon counterparts. The sequence predicted from the cDNA confirms our proposed sequence of isolated rat MCH.

An oligonucleotide probe was synthesized representing the most conserved region between salmon and rat MCH and was used to screen a

human hypothalamic cDNA library. Two clones were isolated and sequenced and found to be identical except at their 5' and 3' untranslated ends. The human preproMCH also comprises 165 amino acids, with a precursor of 144 amino acids; as shown in Figure 3, rat and human MCH are identical to one another.

Figure 3. Schematic of the salmon, rat and human MCH mRNA's. % Identities of the amino acid sequences shown between each precursor. Vertical lines denote basic residues. Putative peptides, NEV, NGE and NEI follow convention of Tatamoto and Mutt (32) of naming neuropeptides as N (neuropeptide)-first amino acid-last amino acid.

ADDITIONAL PUTATIVE NEUROPEPTIDES ARE ENCODED IN THE RAT MCH PRECURSOR

MCH is presumably generated by cleavage of double basic residues, Arg¹⁴⁵-Arg¹⁴⁶, this would also expose the Gly¹⁴⁴ as a potential amidation site yielding Ile-NH₂ as the C terminal of a peptide, NEI cleaved N-terminally prior to Glu⁺ at double basics, Lys¹²⁹-Arg¹³⁰. NEI (preproMCH (132-144), therefore is the tridecapeptide,

E-I-G-D-E-E-N-S-A-K-F-P-I-, and is completely conserved between human and rat. Also conserved with identities at 16 of 19 positions, is the putative peptide NGE which could be generated by cleavages at Lys¹²⁹-Arg¹³⁰ on the C-terminus and at single basic amino acids at either Lys¹⁰⁹ (yielding NGE, preproMCH (110-128)) or at Lys¹⁰³ (yielding NPE, preproMCH(104-128). Although neither NEI nor NGE (or NPE) have been

identified biochemically and thus the processing of the precursor has not been defined, immunologic evidence consistent with the presence of these peptides in brain tissues has accumulated by numerous investigators. We have recently found that antisera raised towards rat/human MCH and rat/human NEI react with the same cell bodies in the lateral hypothalamus (Figure 4).

Figure 4. Immunohistochemical localization of MCH and NEI in the rat hypothalamus with anti rat/human MCH or NEI raised against synthetic peptides according to methods described previously (33)(29).

We propose that antisera raised towards alpha MSH, rat CRF and certain human GRF fragments have in fact been recognizing epitopes in the MCH precursor (Figure 5). As stated previously, antisera towards alpha MSH and rat CRF react with the same cells that are recognized by salmon MCH and now by rat/human MCH and NEI antisera. Furthermore, we have shown that adsorption of alpha MSH or rat CRF antisera with NEI eliminates their staining of the large stellate cells of the lateral hypothalamus and zona incerta. These polyclonal antisera contain antibodies directed towards the amidated C-terminal of CRF and alpha MSH as demonstrated by our finding that adsorption with the dipeptides, Pro-Ile-NH₂ or Pro-Val-NH₂ can also prevent this staining. Some antisera raised against human GRF fragments by Bugnon, Fellman and colleagues (25) have been shown to react with the same cells. We have found that an antiserum raised by Fellman and generously provided to us by Dr. Nick Ling

indeed stains the lateral hypothalamus and that this staining can be suppressed by adsorption with NGE. The modest structural similarities observed among the neuropeptide encoding regions of preproMCH and CRF, GRF and alpha MSH do not necessarily imply functional or even evolutionary relationships, however, such similarities have apparently been sufficient to yield misleading results with polyclonal antisera.

PREPROrMCH C-TERMINUS

Figure 5. Schematic of the regions within the rat and human MCH precursors that have epitopes recognized by antibodies directed towards alpha MSH, rat CRF (rC-70) or human GRF (25).

The MCH precursor does have significant resemblance to two other precursors, the Aplysia Peptide A precursor (34), a member of the Egg Laying Hormone family, and the Atrial Natriuretic Factor precursor (35).

We have explored the actions of salmon and rat MCH on the basal and CRF stimulated secretion of ACTH by cultured anterior pituitary cells and have observed no effect over a 4 hour period. Although the rat MCH positive, dorsolateral hypothalamic cells project widely to various brain regions as well as to the posterior pituitary, there is only sparse innervation of the external lamina of the median eminence (Sawchenko and Vale unpublished results). The products of the brain MCH precursor gene are more likely to be involved as neuroregulators within the central nervous system than as hypophysiotropic hormones.

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The Rat Melanin-Concentrating Hormone Messenger Ribonucleic Acid Encodes Multiple Putative Neuropeptides Coexpressed in the Dorsolateral Hypothalamus*

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ABSTRACT. The melanin-concentrating hormone (MCH) is a cyclic neuropeptide, first isolated from salmon pituitary glands, which regulates melanin dispersion in the skin and perhaps the activity of the pituitary-adrenal axis in teleost fish. We have recently purified and characterized rat MCH (rMCH) and report here the cloning and sequencing of specific MCH cDNA isolated from a rat hypothalamic library. The sequence of rMCH found by DNA sequencing confirms the sequence deduced from the purified peptide. rMCH is located at the C-terminus of a protein precursor of 165 amino acid residues. Comparison of the amino acid sequence of prepro-MCH and that of the *Aplysia* peptide-A prohormone suggests that these proteins as well as other precursors may be evolutionarily related. Besides rMCH, two pu-

tative neuropeptides, termed NGE and NEI, might be generated from the same precursor. The rMCH precursor shared sequence identities with human GH-releasing factor and mammalian CRF in the regions encoding NGE and NEI. By immunohistochemical studies we have established that the amidated C-terminus of NEI is recognized by some α MSH and rat CRF antisera and that the C-terminal portion of NGE is responsible for the cross-reactivity revealed with one hGRF-(1-37) antiserum. Our results explain the staining of a discrete population of dorso-lateral hypothalamic neurons by heretofore seemingly unrelated antisera and provide evidence for the production of multiple novel neuropeptides from a common precursor. (*Endocrinology* 125: 2056-2065, 1989)

MELANIN-concentrating hormone (MCH) is a cyclic heptadecapeptide originally isolated from salmon pituitary glands (1). Salmon MCH (sMCH) induces melanosome aggregation within melanophores of teleost fish, while it stimulates melanin dispersion in frogs and lizards (1; reviewed in Refs. 2 and 3). These differential activities appear to be attributable to distinct regions of the hormone (4). In addition, sMCH is a potent inhibitor of CRF-induced α MSH and ACTH secretion *in vitro* by teleost pituitary glands and is also reported to have a similar, albeit weaker, activity on the rat

pituitary (reviewed in Ref. 2; 5). Therefore, the MCH system may play roles in the regulation of skin pigmentation and certain aspects of the stress response.

Immunological studies have revealed the presence of putative MCH-like peptides in the brains of several classes of vertebrates including mammals (2, 3). MCH immunoreactivity was localized in a single population of perikarya located in the dorsolateral regions of rat and human hypothalamus (6, 7). Coexisting within these same perikarya are peptides immunologically related, but not identical, to α MSH (8-11), GH-releasing factor (GRF) (7, 12), and CRF (13-15). Despite considerable research over the last decade (reviewed in Refs. 2 and 3), the epitopes responsible for these putative peptide immunoreactivities in the dorsolateral hypothalamus have remained unknown.

Different groups have recently cloned and determined the structure of mRNAs encoding sMCH (16-18) (Nahon, J. L., *et al.*, submitted). Neither partial nor full-length native salmon-DNA probes could be successfully used to identify cDNA clones encoding mammalian MCH. Recently, our laboratory has succeeded in the isolation and structural determination of the putative rat

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MCH (rMCH) (19), which differed from the salmon peptide by a two-amino acid N-terminal extension and four additional substitutions. Using an oligonucleotide probe designed from the peptidic sequence of purified rMCH, we have screened a rat hypothalamic cDNA library. We present here the sequences of the putative rMCH mRNA and deduced protein precursor, the amino acid sequence comparison analysis, and immunohistochemical studies which allow us to propose that portions of the putative novel tridecapeptide named NEI as well as the other peptide, NGE, encoded in the rMCH precursor, are responsible, respectively, for both α MSH- and CRF-like and human (h) GRF-like immunoreactivities revealed with some antisera (reviewed in Ref. 2).

Materials and Methods

Identification and nucleotide sequence analysis of cDNA encoding rMCH mRNA

The cDNA library was constructed with the cloning vector λ Zap (Stratagene, San Diego, CA) from 5 μ g rat hypothalamic poly(A)⁺ RNA using the RNase-H procedure (20). A single stranded oligodeoxynucleotide of 30 nucleotides, named RMCH-1, was used as hybridization probe. The sequence of RMCH-1 (5'-GCCCAGCATGCACCGCAGCATGTCAAAGTC3') was designed from the rMCH peptide sequence (19) according to the method of Lathe (21). RMCH-1 was 5' end labeled with ³²P by using polynucleotide kinase (SA, 2–10 $\times$ 10⁸ dpm/ μ g). Prehybridization and hybridization were carried out at 42 C in a solution containing 5 $\times$ SSPE [1 $\times$ SSPE is 0.18 M NaCl, 0.01 M NaPO₄ (pH 7.4), and 1 mM EDTA], 20% (vol/vol) deionized formamide, 0.5% sodium dodecyl sulfate, 0.1% (wt/vol) each of BSA serum albumin, Ficoll, and polyvinylpyrrolidone, and 500 μ g/ml (prehybridization) or 100 μ g/ml (hybridization) sonicated denatured calf thymus DNA. The final washing was performed at 60 C in 1 $\times$ SSPE three times for 10 min each. From 480,000 plaques, 4 positive clones were isolated and analyzed by restriction mapping, revealing overlapping maps, and the DNA was used for dideoxynucleotide sequencing (22). All of the rMCH cDNAs were sequenced in both directions.

Amino acid sequence comparison

The search for homology between MCH precursor sequences and protein sequences stored in the NBRF data bank was carried out using the GAP program (23) and the VAX 11/785 computer (The Salk Institute). The significance of the sequence alignment was estimated by determining the probability of n or more matches in a pair of one sequence of length m:

$$P = \sum_{i=n}^m \binom{m}{i} \left(\frac{1}{20} \right)^i \left(\frac{19}{20} \right)^{m-i}, \quad \text{where } \binom{m}{i} = \frac{m!}{i!(m-i)!}$$

Immunohistochemistry analysis

Five adult male albino rats received intraventricular injections of colchicine (50 μ g in 25 μ l saline). Three days later they

were perfused with 4% paraformaldehyde. Hypothalami were postfixed for 4–8 h, infiltrated with sucrose overnight, sectioned at 20 μ m on a freezing microtome, and prepared for immunohistochemistry using a conventional biotin-avidin-immunoperoxidase method. Details of the histological processing were provided previously (34). Polyclonal antisera against α MSH (Incstar Corp., Stillwater, MN), hGRF (12) rCRF (serum rC70); (25), and synthetic sMCH (19) were used for staining at dilutions of 1:2000. Cross-reactivity tests were carried out by preadsorbing the sera (diluted at 1:200) with the homologous and heterologous synthetic peptides over a half-order of magnitude series of dilutions overnight at 4 C. The sera were then diluted to their working concentrations for staining.

Results

Characterization and nucleotide sequence analysis of rMCH cDNAs

Using the oligonucleotide RMCH-1 as a probe (shown in *Materials and Methods*), we isolated and fully characterized four rMCH clones from a rat hypothalamic cDNA library. All of these clones were identical, except at their 5' ends where they extended to different positions, and at their 3' ends where they had different sizes of poly(A) (not shown). The largest cDNA clone (pRMCH11) comprises the complete coding region of the MCH precursor (495 nucleotides), 41 nucleotides of the 5' nontranslated sequence, and all 3' nontranslated region (192 nucleotides) including a single putative polyadenylation sequence (26) and followed by a poly(A) tract (Fig. 1). The first ATG codon (designed 1 in Fig. 1) initiates an open reading frame of 165 amino acids. The sequence context surrounding this codon, CAGGATGG, is homologous to the optimal context for initiation as defined by Kozak (27). Another in-frame ATG codon located at position 4 could be also used as an initiation codon. Direct biochemical studies have to be performed to establish the actual translation initiation codon. A sequence showing common features with the signal peptide (28) is located after the first in frame ATG (Figs. 1 and 2). A cleavage site by signal peptidases is predicted (28) at Gly²¹, generating a pro-MCH precursor of 144 amino acids. The 19-amino acid MCH peptide is located at the carboxy-terminus of this precursor (Fig. 1), as found for the salmon counterparts (Fig. 2) (16–18) (Nahon, J. L., *et al.*, submitted). The MCH sequence predicted by the cDNA confirms our proposed peptide sequence of isolated rMCH (19). Comparison of the rat and salmon MCH cDNA sequences revealed DNA identities in the region already reported (18) as sharing similarity with the human 7SL RNA (not shown).

Several putative neuropeptides are encoded in the rMCH precursor

The rMCH precursor and the sMCH precursor deduced from our cDNA sequence (Nahon, J. L., *et al.*,

submitted) display several homologous regions. The highest conservation of amino acid sequence is found in the region encoding MCH (Fig. 2). Aside from the N-terminal extension, rMCH exhibits four additional differences from sMCH, although three of these are conservative changes. The protein sequence is strongly conserved within the central portion of MCH (between the two cysteines circled in Fig. 2), which is necessary for full potency of sMCH in the melanotropic concentrating activity in teleosts (reviewed in Ref. 2).

In addition to the similarities between rat and salmon MCH, several moderately conserved regions were found flanked by mono- or dibasic residues, which are thought to serve as potential cleavage sites to generate mature hormones from polyfunctional proteins (29). Seventeen of the 35 residues immediately preceding the MCH sequence are identical or represent conservative substitutions between rat and salmon MCH. Two peptides could be generated from those 35 residues (Fig. 2). Following the convention proposed by Tatemoto and Mutt (30), we refer to rat prepro-MCH-(110-128) as neuropeptide (N) glycine (G)-glutamic acid (E) or NGE; this peptide would be generated from cleavages involving Lys¹⁰⁹ and Lys¹²⁹-Arg¹³⁰, but an alternate cleavage site for this region could involve Lys¹⁰³ rather than Lys¹⁰⁹. Rat prepro-MCH-(132-144)-amide, termed neuropeptide (N) glutamic acid (E) isoleucine (I) amide or NEI, could be produced by cleavages at Lys¹²⁹-Arg¹³⁰ and Arg¹⁴⁵-Arg¹⁴⁶; the finding of a Gly¹⁴⁴ preceding the 2 basic residues on the C-terminus of NEI led us to propose that this putative peptide is C-terminally amidated (31). Presently, neither NEI nor NGE has been identified biochemically, and therefore, the extent to which the MCH precursor is actually processed is not defined. We have recently cloned and sequenced the hMCH precursor (Presse, F., *et al.* in preparation) and found that the MCH and NEI coding portions are identical to those in the rat, while the NGE region differs by only 3 amino acids, with 2 conservative substitutions.

Similarities in peptide-encoding regions between rMCH and Aplysia peptide-A precursors

Besides the rMCH, NEI, and NGE peptide sequences, other areas of the precursor are flanked by single basic residues that may also be used as a cleavage site, albeit less frequently than dibasic residues (29). Computer searches of the protein data base revealed few but significant similarities (24% sequence identities) between the precursors encoding rMCH and *Aplysia* peptide-A (32) in these regions (Fig. 3). It is worth noting that the positions of cleavage sites of the propeptide-A, generating distinct sets of peptides in *Aplysia* atrial gland, match perfectly at 5 positions from 6 with mono- or dibasic

residues in the rMCH precursor (Fig. 3). The degree of identities between those 2 sequences is far above random expectations, and the fact that amino- and carboxy-termini were juxtaposed, introducing few gaps, strengthened the significance of such alignments. Indeed, when the N-termini of rMCH and *Aplysia* peptide-A precursors are aligned, a stretch of 17 residues containing 7 matches (34% identities) was found at the residues 50-66 in the rat (Fig. 3). The probability of getting such positional matches only by chance is $P = 9 \times 10^{-6}$ (see *Materials and Methods*). Further alignment in the C-terminus (at residues 123-149 in the rat; residues 132-158 in *Aplysia*) reveals 9 matches ($P = 2.5 \times 10^{-7}$). The product of the amino- and carboxy-terminal probabilities give a total of $P = 2.2 \times 10^{-13}$. Taking into account the gaps, the probability of aligned sequences being related by chance is less than 10^{-10} . This indicates that such comparison yields alignments that are highly statistically significant.

Interestingly, a single base deletion in the stop codon of rMCH precursor leads to two leucine residue matches in the next four residues between the rat and *Aplysia* sequences; additional single base substitutions would allow two additional matches in that portion of the precursors (Fig. 3, right). These observations are highly reminiscent of the structural difference observed between the precursors encoding peptide-A and -B in *Aplysia* (32). Taken together our results indicate that prepro-rMCH (and the C-terminal portion of prepro-sMCH; not shown) and *Aplysia* prepropeptide-A are distantly yet unambiguously related.

Two novel neuropeptides NEI and NGE share epitopes with α MSH, CRF, and GRF

The existence of regions encoding putative neuropeptides within rat prepro-MCH prompted us to search for peptide sequence similarities with α MSH, CRF, and GRF, antisera against which stain a common group of perikarya in zona incerta and lateral hypothalamic areas (reviewed in Ref. 2). Indeed, we found regions in those neuropeptides exhibiting some similarities with sequences in NGE and NEI, which might explain antigenic cross-reactivity.

Watson and colleagues (8, 11) proposed that the anti- α MSH sera that reacted with the lateral hypothalamic cells were directed against the C-terminal peptide amide portion of α MSH and predicted the existence of a then unknown peptide which they named a-2 antigen which would have a similar C-terminus. The C-terminus of the putative NEI is -Pro-Ile-NH₂ compared to that of α MSH, which is the closely related dipeptide -Pro-Val-NH₂ (Fig. 4).

To evaluate the possibility that cross-reactivity with NEI might account for the hypothalamic α MSH-like

FIG. 3. Relatedness of the rMCH and *Aplysia* peptide-A precursors. Upper line, rMCH precursor sequence (see Fig. 2); lower line, *Aplysia* peptide-A precursor sequence (32). Alignments were performed using the computer program GAP (23), with GAP weight = 3.0. Dashes, highlighting, dot, and arrowheads are explained in Fig. 2. The arrows indicate the putative signal peptide cleavage site. The positions of the putative neuropeptides NGE, NEI, and rMCH in rMCH precursor and peptide-A, peptide-X, peptide-X', egg-laying hormone N-terminus (N-ELH), egg-laying hormone C-terminus (C-ELH), and acidic peptide in *Aplysia* prepropeptide-A are indicated by brackets. Alignment of both sequences after the stop codon in rMCH mRNA is presented on the right.

FIG. 4. Alignment of NGE and NEI from rMCH precursor with hGRF-(30-37) (41), rGRF-(30-37) (42), rat/human CRF-(24-41) (43), and α MSH-(1-13) (44). Identical amino acids are highlighted, and conservative substitutions are marked with a dot. Putative proteolytic processing sites are marked with arrowheads. Known or possible C-terminal amides are marked with a box. An asterisk indicates an acetylation site.

immunoreactivity in the lateral hypothalamus, a series of immunohistochemical analyses was carried out with synthetic NEI and dipeptides (Fig. 5). In accordance with previous reports (8-11), antisera against α MSH stained cells in both the arcuate nucleus (known to contain authentic α MSH) and the extraarcuate grouping scattered primarily through the caudal aspects of the lateral hypothalamic area and zona incerta. Preadsorption of the antiserum with synthetic α MSH abolished staining in both cell groupings, while competition with equimolar concentrations of NEI eliminated staining in the extraarcuate cells, leaving staining of the POMC cell cluster in the arcuate region intact. Strikingly, the α MSH staining of the dorsolateral hypothalamic, but not arcuate, nucleus cell grouping was competed equally by the dipeptides Pro-Ile-NH₂ (PI amide) and Pro-Val-NH₂ (PV amide) (Table 1). Antisera raised against sMCH (or rMCH) stained cells in the lateral hypothalamic/zona incerta grouping and not in the arcuate region. These localizations were prevented by competition by either synthetic salmon or rat MCH, but not with high concentrations of α MSH (not shown). Based on these results we conclude that the α MSH-like immunoreactivity described in the lateral hypothalamus and zona incerta can be explained on the basis of cross-reactivity with a di-

peptide amide sequence at the C-terminus of the novel peptide NEI encoded by the rMCH precursor.

Different laboratories have reported (13-15) the existence in the dorsolateral hypothalamic area of hormone or precursor segments sharing epitopes with rCRF. Furthermore, several of our polyclonal antisera (25) raised against rCRF have reacted with cells in the lateral hypothalamus/zona incerta (33); we have observed previously (33) that we could suppress rCRF-like staining in this region by preadsorption with α MSH. rCRF-(24-41) appears to be related to prepro-rMCH-(116-133), in that 6 of 18 residues are identical ($P = 1.5 \times 10^{-4}$; Fig. 4). Dibasic residues (Lys¹²⁹-Arg¹³⁰) interrupt the prepro-MCH in that region and could generate the two putative neuropeptides defined above as NEI and NGE. Alternatively, the region might remain intact. It is, therefore, conceivable that antisera raised against rCRF might detect peptides (such as the putative NGE and NEI) derived from this segment of prepro-rMCH. Furthermore, both NEI and rat/human CRF have a C-terminal isoleucine-amide, and α MSH has a closely related bulky aliphatic amide (valine amide) at its C-terminus (Fig. 4).

We tested the possibility that our anti-rCRF sera could react with NGE or NEI in the lateral hypothalamus by carrying out immunohistochemical studies (Fig. 6). Using

FIG. 5. Localization and specificity of α MSH and MCH immunoreactivity in the rat hypothalamus. *Upper left*, α MSH immunostaining is found both in the arcuate nucleus (ARH), the acknowledged focus of POMC expression in the hypothalamus, and in cells scattered through the lateral hypothalamic area (LHA) and the zona incerta (ZI). The fornix (fx), optic tract (ot), and third ventricle (V3) are labeled for reference. *Upper right*, Staining of an adjacent section with anti- α MSH adsorbed with synthetic α MSH. *Lower left*, Staining of an adjacent section with anti- α MSH adsorbed with synthetic NEI. *Lower right*, sMCH-immunoreactivity at a level similar to that shown in the other panels. All micrographs, $\times 30$.

TABLE 1. Minimum effective concentrations (millimolar) of peptides required to block α MSH and rCRF immunostaining in dorsolateral hypothalamic neurons

Antiserum	Peptide	Site	
		Dorsolateral hypothalamus	Arcuate nucleus
α MSH	α MSH	7.0	<1.4
	NEI	<1.4	>140.0
	Pro-Ile-NH ₂	<1.4	>140.0
	Pro-Val-NH ₂	<1.4	>140.0
		Paraventricular Nucleus	
rCRF	rCRF	<1.4	<1.4
	α MSH	140.0	>140.0
	NEI	7.0	>140.0
	NGE	>140.0	>140.0
	Val-NH ₂	>140.0	>140.0
	Ile-NH ₂	>140.0	>140.0
	Pro-Ile-NH ₂	<1.4	>140.0
	Pro-Val-NH ₂	7.0	>140.0

Antisera were adsorbed with 1.4-, 7-, 14-, 70-, and 140-mM concentrations of peptides. Values given are the concentrations required for complete abolition of staining. Values less than 1.4 indicate that staining was abolished with this lowest dose tested; values greater than 140 indicate that staining persisted despite the addition of 140 mM peptide.

our rCRF antiserum, two CRF-like cell types were evident at higher magnification in the dorsolateral hypothalamus. One is large, darkly stained, and spiny and is

concentrated in the lateral hypothalamic area and zona incerta (Fig. 6, *upper right*). This cell type is indistinguishable in distribution and form from that stained with anti-MCH (see Fig. 5). The second cell type, smaller, more lightly stained, and nonspiny, is concentrated in the region around the fornix. Other acknowledged sites of CRF production, such as in the paraventricular nucleus of the hypothalamus, were also positively stained (not shown; see Table 1). Preadsorption of the anti-rCRF serum with synthetic NEI eliminated staining exclusively in the large spiny cell grouping in the dorsolateral hypothalamus, while competition with NGE left intact the staining in all groups, and rCRF blocked staining in all groups (Fig. 6 and Table 1). Thus, our results strongly suggest that the rCRF antiserum used in the present and previous studies (13–15, 33) recognizes epitopes shared by the putative peptide NEI. Because the dipeptides PI amide and PV amide, albeit at higher concentrations (Table 1), also suppress this staining in our experiments, it is likely that this epitope for our antisera is the C-terminal bulky aliphatic amide of NEI.

As shown in Fig. 4, an eight-residue stretch in NGE shares five identical residues with the sequence 30–37 of hGRF ($P = 1.5 \times 10^{-5}$), while adjacent segments display the least similarity to hGRF (not shown). Antisera raised against hGRF-(1–37) detect an uncharacterized neuropeptide sharing an epitope with the 29–37 sequence of hGRF within MCH/ α MSH-like cell groups in the lateral hypothalamus of the rat and human (7, 12). In view of the fact that the anti-hGRF-(1–37) sera do not recognize

FIG. 6. Effects of competition with NEI on rCRF-like immunoreactivity in the dorsolateral hypothalamus. *Upper left*, Low magnification view ($\times 30$) of rCRF-stained cells, distributed broadly through the lateral hypothalamic area and the zona incerta. The level illustrated is somewhat caudal to that shown in Fig. 5. See Fig. 5 for abbreviations. Additional structures labeled for reference include the ventromedial (VMH) and dorsomedial (DMH) hypothalamic nuclei. The remaining panels show a higher magnification view ($\times 75$) of the region around the fornix (fx) in this and two adjacent sections. *Upper right and left*, Numerous apparent CRF-immunoreactive neurons were detected in the dorsolateral hypothalamus, whose distribution and morphology appeared similar to those of cells stained with the anti-MCH serum (see Fig. 5). An additional morphologically distinct grouping of rCRF-stained cells was found centered in the perifornical region of the lateral hypothalamic area. *Lower left*, An adjacent section stained with anti-rCRF that was preadsorbed with rCRF. *Lower right*, Another adjacent section stained with rCRF preadsorbed with NEI.

rGRF, it may be noteworthy that the single residue (Ser³⁴) that is not conserved between the human and rat GRF-(30-37) sequence is present in NGE (Fig. 4). We propose, therefore, that those antibodies against hGRF-(1-37) may be reacting with a sequence within NGE. Using the hGRF-(1-37) antiserum (12), kindly provided to us by Dr. N. Ling, we have found that the specific staining observed in the lateral hypothalamus can be totally suppressed by preadsorption with NGE (not shown). Taken together, our results strongly suggest that the C-terminal portion of NGE shares the epitope recognized by some hGRF-(1-37) antisera (7, 12).

Discussion

We have cloned and sequenced cDNAs encoding the putative rMCH mRNA expressed in rat hypothalamus. In contrast to the situation found in chinook and chum salmon species (16, 17), our results do not provide evidence for the existence of two kinds of MCH-encoding mRNA in rat. However, our Southern blot analysis does not preclude the possibility that two MCH genes are present in mammals (Presse, F., *et al.*, in preparation).

The rat and salmon precursors exhibit only 28% identities (54% similarities), although this varies at different regions of the precursor. Indeed, the signal peptide and central parts (until residues 108 in the rat and 81 in salmon) have diverged extensively (17% identities), while

the C-terminal region shows significant sequence conservation (42% identities). Interestingly, the most conserved regions are bounded by basic residues, the cleavage sites for trypsin-like processing enzymes (29). Hence, besides the nonadecapeptide rMCH, two novel putative peptides, NGE and NEI, might be generated from the rMCH prohormone. Interestingly, although NEI in the rat is probably amidated, the counterpart in salmon, named NEV according to the same convention, lacks the C-terminal glycine replaced by a valine residue. In addition, NEI and NEV display only 30% sequence identities (69% sequence similarities), suggesting, therefore, that weak structural and/or functional constraints have acted on those peptide-encoding sequences during evolution from fish to mammals.

Although the precise processing of the rMCH precursor has not been established by purification and characterization, our isolation and sequencing of a rMCH with an aspartic acid N-terminus (19) support the existence of a cleavage between the putative NEI and MCH. The immunohistochemical evidence is consistent with the presence of a peptide whose C-terminus is an aliphatic amino acid amide in neurons staining with anti-MCH sera. The most likely source for this peptide is from cleavage and amidation of the peptide on the N-terminal side of MCH.

Search of a protein data base reveals homologies between rMCH precursor and other proteins. The most

striking resemblance was found with the precursor encoding *Aplysia* peptide-A. The observed relatedness has been estimated to be statistically significant (see *Results*). In contrast to the poor conservation of sequence observed between rat and salmon pro-MCH in the N-terminal and central parts, there is a dramatic conservation in peptide-encoding portions within these regions of rMCH and *Aplysia* peptide-A precursors. This surprising observation makes it likely that strong structural or functional constraints have acted selectively during evolution on the N-terminus of the rMCH precursor. In this context we also found significant sequence identities between rMCH and rat atrial natriuretic factor (rANF) (34) precursors, mostly in the N-terminal part encoding the γ ANF peptide (not shown). *Aplysia* peptide-A and γ ANF are expressed specifically in extracerebral areas (atrial gland and heart, respectively) and participate in complex behavioral and physiological arrays subserving to egg laying (32) and cardiovascular homeostasis (34). We are now investigating the possibility that the gene encoding rMCH is also transcribed in tissues other than brain and might be involved in the control of specific goal-oriented behaviors and homeostatic functions. The increase observed by Zamir *et al.* (35) in hypothalamic and pituitary MCH-like immunoreactivity after osmotic challenges supports such potential ties.

Short stretches of NGE and NEI peptide sequences exhibit significant similarities with known neuropeptides such as CRF or GRF. Although the similarities between prepro-MCH and CRF or GRF are slight, they may reflect common ancestry, in fact rat/human CRF and hGRF themselves share a hexapeptide sequence with four identities and one conservative substitution. These sequence similarities and the others noted already for the case of *Aplysia* peptide-A and rANF precursors strengthen the argument that an evolutionary relatedness exists among genes encoding neuropeptides which appear coregulated under physiological circumstances such as stress (in the case of CRF, ACTH, vasopressin, FMRF amide) (36, 37) or osmotic challenge (in the case of peptide-A, rANF, and rMCH precursors shown here). Interestingly, we found a striking homology between salmon and rat MCH mRNA sequences and the 7SL RNA in region encompassing the biologically active peptide (18) (not shown). The RNA sequences corresponding to the regions aligned between the rMCH and the *Aplysia* peptide-A and rANF precursors also display significant similarities with the 7SL RNA (our unpublished observations). On the basis of these observations it is tempting to speculate that the ancestral gene from which these neuropeptides precursors derived might be the 7SL RNA. This hypothesis seems plausible for two main reasons: 1) the 7SL RNA is transcribed from a moderately repeated gene family which appeared very early during

evolution (38); and 2) this structural RNA is functionally related to neuropeptide genes by the evidence that it is an essential component of the signal recognition particle (39) which is involved in targeting secretory proteins to the endoplasmic reticulum membrane (reviewed in Ref. 40). We are currently analyzing numerous mRNA sequences to determine the occurrence of 7SL-like sequence within the transcripts encoding secretory proteins.

The modest structural similarities observed among the neuropeptide-encoding regions of prepro-MCH and CRF, GRF, and α MSH do not imply functional relationships. However, such similarities may be sufficient to yield misleading results with immunohistochemical techniques using polyclonal antisera. In this context, the present work clarifies the observations of MCH-, α MSH-, CRF-, and GRF-like immunoreactivities within the same cells in the lateral hypothalamic area (reviewed in Ref. 2). Indeed, we present evidence that sequences within the novel putative neuropeptides NEI and NGE, which are generated from the rMCH precursor, might be detected by some α MSH, CRF, and GRF antibodies. In fact, the amidated dipeptide present at the C-terminus of NEI appears to be sufficient to cross-react with some α MSH and rCRF antibodies. Recently, Antoni and Linton (15) proposed that a Met-Glu sequence shared by both α MSH and CRF C-terminal regions might be responsible for the cross-reaction revealed in the arcuate nucleus and lateral hypothalamus with rCRF serum (rC70). By contrast, our results suggest that the cross-reacting antibodies are directed against the C-terminal isoleucine amide. Interestingly, the same dipeptide amide might be responsible for the cross-reaction of this rCRF serum with PHI (15), which has a Leu-Ile-NH₂ sequence at its C-terminus.

It should be noted that we identified rCRF-containing perikarya in a perifornical region that appeared to be morphologically, distributionally and immunologically distinct from those containing MCH immunoreactivity. The authenticity of these CRF perikarya is supported by *in situ* hybridization experiments with rCRF cRNA probes which reveal the presence of rCRF mRNA in cells of the perifornical region (Imaki, T., J. Imaki, and W. Vale, personal communication).

In contrast to many other hypothalamic neuropeptides, neuroanatomical studies have indicated that the median eminence is only moderately innervated by the pro-MCH neuronal system (reviewed in Ref. 2). A small contingent of cells in this grouping have been shown to project to the posterior pituitary (6, 13), an observation supported by our finding of scattered MCH-like immunoreactive fibers in the posterior lobe (data not shown). From a functional standpoint, sMCH has been reported to antagonize CRF-stimulated corticotropin secretion by fish and, to a lesser extent, rat pituitary glands (5); we

have been unable to confirm this action of sMCH on rat cells (Yamamoto and W. Vale, unpublished data), but have observed instead that rMCH is an ACTH secretagogue in some, but not all, *in vitro* systems (Boyle, *et al.*, in preparation).

The available evidence on the central distribution of the MCH system would indicate that its expression in perikarya is restricted to the dorsolateral hypothalamus and zona incerta, while terminal distributions are widespread and diffuse, including aspects of the olfactory bulb, basal ganglia, neocortex, hippocampal formation, and broad expanses of the brainstem and spinal cord (reviewed in Ref. 2). The lateral hypothalamic area and zona incerta have often been associated with the control of specific goal-directed behaviors and homeostatic functions. Nonetheless, the convergence of information from a number of sensory modalities on units in this region and the generalized sensorimotor effects that have been described in focal lesion or stimulation experiments have fostered the notion that at least some cells in the lateral hypothalamus may serve generally to modulate levels of arousal and/or motor activity (10). Indeed, the distribution of pro-rMCH-related peptides would seem most compatible with their having a place in the context of more general behavioral and physiological aspects of lateral hypothalamic functions.

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The Melanin-Concentrating Hormone System of the Rat Brain: An Immuno- and Hybridization Histochemical Characterization

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ABSTRACT

In addition to a nonadecapeptide homologous to the teleost melanin-concentrating hormone (MCH), the amino acid sequence predicted from a rat prepro-MCH (ppMCH) cDNA suggested that at least one (neuropeptide EI, or NEI), and possibly a second (NGE), additional neuropeptide may be encoded by this precursor. Cross-reactivity with epitopes of NEI or NGE can account for reported localization of α -MSH, rat CRF, and human GRF in rat dorsolateral hypothalamic neurons. We have used antisera raised against rat MCH and NEI in immunohistochemical studies at the light and electron microscopic levels, along with hybridization histochemical localization of ppMCH mRNA, to define the organization of this system. As expected, ppMCH mRNA is prominently expressed in cells in the lateral hypothalamic area and zona incerta. The MCH and NEI peptides were extensively colocalized in neurons in both of these areas. In addition, smaller cell groups in the olfactory tubercle and pontine tegmentum were also positively hybridized for ppMCH mRNA and immunostained for MCH and NEI. Fibers stained for MCH and NEI were similarly, and very broadly, distributed throughout the central nervous system in patterns that generally conformed with known projection fields of the lateral hypothalamic area and zona incerta. A differential distribution was seen in at least one region, the interanterodorsal nucleus of the thalamus, which contained a prominent terminal field stained for MCH but not NEI. At the electron microscopic level, MCH-stained perikarya displayed a prominent staining associated with the Golgi apparatus; this was not encountered in NEI-stained cells. Both peptides were distributed similarly in terminals in the lateral hypothalamic area and median eminence, with staining associated principally with dense-cored vesicles. The results suggest that ppMCH-derived peptides may serve as neurotransmitters or modulators of prominence in a surprisingly expansive projection field of incerto-hypothalamic neurons. The terminal distributions of this system seem most compatible with functional roles in generalized arousal and sensorimotor integration, processes previously implicated as being subject to modulation by the lateral hypothalamic area. © 1992 Wiley-Liss, Inc.

Key words: hypothalamus, lateral hypothalamic area, neuropeptides, neurosecretory neurons, zona incerta

Melanin-concentrating hormone (MCH) is a cyclic heptadecapeptide isolated from the pituitary of teleosts, which plays a role in regulating adaptive changes in pigmentation in fishes, amphibians, and reptiles (Kawauchi et al., '83). In teleosts, MCH promotes melanin granule aggregation, while opposing actions (i.e., melanin dispersion) have been reported in amphibians and reptiles (see Baker, '88, '91; and Eberle, '88, for reviews). MCH is also a potent inhibitor of corticotropin-releasing factor (CRF)-induced adrenocorti-

cotrophic hormone (ACTH) secretion in teleost pituitary, in vitro (see Baker, '88, '91; Eberle, '88), and may have a similar, though much weaker, action in the rat (Baker et al., '85).

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Fig. 1. 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 positions of basic residues representing possible proteolytic cleavage sites are represented by vertical lines. C, cysteine residues; ■, amidation site.

Antisera raised against salmon MCH (sMCH) have been used to demonstrate the presence of immunologically related peptides in the central nervous systems of several vertebrate classes, including mammals. In the best-studied species, the rat, sMCH-immunoreactive (ir) cells have been localized to a large population of neurons found exclusively in the diencephalon and centered in distinctive territories of the lateral hypothalamic area and zona incerta (e.g., Fellmann et al., '87; Nahon et al., '89a; Saper et al., '86; Skofitsch et al., '85; Zamir et al., '86). Ostensibly in keeping with the above-mentioned endocrine roles of MCH in lower vertebrates, most of these neurons were also reported to stain positively with antisera directed against α -melanocyte stimulating hormone (α -MSH), rat CRF (rCRF), and human growth hormone-releasing factor (hGRF) (Fellmann et al., '85, '86, '87; Guy et al., '80; Kawano et al., '88; Naito et al., '86; Pelletier et al., '87; see also Saper et al., '86; Watson and Akil, '79, '80). Recent cDNA cloning of the rat hypothalamic MCH precursor predicted the existence of a C-terminal nonadecapeptide (rMCH) whose structure is highly related to that of sMCH (Nahon et al., '89a; see also Fellmann et al., '89; Vaughan et al., '89). In addition, however, rat prepro-MCH (ppMCH) also appears to encode at least one, and perhaps two, additional neuropeptides (see Fig. 1). Immediately preceding the rMCH homologue is a 14-amino acid sequence flanked by paired basic residues and bearing a C-terminal amidation site; by convention (Tatemoto and Mutt, '81), this putative peptide was termed neuropeptide EI, or NEI (Nahon et al., '89a), and recent evidence indicates that this peptide is indeed processed from ppMCH (Parkes et al., '91). Preceding the NEI sequence is a 19-residue stretch bordered by mono- and dibasic residues that might be processed to yield an additional peptide, NGE. Structural similarities were noted between portions of the NEI or NGE sequences and those of each of the three peptides which had been reported to cohabit MCH-ir neurons in the rat, and the results of immunohistochemical competition experiments suggested that cross-reactivity with epitopes on the rMCH precursor could account for α -MSH-, rCRF-, and hGRF-ir described in the dorsolateral hypothalamus (Nahon et al., '89a,b).

While the function of any of the peptides that may be processed from ppMCH in mammals is quite obscure, it seems clear that peptide(s) generated from this precursor provide a neurochemical signature for a large population of cells centered in the lateral hypothalamic area, which has been implicated in a wide variety of functions, ranging from ingestion to sensorimotor integration to arousal to autonomic control (see, e.g., Hosoya and Matsushita, '83; Luiten et al., '86; Swanson, '87). These have proven difficult to sort out experimentally, largely because lateral hypothalamic

neurons are interdigitated among fibers of the medial forebrain bundle (MFB), arguably the most complex fiber system in the mammalian central nervous system (CNS) (Nieuwenhuys et al., '82; Veening et al., '82). Moreover, the strong indications that at least two biologically active peptides (rMCH and rNEI) are generated from a common precursor poses questions as to whether these are necessarily co-distributed within the CNS.

In the present study we employed immunohistochemical and hybridization histochemical methods to provide a general overview of the organization of the prepro-MCH neuronal system(s) in the rat brain. Portions of these data have been reported in abstract form (Bittencourt et al., '90).

MATERIALS AND METHODS

Animals

Young adult male albino (Sprague-Dawley) and pigmented (Long-Evans) rats were used in all experiments. They were group-housed in a vivarium with a 12:12 hour light:dark cycle and had free access to food and water at all times.

Immunohistochemistry

Intact animals, or rats pretreated 48–72 hours earlier with intraventricular or intracisternal injections of colchicine (50 μg in 25 μl saline), were anesthetized with chloral hydrate (35 mg/kg, i.p.) and perfused transcardially with saline followed by 500–700 ml ice-cold fixative. While a variety of different fixation and postfixation protocols were screened, the best results were obtained by using either 4% paraformaldehyde in 0.1 M borate buffer or Zamboni's fixative (Zamboni and De Martino, '67) coupled with short (0–2 hour) postfixation times. Brains were then placed in 10% sucrose in 0.1 M phosphate buffer overnight at 4°C. They were then frozen in dry ice, and multiple 1-in-5 or 1-in-10 series of 30 μm thick frontal sections throughout the brain were saved from a total of 33 animals: 7 normal rats and 6 colchicine-treated rats perfused with Zamboni's fixative, and 9 normal rats and 11 colchicine-treated rats perfused with 4% paraformaldehyde. Eight representative levels of the spinal cord were sampled in five of these animals. Additional, more closely spaced, series of sections through regions of interest or ambiguity were available from some 30 animals exposed to varying pretreatment and fixation schedules.

Typically, one series of sections was stained by conventional avidin-biotin immunoperoxidase or immunofluorescence protocols (Gerfen and Sawchenko, '84; Sawchenko et al., '90a) for rMCH, one for NEI, and an intervening one with thionin for reference.

Abbreviations

III	occulomotor nucleus	MG	medial geniculate complex
v	trigeminal nerve	MH	medial habenula
VII	facial nucleus	MidThal	midline thalamic nuclei
XII	hypoglossal nucleus	mlf	medial longitudinal fasciculus
AAA	anterior amygdaloid area	MM	medial mammillary nucleus
ac	anterior commissure	MML	medial mammillary nucleus, lateral part
Acb	nucleus accumbens	MMM	medial mammillary nucleus, medial part
AD	anterodorsal nucleus (thalamus)	MoV	motor nucleus of the trigeminal nerve
AHA	anterior hypothalamic area	mp	mammillary peduncle
AM	anteromedial nucleus (thalamus)	MPO	medial preoptic area
Amyg	amygdala	mr	mammillary recess (third ventricle)
AON	anterior olfactory nucleus	MR	median raphe nucleus
ARH	arcuate nucleus	MS	medial septal nucleus
aq	aqueduct of Sylvius	mtt	mammillothalamic tract
AT	axon terminal	MV	medial vestibular nucleus
AV	anteroventral nucleus (thalamus)	N	nucleus
ax	axon	NA	nucleus ambiguus
Bar	Barrington's nucleus	Nu	nucleolus
BLA	basolateral nucleus (amygdala)	NEI	neuropeptide EI
BST	bed nucleus of the stria terminalis (p, posterior part)	NTB	nucleus of the trapezoid body
bp	brachium pontis	NTS	nucleus of the solitary tract
C	cuneate nucleus	OB	olfactory bulb
CA1-3	hippocampal pyramidal cell fields	och	optic chiasm
CeA	central nucleus (amygdala)	ot	optic tract
CeM	central medial nucleus (thalamus)	OT	olfactory tubercle
CBL	cerebellum	OVLt	vascular organ of the lamina terminalis
cc	corpus callosum	PB	parabrachial nuclei
CG	central gray	PF	parafascicular nucleus (thalamus)
CLA	claustrum	PG	pontine gray
CN	cochlear nuclei	PH	prepositus nucleus
Cnf	cuneiform nucleus	PHA	posterior hypothalamic area
CoA	cortical nucleus (amygdala)	Pir	piriform cortex
CoAp	posterior cortical nucleus (amygdala)	PMv	ventral premammillary nucleus
cp	cerebral peduncle	Po	posterior nuclear complex (thalamus)
CP	caudoputamen	POA	preoptic area
CTF	central tegmental fields	PP	posterior pituitary
CTX	cerebral cortex	PPN	pedunculopontine nucleus
D	dendrite	PreCBL	precerebellar nuclei
DBB	nucleus of the diagonal band	Pretect	pretectal area
DCV	dense-cored vesicle	PSV	principal sensory nucleus of the trigeminal nerve
DG	dentate gyrus	PT	parataenial nucleus (thalamus)
DHA	dorsal hypothalamic area	PVa,p	periventricular nuclei (anterior, posterior)
DLG	dorsal lateral geniculate	PVH	paraventricular nucleus (hypothalamus)
DLL	dorsal nucleus of the lateral lemniscus	PVT	paraventricular nucleus (thalamus)
DMH	dorsomedial nucleus (hypothalamus)	py	pyramidal tract
DR	dorsal raphe nucleus	rb	restiform body
DTN	dorsal tegmental nucleus	Re	nucleus reuniens (thalamus)
EC	external cuneate nucleus	rer	rough endoplasmic reticulum
EPi	endopiriform nucleus	RM	nucleus raphe magnus
FF	fields of Forel	RN	red nucleus
fi	fimbria	RT	reticular nucleus (thalamus)
fm	forceps minor	SC	superior colliculus
fr	fasciculus retroflexus	SCN	suprachiasmatic nucleus
fx	fornix	SEP	septal nuclear complex
G	gracile nucleus	SF	septo-fimbrial nucleus
Go	Golgi apparatus	SI	substantia innominata
GP	globus pallidus	sm	stria medullaris
gvii	genu of the facial nerve	SN	substantia nigra
Hab	habenula	SNr	substantia nigra, pars reticulata
HF	hippocampal formation	SNV	spinal nucleus of the trigeminal nerve
HYP	hypothalamus	SO	supraoptic nucleus (r, retrochiasmatic part)
IAD	interanterodorsal nucleus (thalamus)	SOC	superior olivary complex
ic	internal capsule	Sp.Cd.	spinal cord
IC	inferior colliculus	SpV	spinal vestibular nucleus
IO	inferior olivary complex	st	stria terminalis
IPN, IP	interpeduncular nuclear complex	STN	subthalamic nucleus
LA	lateral nucleus (amygdala)	stv	spinal tract of the trigeminal nerve
LD	laterodorsal nucleus (thalamus)	SubThal	subthalamic region
lfp	longitudinal fasciculus of the pons	SUM	supramammillary nucleus
LDT	laterodorsal tegmental nucleus	tb	trapezoid body
LH	lateral habenula	TM	tuberomammillary nucleus
LHA	lateral hypothalamic area	TRN	tegmental reticular nucleus
LM	lateral mammillary nucleus	TT	taenia tecta
lot	lateral olfactory tract	uD	unlabeled dendrite
LPO	lateral preoptic area	V3,4	third, fourth ventricle
LRN	lateral reticular nucleus	Vest	vestibular nuclei
LS	lateral septal nuclei	VL	ventrolateral nucleus (thalamus)
LV	lateral ventricle	VLL	ventral nucleus of the lateral lemniscus
Mam	mammillary complex	VLG	ventral lateral geniculate
MCH	melanin-concentrating hormone	VMH	ventromedial nucleus (hypothalamus)
MD	mediodorsal nucleus (thalamus)	VTA	ventral tegmental nucleus
me	median eminence	wm	white matter
MeA	medial nucleus of the amygdala	ZI	zona incerta
MePO	median preoptic nucleus		

Sequential Dual Immunofluorescence Labeling

This procedure was used to evaluate the extent to which rMCH and rNEI might be colocalized in individual hypothalamic neurons. Fixation and tissue processing protocols were as described above, save that thinner sections (10–15 μm) were cut, and were exposed to an initial primary antiserum for only 1 hour at room temperature. The primary antisera were localized by exposing the sections for 1 hour to a 1:200 dilution of affinity purified, fluorescein-conjugated goat anti-rabbit IgG (Tago, Inc., Burlingame, CA). The distribution of labeled cells in the hypothalamus was recorded photographically at $25\times$ in each section with Ilford XP-1 film. The reagents were then eluted from the tissue by a minor modification of the method of Tramu et al. ('78) described elsewhere (Sawchenko et al., '84, '90b). The material was then re-incubated in the secondary antiserum and re-examined. Any signal in cells or fibers was taken as evidence that the elution had been ineffective, and the material was either re-eluted or discarded. Material from which the immunoreagents were effectively removed was then incubated first in the polyclonal antiserum to which it had not yet been exposed (rMCH or rNEI), and then in the anti-rabbit secondary antiserum. The distribution was re-photographed.

To control for diminution of reactivity as a function of subsection to the elution protocol, the sequence with which the antisera were applied to sections was varied systematically within animals.

Colocalization patterns were analyzed photographically. Enlargements (5×7 inches) of each frame from good experiments were made, and each cell was scored for its peptide complement. Estimated counts were extrapolated from the number of labeled cells detected on one side of the brain in a 1-in-5 series of 10 or 15 μm thick sections, and corrected for double-counting errors (Abercrombie, '46).

Concurrent Dual Immunofluorescence Labeling

Because the distribution of ppMCH-derived peptide overlapped in part with those of other neurochemically defined systems, possible colocalization was evaluated in selected instances. This involved staining with a mouse-derived monoclonal antibody against serotonin (Dako, Inc., Carpinteria, CA) or with a rat-derived monoclonal against choline acetyltransferase (CAT; a marker for cholinergic neurons; Eckenstein et al., '81; Eckenstein and Thoenen, '82). Each was applied at a 1:500 dilution in a cocktail with one of the rabbit-derived polyclonal antisera to a ppMCH-derived peptide. Localization was achieved with a mixture of the fluorescein-labeled goat anti-rabbit IgG and species-specific, affinity-purified, rhodamine-labeled goat anti-mouse (or rat) IgGs (American Qualex, Inc., La Mirada, CA). Controls to ensure non-cross-reactivity of secondary antisera with the inappropriate primary, or with each other, were carried out as described (Sawchenko et al., '85, '90b), and were negative.

Ultrastructural Immunolabeling

The perfusion-fixation method employed was based on a protocol described by Pickel and colleagues ('86). Rats were deeply anesthetized and then perfused through the ascending aorta over the course of 25 minutes with 50–75 ml saline, approximately 100 ml of 3.75% acrolein, 2.0%

paraformaldehyde, and 0.1–0.25% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4, and then 500 ml of 2.0% paraformaldehyde in the same buffer, with the aid of a peristaltic pump. Hypothalamic blocks were excised, bisected along the midline, and postfixed in 2.0% paraformaldehyde for 2–3 hours. The blocks were rinsed in buffer, and at least two series of 50 μm thick coronal sections were cut through the hypothalamus from each block on a vibratome and collected in 0.05 M potassium phosphate buffered saline (KPBS).

The sections were pretreated first in 0.3% hydrogen peroxide for 20 minutes, followed by 5×5 minute rinses in KPBS, and then in 1.0% sodium borohydride for 15 minutes, followed by $6-8 \times 10$ minute rinses in KPBS. Avidin-biotin-immunoperoxidase staining was again carried out as described (Gerfen and Sawchenko, '84; Sawchenko et al., '90a), with the reaction product developed by a nickel-enhanced glucose oxidase method (Shu et al., '88). After final (5×5 minute) rinses in buffer, the sections were postfixed in 0.75% osmium tetroxide for 45 minutes, dehydrated, and infiltrated with Spurr's resin. Sections were flat-embedded overnight at 60°C between two tightly clamped slides, one of which was coated with mold-release compound. Useful sections were reembedded in Epon 812 and trimmed, and pale gold ultrathin sections were taken, collected on grids, counterstained with uranyl acetate and lead citrate, and examined on a Zeiss EM 109 transmission electron microscope. Observations reported here are based principally on analysis of thin sections obtained from the best 4 of over 20 animals initially perfused for fine structural examination.

Antisera and Controls

Three antisera against rMCH were raised in rabbits by immunization with synthetic peptide conjugated to human α -globulins via glutaraldehyde. Three antisera were raised against Tyr-Gly-rNEI conjugated to the same carrier by bisdiazotized benzidine. The conjugation and injection protocols have been described in detail elsewhere (Vale et al., '86). All antisera were preadsorbed against the human α -globulin carrier (5 mg/ml serum for 24 hours at 4°C) prior to use. While all antisera proved effective in specifically labeling cells in expected locations in the dorsolateral hypothalamus, the most effective of these sera—PBL-234 (MCH) and PBL-237 (NEI)—in initial screenings were used throughout the remainder of the study. For all applications, both antisera were used at a dilution of 1:10,000. Controls for specificity involved incubating primary antisera overnight at 4°C with 4–400 μM concentrations of their respective synthetic immunogens, or of various structurally related peptides, some of which have been reported to be localized in the zona incerta and lateral hypothalamic area. The results of these competition studies are given in Table 1.

Hybridization Histochemistry

Hybridization histochemical localization of ppMCH mRNA was accomplished with ^{35}S -labeled cRNA probes. Perfusion (4% paraformaldehyde only) and histology were carried out as described above, and full or partial series of sections through the brains and spinal cord of over 30 animals were available for analysis. Techniques for probe synthesis, hybridization, and autoradiographic localization of mRNA signal have been described previously (Simmons et al., '89). Briefly, tissue was processed as described above,

TABLE 1. Competition Studies to Characterize rMCH and rNEI Antisera¹

Antiserum	Competing peptide	Concentration		
		400 μ M	40 μ M	4 μ M
rMCH	rMCH	-	-	-
	sMCH	++	++	++++
	rNEI	++++	++++	++++
	rNGE	++++	++++	++++
	rCRF	++++	+++	++++
	α MSH	++++	++++	++++
	hGRF ₁₋₄₀	++++	++++	++++
	rANF ₁₋₂₈	++++	++++	++++
	rNEI	-	-	+
	rMCH	++++	++++	++++
rNEI	rNGE	++++	++++	++++
	rCRF	++++	++++	++++
	hGRF ₁₋₄₀	++++	++++	++++
	α MSH	+++	+++	++++
	rANF ₁₋₂₈	++++	++++	++++
	Val-NH ₂	++++	++++	++++
	Ile-NH ₂	++++	+++	++++
	Pro-Val-NH ₂	++++	++++	++++
	Pro-Ile-NH ₂	+++	++++	++++
	rMCH	++++	++++	++++

¹Staining intensity was rated blind by using a 5-point scale, with ++++ denoting labeling indistinguishable from that achieved with control (non-preadsorbed) antisera, and - indicating a complete absence of specific labeling.

and then mounted onto poly-L-lysine-coated slides. These sections were then digested with 10 μ g/ml of proteinase K for 30 minutes, at 37°C. Antisense and sense (control) ppMCH probes were generated from a 740 bp cDNA (pRMCH11; see Nahon et al., '89a) encompassing the complete coding region of the MCH precursor and all of the 3'- and a portion of the 5'-untranslated regions, that was ligated into a Bluescript SK (Stratagene) transcription vector that contains the bacteriophage T3 and T7 promoters in opposite orientations. Labeled antisense and sense probes were synthesized by linearizing the plasmid with Sma I or Hind III, respectively, and then using the appropriate RNA polymerases (T3 for antisense, T7 for sense) and ³⁵S-UTP.

The probes were used at concentrations of about 10⁷ cpm/ml. Probes were applied to sections overnight at 56–58°C in a solution containing 50% formamide, 0.3 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 0.05% tRNA, 10 mM dithiothreitol, 1 $\times$ Denhardt's solution, and 10% dextran sulfate, after which they were treated with 20 μ g/ml of ribonuclease A for 30 minutes, at 37°C, and washed in 15 mM NaCl/1.5 mM sodium citrate, at 55–60°C. Sections were then dehydrated and exposed to X-ray films for 1–4 days. Sections were defatted in xylene, rinsed in absolute ethanol, air dried, coated with Kodak NTB-2 liquid autoradiographic emulsion, and exposed at 4°C, in the dark and desiccated, for 4 days to 3 weeks. They were then developed with Kodak D-19 for 3.5 minutes at 14°C, rinsed briefly in distilled water, fixed with film strength Kodak rapid fixer for 2 minutes at 14°C, and washed in distilled water for at least 1 hour. Adjacent sections were stained with thionin and used to identify cytoarchitectonic features for reference purposes.

RESULTS

Antiserum Specificity

The results of competition studies used to characterize the rMCH and rNEI antisera are given in Table 1. Staining for rMCH was completely abolished by preincubation of the antiserum (code PBL 234) with concentrations of the synthetic immunogen in the low micromolar range. Surprisingly, sMCH, which shares 13 of 19 residues in common

with rMCH (68% homology; Nahon et al., '89a), was relatively ineffective in blocking staining, suggesting that epitopes at or near the N-terminus of rMCH are important for staining using this antiserum. None of the peptides previously colocalized with sMCH in rat dorsolateral hypothalamic neurons competed effectively. Atrial natriuretic factor₁₋₂₈, which shares 9 of 19 amino acids in common with rMCH (Presse et al., '91) and has been localized in some cells in the lateral hypothalamic area, was also inert in these competition studies.

The anti-NEI (code PBL 237) serum employed here also proved to be quite specific. Only α -MSH, which shares a C-terminal bulky aliphatic amide motif in common with rNEI, interfered at all with staining for NEI-ir, and this was manifest as a mild attenuation at concentrations at which NEI completely abolished staining. Consistent with this, the amidated C-terminal dipeptides of α -MSH (Pro-Val-NH₂) or rNEI (Pro-Ile-NH₂), which effectively block α -MSH immunostaining in the dorsolateral hypothalamus (Nahon et al., '89a; see also Khachaturian et al., '86), also failed to interfere significantly with localizations of rNEI-ir. Nonetheless, using anti-NEI we did note in the brains of some colchicine pretreated, but not unmanipulated, rats faint staining of cells in the arcuate nucleus. This suggests that our anti-NEI serum contains antibodies that cross-react with the C-terminal aliphatic amide of α -MSH. For this reason, and because pretreatment with colchicine did not ostensibly affect our ability to detect NEI-ir perikarya in the hypothalamus, the bulk of our analyses made use of material from nonpretreated rats. As an additional control, we compared staining for NEI in adjacent series of sections from colchicine-treated and nonpretreated rats prepared with antiserum that was or was not preadsorbed with 40 μ M synthetic α -MSH. Apart from the abolition of staining of presumed POMC cells in the arcuate nucleus of colchicine-treated animals, no other differences in the pattern or relative intensities of perikaryal or fiber staining were noted using adsorbed and nonadsorbed anti-NEI preparations.

Cellular Localization of ppMCH Derived Peptides and mRNA

Antisera raised against rMCH and rNEI proved effective in robustly labeling cell bodies in the hypothalamus and zona incerta whose distribution was similar to that described previously using antisera raised against teleost MCH (Fellmann et al., '87; Nahon et al., '89a; Skofitsch et al., '85; Zamir et al., '86). Perikarya stained for either peptide were equally well visible in colchicine-pretreated and nonpretreated rats, though their morphological appearance differed under these conditions. In unperturbed rats, hypothalamic cells stained for either peptide (Fig. 2) were medium-sized and multipolar to fusiform in shape, typically giving rise to 2–5 primary dendrites that were either smooth or only very sparsely invested with dendritic spines; secondary dendritic branching was seen commonly, while third order division was more rarely encountered. Where evident, varicose axons arose typically from labeled somata or the proximal portion of primary dendrites. In rats pretreated with colchicine, MCH- and NEI-stained cells assumed a very different appearance (Fig. 2). Stained cells were more irregularly shaped, and moderately invested with somatic and dendritic protuberances; those on the dendrites resembled large dendritic spines. Such colchicine-dependent alterations in morphology are reminiscent of

Fig. 2. Hypothalamic MCH-ir perikarya in normal and colchicine-treated rats. Brightfield photomicrographs of avidin-biotin-immunoperoxidase material, showing rMCH-ir cells in the lateral hypothalamic area of nonmanipulated (A, C) and colchicine-pretreated (B, D) rats. Drug treatment results in the appearance of dendritic and somatic spines and other protuberances. A and B, $\times 300$; C and D, $\times 750$.

those described in parvocellular neurosecretory neurons by Rho and Swanson ('89).

Hypothalamus and zona incerta. Diencephalic ppMCH-expressing cells were distributed continuously in the rostro-caudal dimension from the level of the caudal aspect of the paraventricular nucleus (PVH) to just beyond the mammillary recess (Fig. 3). They nonetheless could be localized to at least six cytoarchitectonically distinct foci; the rostromedial part of the zona incerta, three distinct aspects of the lateral hypothalamic area, the anterior periventricular nucleus, and a portion of the tuberomammillary complex.

At the most rostral levels of the ppMCH distribution, stained cells within the medial aspect of the zona incerta predominate. At their medialmost extreme these were found to abut, but never fall within the morphological borders of the PVH. Just caudal to this, at the level of the dorsomedial nucleus (Fig. 3C), three additional features of the distribution become apparent. First is a relatively small population of cells that lies within the anterior periventricular nucleus, and is concentrated in its dorsal aspect (Fig. 3C-E). Second is a population of larger cells that lie just medial to the medial edge of the internal capsule, and correspond to a cluster of darkly stained neurons in adjoining Nissl preparations (Fig. 3C,D). Third is a more diffusely organized group of cells in the dorsolateral part of the lateral hypothalamic area (LHA), just ventral to the zona incerta, that persists throughout the remainder of the rostrocaudal extent of the distribution (Fig. 3C-F). At more caudal levels, these cells lie just medial to the subthalamic

nucleus. This dorsolateral hypothalamic group appears to comprise the numerically predominant grouping of ppMCH-expressing cells in the diencephalon. Finally, a group of cells in the perifornical region is evident throughout much of the middle half of the rostrocaudal extent of the ppMCH distribution.

Near the caudalmost extension of the ppMCH distribution, a feature not previously recognized using antisera against sMCH (Skofitsch et al., '85) was noted. This was a cluster of labeled cells localized just lateral to the dorsal tip of the third ventricle just rostral to the level of the mammillary recess (Fig. 3F), corresponding to a portion of the tuberomammillary complex (the tuberal magnocellular nucleus of Bleier et al., '79; see also Fig. 1 of Staines et al., '87). This cell group was apparent in immunohistochemical preparations stained with either anti-rMCH or -rNEI and in hybridization histochemical material. Never, however, were positively immunostained or hybridized cells apparent in what is now commonly known as the tuberomammillary nucleus, proper (the caudal magnocellular nucleus of Bleier et al., '79), i.e., the thin sheet of cells lying just deep to the pial surface at and around the level of the supramammillary nucleus (see Köhler et al., '85).

In addition to these localizations in more-or-less well defined regions, scattered cells positively stained or hybridized for ppMCH markers were invariably detected in the medial zone of the hypothalamus, where they typically lay in the neuropil surrounding the ventral aspect of the PVH, the dorsolateral margin of the ventromedial nucleus, the

Fig. 3. Diencephalic distribution of cells expressing ppMCH-derived peptides. Brightfield photomicrographs of immunoperoxidase material stained for NEI-ir, and adjoining Nissl sections, to show key features of the distribution of labeled perikarya. The distribution of MCH-ir, as seen in adjacent series of sections, was indistinguishable from that of NEI. All micrographs $\times 30$.

ventrolateral part of the dorsomedial nucleus, and within the posterior hypothalamic area, proper. At all levels within the diencephalon, the distribution and density of cells positively immunostained for MCH- and NEI-ir or positively hybridized for ppMCH mRNA were similar (Fig. 4).

Olfactory tubercle. In addition to these generally predictable sites of ppMCH-derived peptide and mRNA expression in the diencephalon, two additional, and much smaller, populations were seen consistently in immunostained material obtained from colchicine-treated animals and in hybridization histochemical material from nonpretreated rats (Fig. 5). One was centered in the olfactory tubercle, and consisted of small multipolar cells scattered principally

through the polymorph (or multiform) layer of Millhouse and Heimer ('84). A smaller number of positively stained or hybridized cells was seen at the periphery of the dense cell layer; only very rarely were positively immunostained or hybridized cells found clearly within the substance of the islands of Calleja. This basal forebrain ppMCH-expressing cell group was not strictly limited to the olfactory tubercle, but spread dorsally and caudally to include small numbers of cells in the substantia innominata and the anterior amygdaloid area, respectively.

Pons. A second unexpected site of ppMCH gene and peptide expression was encountered in a nondescript region of the paramedian pontine reticular formation (Fig. 5). A

Figure 3 continued

cluster of positively immunostained or hybridized neurons was seen straddling the midline and embedded within the decussation of the superior cerebellar peduncle beginning at the level of the caudal pole of the ventral tegmental nucleus of Gudden and extending in an analogous position caudally to the level of the caudal aspect of Barrington's nucleus. For the most part, these MCH-expressing cells were not localizable to any recognized cell group in the region, though isolated cells were seen within the confines of the dorsal raphe nucleus and the pontine central gray. Near its caudal extreme, a finger-like dorsolateral extension of this cell cluster comprising 3–6 cells per section per side was reliably seen in the pontine central gray between Barrington's nucleus and the dorsal tegmental nucleus. Because of their proximity to major groupings of pontine

serotonergic and cholinergic neurons, concurrent dual immunofluorescence staining was carried out in series of sections through the pons obtained from colchicine-treated rats using (rabbit) antisera against rMCH or rNEI with monoclonal antibodies against serotonin ($n = 3$) or choline acetyltransferase ($n = 2$). No individual cells staining for a ppMCH-derived peptide and either "classical" neurotransmitter were identified.

The basal forebrain (olfactory tubercle) ppMCH-expressing cell group was best visualized in animals pretreated with colchicine via the lateral ventricle, though a fraction of this complement was seen occasionally in nonpretreated rats. The pontine immunolocalizations were detected only in colchicine-treated rats, and were most effectively visualized in animals treated with the drug via the cisternum

Fig. 4. Comparison of the hypothalamic distributions of MCH- and NEI-ir and ppMCH mRNA. Brightfield photomicrographs of adjoining sections stained for MCH- and NEI-ir and a darkfield photomicrograph of a section through a comparable level of the hypothalamus, showing cells positively hybridized by using an antisense probe for ppMCH mRNA. The essential features of all three distributions are similar. These include positively labeled cells of comparable relative densities lining the medial edge of the internal capsule, in the dorsolateral and perifornical regions of the lateral hypothalamic area, and in the anterior periventricular nucleus. All micrographs $\times 35$.

magnum. In any given preparation, MCH- and NEI-ir cells were detected in comparable numbers and with similar distributions in both the olfactory tubercle and pons (Fig. 5). Compatible with these indications that the basal forebrain and pontine cell groups represent relatively low abundance sites of MCH and NEI expression, hybridization signal for ppMCH mRNA in both regions was consistently

lower than that seen in the hypothalami obtained from the same animals, and frequently required longer exposures (14–21 days) for optimal detection in autoradiograms.

Colocalization of MCH- and NEI-ir in the Hypothalamus

A sequential dual immunofluorescence staining method was used to examine the extent to which MCH- and NEI-ir are colocalized in the lateral hypothalamus and zona incerta. Complete series of sections from the level of the paraventricular nucleus of the hypothalamus to the mammillary recess were analyzed quantitatively in 4 rats, and partial series from 6 others were used to confirm the essential pattern of colocalization. We consistently noted a decrement (9–20%) in the number of cells that could be stained with the antiserum applied to tissue that had undergone the elution procedure. As a consequence, we evaluated the percentage of cells that were detected using the antiserum that was applied to tissue second (i.e., after elution) that also stained positively with the antiserum that was applied first (before elution). Using this approach, nearly all cells ($96 \pm 3\%$, mean $\pm$ SEM, $n = 6$ series obtained from 4 rats; 4 with MCH stained first, 2 with NEI stained first) stained positively for both MCH- and NEI-ir (Fig. 6). Those few that did not displayed no distinctive topography, and we are inclined to view these isolated instances of singly labeled cells as attributable to technical limitations, such as a failure to precisely duplicate the plane of focus for photomicrographs used for analysis, rather than to an inability of any numerically significant or topographically distinct population of dorsolateral hypothalamic neurons to express both peptides.

Distribution of MCH- and NEI-ir Fibers in Brain and Pituitary

Every commonly recognized cell group and cortical field in the rat brain was found to contain at least some MCH- and NEI-ir fibers. The relative abundance of axons and varicosities stained for these peptides in a number of brain regions is summarized in Table 2. Adjacent series of sections from individual animals stained separately for MCH and NEI revealed nearly identical staining patterns and intensities (see also Fig. 8, below). As a consequence, a series of low magnification photomicrographs of only one of the peptide distributions (NEI) is shown to provide an overview of the system (Fig. 7). The narrative that follows is not intended to provide a detailed recantation of the distribution of MCH- and NEI-ir fibers in every cell group of the rat brain, but rather to highlight general organizational features.

Comparison of MCH and NEI innervation patterns. As is evident from the data summarized in Table 2, the nuclear and subnuclear distributions, and relative densities, of MCH- and NEI-ir fibers in nearly all regions of the rat brain are indistinguishable. Some examples of adjoining sections from individual animals stained separately for the two peptides are provided in Figure 8. Only a single cell group was encountered in which the densities of MCH- and NEI-ir fibers were clearly discrepant. This occurred in the interanterodorsal nucleus of the thalamus (this cell group corresponds to Gurdjian's [27] nucleus of the interanterodorsal commissure), which contained a discrete and moderately dense field of punctate MCH-ir terminals and only widely scattered NEI-ir fibers (Fig. 9). To provide an additional assessment of the validity of MCH-ir at this

Fig. 5. Novel sites of ppMCH gene and peptide expression in the basal forebrain and pons. **Top:** Olfactory tubercle. Darkfield photomicrograph showing ppMCH mRNA signal in the olfactory tubercle (A); an adjoining Nissl-stained section is shown for reference (A'). Panel B shows, at somewhat higher magnification, MCH-ir perikarya at a similar locus. **Bottom:** Pons. Positively hybridized cells are seen (C) just deep to the pontine central gray at the level of the laterodorsal and dorsal tegmental nuclei (C'). Cells immunostained for NEI-ir (D) are detectable in a comparable region in material from a colchicine-treated rat. A and A', $\times 35$; B, $\times 75$; C and C', $\times 70$; D, $\times 115$.

Fig. 6. Colocalization of MCH- and NEI-ir in the diencephalon. Fluorescence photomicrographs of a single field near the rostral extreme of the ppMCH distribution in the rat diencephalon that was stained sequentially for MCH-ir and then NEI-ir. Arrows indicate examples of individual cells stained for both peptides. The third ventricle is at the lower left. Both micrographs $\times 100$.

locus, we compared in two animals the staining for MCH-ir fibers in the interanterodorsal nucleus and elsewhere in the diencephalon using antisera that were preadsorbed with five different concentrations of synthetic rMCH ranging in half order of magnitude increments between 4 and 400 μ M, and found no differences. That is, the degree of competition by each of these concentrations of rMCH for MCH immunostaining within and beyond the interanterodorsal nucleus was similar, supporting the specificity of MCH staining at this site.

Medial forebrain bundle. Ascending and descending components of the medial forebrain bundle far and away comprise the predominant fiber path taken by axons stained for ppMCH-derived peptides. The ascending fibers of incerto-hypothalamic origin occupy a broad expanse of the MFB, though they are preferentially concentrated in the ventromedial aspect of compartment "c" of the MFB as described by Nieuwenhuys et al. ('82). Throughout the initial course of their ascent, axons exit laterally via the ansa peduncularis to reach the amygdala, basal ganglia, and substantia

TABLE 2. Relative Densities¹ of MCH- and NEI-ir Fibers in Rat Brain Cell Groups²

Cell group	MCH	NEI	Projection Field of (ref.) ^{3,4}		Cell group	MCH	NEI	Projection Field of (ref.) ^{3,4}	
			LHA	ZI				LHA	ZI
I. Forebrain									
A. Isocortex			2,5,6		3. Bed n. stria terminalis			1,4,5,7	
I	++	++			Rostromedial region	+++	+++		
II	++	++			Rostrolateral region	++	++		
III	+	+			Posterodorsal region	++	++		
IV	+	+			Posteroventral region	+++	+++		
V	++	++			4. Bed n. anterior commissure	++	++		
VI	++	++			5. Septofimbrial nucleus	+++	+++		
Clastrum	+++	+++	5,7		6. Triangular nucleus	+++	+++		
B. Olfactory regions					7. Subfornical organ	+	+		
1. Main bulb			7		F. Basal ganglia				
Olfactory nerve layer	-	-			1. Caudoputamen	+	+	5,7	
Periglomerular layer	+	+			Posteroventral part	++	++		
Outer plexiform layer	+	+			Nucleus accumbens	++	++	5,7	
Mitral layer	+	+			Fundus of striatum	+++	+++		
Inner plexiform layer	+	+			2. Globus pallidus	++	++	4,5,7	3
Granular layer	++	++			Entopeduncular n.	++	++		
2. Anterior olfactory n.	++	++	7		Substantia innominata	++	++	1,4,5,7	
3. Olfactory tubercle			5		Magnocellular preoptic nucleus	++	++	4,5	
I	++	++			3. Subthalamic nucleus	++	++	4,5,2	
II	+	++			4. Substantia nigra				
III	++	++			Compact part	++	++	4,5,6,8	
4. Piroform cortex			5		Reticular, lateral parts	+	+	4,5,6,8	
I	++	++			Ventral tegmental area	+++	+++	1,2,4,5,6,8	
II	++	++			G. Thalamus				
III	++	++			1. Medial habenula	++	++		
Endopiriform nucleus	+++	+++			2. Lateral habenula	++	++	1,2,7	
5. Taenia tecta	++	++	5		3. Anterior group			1	
C. Hippocampal formation (cortex)					Anteroventral n.	++	++		
1. Entorhinal area (lateral and medial)			5,6,7		Anteromedial n.	++	++		
I	++	++			Anterodorsal n.	+	+		
II	+	+			Interanteromedial n.	++	++		
III	+	+			Interanterodorsal n.	+	+++		
IV-VI	++	++			4. Mediodorsal nucleus			2	
2. Subiculum (dorsal)			5		Medial part	++	++		
Molecular layer	++	++			Central part	++	++		
Stratum radiatum	++	++			Lateral part	+	+		
Pyramidal layer (deep)	++	+			5. Lateral group			1	
Pyramidal layer (super)	++	++			Lateral dorsal n.	++	++		
Subiculum (ventral)			5		Lateral posterior n.	+	+		
Molecular layer	++	++			6. Midline group				
Stratum radiatum	+	+			Paraventricular n.	++	++	1,2,4,5,7	3
Pyramidal layer	+	++			Parastaenial n.	++	++	7	
3. CA ₁			2,5,7		N. reuniens	++	++	7	
S. lacunosum-moleculare	++	++			Rhomboid n.	+++	+++	1,2	
Stratum radiatum	++	++			N. gelatinosa	++	++		
Pyramidal layer	+	+			7. Ventral group				3
Stratum oriens	++	++			Ventral anterior/v. lat.	+	+		
4. CA ₃			2,5,7		Ventral medial	++	++		
S. lacunosum-moleculare	++	++			Ventral posterior	+	+		
Stratum radiatum	++	++			Gustatory nucleus	+	+		
Pyramidal layer	++	+			8. Posterior complex	++	++		
Stratum oriens	+++	+++			9. Medial geniculate n.				
5. Dentate gyrus			2,5,7		Dorsal part	+	+		
Molecular layer	+	+			Ventral part	+	+		
Granular layer	+	+			Medial part	++	++		
Polymorph layer	++	++			10. Lateral geniculate n.				
6. Induseum griseum/fasciola cinerea	++	++			Dorsal part	++	+		
D. Amygdala					Intergeniculate leaflet	++	++		
1. Medial nucleus			1,2,5,7		Ventral part	+++	++	1	
Anterior part	+	++			11. Intralaminar nuclei			1,2,5,7	3
Posterodorsal part	+	+			Central medial n.	++	++		
2. Amygdalohippocampal area	++	++	5		Paracentral n.	+	+		
3. Cortical nucleus			2,5		Central lateral n.	+	+		
Anterior part	++	++			Parafascicular n.	++	++		
Posterior part	++	++			12. Reticular nucleus	++	++	1,7	
4. N. lat. olfactory tract	++	++			13. Zona incerta			2,5,7	3
5. Anterior amygdaloid area	++	++	7		Rostral	+++	+++		
6. Central nucleus	++	++	1,5,7		Caudal	++	++		
7. Lateral nucleus	++	++	2,5		14. N. fields of Forel	+	+		
8. Basolateral nucleus	++	++	2		15. Pineal	+	+		
9. Basomedial nucleus	++	++			H. Hypothalamus				
10. Intercalated nuclei	+++	+++			1. Periventricular zone				
E. Septum					Median preoptic n.	+	+		
1. Lateral nucleus			1,5		Anteroventral periventricular n.	+++	+++		
Dorsal part	++	+++			Preoptic periventricular nucleus	++	++	1	
Intermediate part	++	++			Suprachiasmatic n.	++	++		
Ventral part	+++	+++			Supraoptic nucleus	+	+		
2. Medial n. n. diagonal band	++++	++++	1,4,5,7		Paraventricular n.			1,7	
					Autonomic part	++	++		
					Parvicellular part	++	++		
					Magnocellular part	+	+		

(continued)

TABLE 2. Relative Densities¹ of MCH- and NEI-ir Fibers in Rat Brain Cell Groups² (continued)

			Projection Field of (ref.) ^{3,4}					Projection Field of (ref.) ^{3,4}	
Cell group	MCH	NEI	LHA	ZI	Cell group	MCH	NEI	LHA	ZI
H. Hypothalamus (continued)					4. Vestibular				
Anterior periventricular nucleus	++	++	1,4,5,7		Medial n.	++	++		
Arcuate nucleus	++	++	5,7		Lateral n.	++	++		
Posterior periventricular n.	++	++			Superior n.	++	++		
Median eminence			4,7		Spinal n.	++	++		
Internal lamina	++	++			5. Gustatory				
External lamina	+	+			N. solitary tract, ant.	++	+++	1,2	
2. Medial zone					6. Visceral				
Medial preoptic area	++	++	4,5,7		N. solitary tract			1,2,6,8	
Medial preoptic n.			4,5		Medial part	+	+		
Lateral part	++	++			Commissural part	++	++		
Medial part	++	++			Lateral part	++	++		
Central part	+	+			Area postrema	+	+	2	
Anterior hypo. n.			4,7		Parabrachial n.			1,2,4,6,8	3
Anterior part	++	++			Lateral	++	++		
Central, posterior parts	+++	+++			Medial	+++	+++		
Retrochiasmatic area	++	++	4		Kölliker-Fuse n.	++	++		
Ventromedial n.	+	+	4,5,7		B. Motor				
Dorsomedial n.	+++	+++	1,4,5,7		1. Eye				
Tuberomammillary n.	+++	+++	1,4		Oculomotor (III)	++	++	4	
Premammillary n.			1,4,7,8		Trochlear (IV)	++	++		
Dorsal part	++	++			Abducens (VI)	+	+		
Ventral part	+	+			2. Jaw				
Supramammillary n.			1,4,8		Motor n. V	+	+	2	
Lateral part	+++	+++			3. Face				
Medial part	+++	+++			Facial n. (VII)	++	++	2	
Lateral mammillary n.	+	+	1,4,6		4. Pharynx/larynx	+	+	2	
Medial mammillary n.			1		N. ambiguus				
Median part	++	++			5. Tongue				
Medial part	++	++			Hypoglossal n. (XII)	++	++	2	
Lateral part	++++	++++			6. Viscera				
Posterior part	++	++			Dorsal motor n. X	+	+	3	
3. Lateral zone					C. Reticular core (including central gray and raphe)				
Lateral preoptic area	+++	+++	1,4,5,7	3	1. Periaqueductal gray—assoc.				
Lateral hypothalamic a.	++++	++++	1,4,5,7,8	3	w/PAG	+++	+++	1,2,4,5,6,8	3
Posterior hypo. area	+++	+++	1,4,5,7,8		Interstitial n. of Cajal	++	++		3
I. Pituitary gland					N. Darkschewitsch	++	++		3
1. Anterior lobe	—	—			Dorsal tegmental n.	+	+		
2. Intermediate lobe	+	+			Ventral tegmental n.	+++	+++	8	
3. Posterior lobe	++	++			N. incertus	+++	+++		
II. Brain stem					Laterodorsal teg. n.	++	++	1	3
A. Sensory					Barrington's n.	++	+		
1. Visual					Locus coeruleus	++	+	2,4,6,8	
Superior colliculus			1,2,5,6,8	3	2. Raphe				
I	+	+			Interfascicular n.	++	++		
II	+++	+++			Rostral linear n.	++	++	6	
III	++	++			Dorsal raphe	+++	+++	1,2,4,6,8	
IV	++	+			Median raphe	+++	+++	1,2,4,6,8	
V-VI	++	++			N. raphe pontis	++	++	2,6,8	
Parabigeminal n.	+	+			N. raphe magnus	++	++	1,2,6,8	
Pretectal region			2,8	3	N. raphe obscurus	+	+	1,2,6,8	
Olivary n.	++	++			N. raphe pallidus	+	+	8	
N. optic tract	++	++			3. Interpeduncular n.			1,2,5,6	
Anterior n.	++	++			Rostral subnucleus	++	++		
Posterior n.	++	++			Apical subnucleus	+++	+++		
Medial pretectal area	++	++			Dorsomedial subnucleus	+	+		
N. posterior commissure	++	++			Lateral subnucleus	+++	+++		
Medial terminal n.	++	++			Intermediate subnucleus	++	++		
2. Somatosensory					Central subnucleus	+	+		
Mesencephalic n. V	++	++	6		4. Reticular formation				
Principal sensory, n. V	++	++			Central teg. field			1,2,4,6,8	
Spinal n. V			2,8		Subcuneiform part	++++	++++		
Oral part	++	++			Retrobulbar part	+++	+++		
Interpolar part	++	++			Peripeduncular n.	++	++		
Caudal part	++	++			Pedunculopontine n.	+++	+++	8	3
Dorsal column n.	+	+			Cuneiform n.	+++	+++	1,2,6,8	
External cuneate n.	+	+			Pontine reticular	++	++	6,8	
3. Auditory					Linear n. medulla	++	++		
Cochlear nuclei					Parvicellular ret. field	+++	+++	2,8	
Dorsal	++	++			Gigantocellular ret. field	++	++		
Ventral	+	+			Lat. paragigantocellular	++	++	2,8	
N. trapezoid body	+	+			Intermediate ret. field	++	++	2	
Superior olive	+	+			Paramedian reticular n.	++	++		
N. lateral lemniscus					D. Pre- and postcerebellar				
Ventral	+	+			1. Pontine gray	++	++	2,8	3
Dorsal	++	++			2. Tegmental reticular n.	++	+++	1,8	3
Inferior colliculus			2,6		3. Inferior olive	++	++	6	3
External	+++	+++			4. Lateral reticular n.	++	++		
Dorsal	++	++			5. Red nucleus	++	++	2	3
Central	++	++			6. N. Roller	++	++	6	
N. brachium inf. coll.	++	++			7. N. prepositus	+++	+++		
N. sagulum	++	++							

(continued)

TABLE 2. (continued)

Cell group	MCH	NEI	Projection Field of (ref.) ^{3,4}		Cell group	MCH	NEI	Projection Field of (ref.) ^{3,4}	
			LHA	ZI				LHA	ZI
III. Cerebellum					IV. Spinal cord				
A. Deep nuclei	+	+	2,8		A. Marginal zone	++	++	8	
B. Cortex					B. Substantia gelatinosa	++	++	8	
Molecular layer	+/-	+/-			C. Intermediate gray	+	+	8	
Purkinje layer	+/-	+/-			D. Intermediolateral column	+	+		
Granular layer	+/-	+/-			E. Ventral horn	++	++	8	
					F. Central gray	++	++	8	

¹Density of labeled axons and varicosities evaluated in 7 brains in which adjoining series of sections were stained for MCH and NEI. A 5-point scale was used in which ++++ represents the highest density observed in these experiments, + widely scattered elements, and - a complete absence of staining. The +/- designation used for the cerebellar cortex indicates an extremely sparse innervation.

²The nomenclature and tabular scheme employed here are based on those employed by Wada et al. ('89).

³Citations to document the existence of projections from the lateral hypothalamic area and/or zona incerta to the indicated projection field of MCH- and NEI-ir neurons are numbered as follows: 1—Berk and Finkelstein, '82; 2—Hosoya and Matsushita, '81; 3—Ricardo, '81; 4—Saper et al., '79; 5—Saper, '85; 6—Villalobos and Ferresini, '87a; 7—Villalobos and Ferresini, '87b; 8—Hosoya and Matsushita, '83.

⁴A further note on the selection of data describing efferent projections of the lateral hypothalamic area and zona incerta is warranted. We have endeavored to restrict our listing of anatomically documented projection fields of the lateral hypothalamic area to studies, or particular experiments within a given study, in which the regions containing MCH- and NEI-ir perikarya seem likely to have been involved by tracer deposits. The study of Ricardo ('81) provides the only thorough characterization of the efferent projections of the zona incerta based on modern methods; unfortunately this study did not include injections that sampled the particular (rostromedial) aspect of the zona incerta in which ppMCH-expressing neurons are concentrated. We nonetheless adopted Ricardo's ('81) descriptions as being representative of the outputs of the zona incerta as a whole. It is also worthy of mention that for use in this table we have taken some license in attributing projections to various authors based on their illustrations, where the authors themselves might not have commented upon them.

innominata (Fig. 7C–E). At the level of the preoptic recess (Fig. 7B), the principal ascending pathway occupies the most medial aspect of the nucleus of the diagonal band; at this level, a large contingent of fibers sweeps dorsomedially to enter the septal region and its associated fiber systems. Labeled axons traveling with the ascending limbs of the anterior commissure reach the anterior olfactory nuclei and the olfactory bulbs, proper.

Other discrete diencephalic fiber systems also participate in the distribution of MCH- and NEI-ir axons within the forebrain. For example, a relatively small cohort of axons ascends through the periventricular zone to contribute to the innervation of the midline thalamic nuclei (Fig. 7C–E). These are joined by dorsomedially directed projections that appear to arise principally from labeled cells in the zona incerta (see also Ricardo, '81). In addition, fibers of MFB origin enter the stria medullaris at the level of the preoptic area (Fig. 7C) and distribute to (and/or through) the habenula. The descending columns of the fornix, mammillothalamic tract, and fasciculus retroflexus each contain moderate complements of labeled fibers which appear to contribute, to uncertain extents (relative to fibers of MFB origin), to the substantial projections to the mammillary and interpeduncular nuclear complexes. The stria terminalis is joined at preoptic levels by a small number of MCH- and NEI-ir fibers apparently destined for the amygdaloid complex.

The principal descending component of MCH- and NEI-ir fibers arising from cell groups in the diencephalon congeals at the level of the diencephalic-mesencephalic transition region into a relatively discrete array of obliquely oriented fibers wedged between the substantia nigra and the medial lemniscus. At the level of the red nucleus (Fig. 7G), the bulk of this contingent arches dorsomedially to descend through the central tegmental fields and the subcuneiform region of the rostral pons (Fig. 7H). Throughout the length of this descent, axons distribute medially to innervate the mesencephalic and pontine central gray, and, to quite variable degrees, the more discrete nuclei associated with it (Fig. 7G–I). The now minor ventral component also proceeds caudally, ostensibly to contribute to the innervation of the ventral tegmental area, the interpeduncular nuclear com-

plex, the raphe nuclei, and the pontine gray and tegmental nuclei. Through the caudal pons and medulla, the principal descending path comprises a relatively discrete, nearly vertically oriented, strip of fibers hard upon the medial edge of the trigeminal complex and traversing principally the medullary parvicellular reticular field (Fig. 7I–L). Immuno-reactive fibers descend through the dorsolateral funiculi to all levels of the spinal cord.

Our inability to visualize substantial numbers of MCH- or NEI-stained cells in the pons or basal forebrain in non-colchicine-pretreated rats thwarted any attempt to independently assess the trajectories and targets axons arising specifically from these regions.

Cortical innervation. Although the distribution of MCH- and NEI-ir fibers within individual cortical fields is quite diffuse (Fig. 7A–F), clear and pervasive differences in the laminar and regional distributions of these peptidergic afferents within the cortical mantle could be discerned (Fig. 10). Characteristic of the general scheme of the neocortical innervation are relatively dense accumulations of labeled fibers in layers I and VI. Somewhat lesser concentrations of immunoreactive axons were seen in layers II and Vb, though these were less consistently observed and were quite variable from field to field. Layers III, IV, and Va were consistently found to be the most sparsely innervated. While all neocortical areas were found to receive MCH- and NEI-ir inputs, considerable variability in the strength of innervation from field to field was encountered. Infralimbic, prelimbic, anterior cingulate, and agranular insular regions were among the most densely innervated, while some primary sensory cortices (somatosensory, auditory, visual) were among the least.

The distribution of MCH and NEI projections in hippocampal (entorhinal and subicular) cortices generally conformed to the organizational plan seen in isocortex, though the pattern in Ammon's horn merits additional comment. The most distinctive feature here was a rather prominent projection to the stratum oriens that is most dense in the CA3 subfield (Fig. 7D–F). The pyramidal and molecular layers received a less pronounced and generally diffuse input. Apart from a moderate accumulation of fibers in the

Fig. 7. Distribution of MCH- and NEI-ir fibers in the rat brain. A series of darkfield photomicrographs arranged from rostral (A) to caudal (F) to show the gross features of the distribution of axons and terminals stained for NEI-ir. Note that in a few instances (e.g., the

lateral olfactory tract, the optic tract, the trapezoid body, the cerebellar peduncles) refringence of myelinated fiber tracts conveys a misleading impression of positive labeling. All micrographs $\times 7.5$.

Figure 7 continued

Fig. 8. Comparison of the distribution and density of MCH- and NEI-ir fibers in selected regions. Darkfield photomicrographs of adjacent sections showing MCH- (left column) and NEI- (right column) stained fibers and terminals at the levels of (A) the caudal septal region,

the (B) the ventromedial nucleus of the hypothalamus, and (C) the mammillary bodies. In each of these examples the distribution and density of elements stained for each peptide are similar. All micrographs $\times 50$.

hilar region, the dentate gyrus was found to receive a sparse and diffusely distributed projection.

The inferences we were able to draw, derived from analyses of immunohistochemical material, as to the pathways by which MCH- and NEI-ir fibers reach the cortical mantle are very much in accord with Saper's ('85) description of hypothalamic projections to cortex based on the autoradiographic method, and the delineation of the cortical α -MSH-ir projections provided by Khachaturian et al. ('85). Three major routes may be discerned. First, a relatively small contingent associated with the fornix at hypothalamic and preoptic levels provides one likely route by which MCH/NEI axons reach the hippocampal formation. A second, and more prominent pathway, derives from axons

of the medial forebrain bundle, and traverses the medial septum/diagonal band complex to innervate medial prefrontal regions and then turns caudally at the genu of the corpus callosum (Fig. 7A), in association with the fimbria/fornix and the cingulum bundle, for distribution to the hippocampal formation and more medially situated cortical fields, respectively. A third, and at least equally prominent, path exits the hypothalamus laterally in association with the ansa peduncularis, and traverses (and distributes to) the amygdala and the substantia innominata to join the external capsule.

Neuroendocrine projections. In light of the functional data to suggest a role for MCH in neuroendocrine, and particularly anterior pituitary, function, additional com-

Fig. 9. Differential distributions of MCH- and NEI-ir fibers in a rostral thalamic cell group. Brightfield photomicrographs of adjacent sections stained for Nissl material, MCH-ir and NEI-ir to show a distinctive terminal field in the interanterodorsal nucleus (IAD) that stained positively for MCH-ir, but not NEI-ir. Note that the two peptide distributions in other regions of the thalamus at this level (e.g., the paraventricular nucleus, or PVT) are similar. All micrographs $\times 45$.

ment on the distribution of MCH- and NEI-ir in the median eminence and pituitary is warranted. Both peptides were reliably localized in the median eminence; the density of each in the internal lamina was moderate, while the external zone was sparsely invested (Fig. 11). Correspondingly, a moderate number of fibers and terminals stained for either peptide were present in the posterior pituitary, and these were preferentially concentrated near the periphery of the gland, suggesting a preferential association with oxytocin-containing terminals (e.g., van Leeuwen et al., '79). Labeled fibers were consistently detected in the intermediate lobe, though the density of these was slight.

Ultrastructural Localization of MCH- and NEI-ir

Adjoining series of vibratome sections throughout the length of the diencephalic ppMCH distribution were prepared for pre-embedding immunoperoxidase localization of MCH or NEI, in order to further characterize the morphology of ppMCH-expressing neurons, and to gain preliminary insight into the subcellular distribution of each peptide. Profiles of perikarya stained for either MCH- or NEI-ir (Fig. 12) typically displayed invaginated nuclei and moderately well-developed Golgi apparatus and rough endoplasmic reticulum, the latter often tending to be arrayed in parallel stacks near the periphery of the cytoplasm. In addition to an ostensibly unremarkable complement of organelles, moderate numbers of dense-cored granules (75–200 nm in diameter) were distributed throughout the cytoplasm. Labeled perikarya were often found in close association with vascular elements (venules), but were invariably segregated from the basal laminae of the perivascular cells by fine interposed (presumably astrocytic) processes. Similarly, both MCH- and NEI-stained cell bodies or primary dendrites shared lengthy stretches of close membrane apposition with unlabeled neurons; again, membranes of adjoining labeled and unlabeled elements were segregated by fine glial processes, and no clear instances of direct membrane apposition or contact were encountered in our material.

One clear feature distinguished MCH- from NEI-stained perikarya at the subcellular level. This was an intense localization of peroxidase reaction product associated with saccules of the Golgi apparatus that was almost invariably seen in MCH-stained perikarya that contained well-developed Golgi profiles, but only extremely rarely in NEI-ir cells (Figs. 12, 13). In MCH-ir cells, as many as four contiguous saccules (more commonly one or two) contained reaction product, and these characteristically lay closer to the inner (or trans) face of the organelle than to its outer (cis) face. Most, but not every, independent iteration of the Golgi complex seen in profiles of MCH-stained cells contained immunoperoxidase reaction product.

Within the hypothalamus, profiles of MCH- and NEI-labeled axons and axon terminals were also observed (Fig. 13). Axons stained for either peptide were thin (0.1–0.2 μm in diameter) and most commonly unmyelinated, though examples of thinly myelinated ones were encountered. Terminals within the hypothalamus stained for NEI were indistinguishable from the MCH-ir ones, and appeared generally characteristic of peptidergic terminals described in other systems. These contained a preponderance of small (30–60 nm diameter) electron lucent vesicles and a variable number (0–7) of larger (80–150 nm) dense-cored vesicles. Reaction product in labeled terminals was distributed diffusely around, but not within, the smaller vesicles, while the larger ones were invariably densely stained. Dense-cored vesicles tended to be preferentially sequestered at the periphery of the terminal, away from the junctional region. Terminals within the lateral hypothalamus and zona incerta were most commonly asymmetric and ended principally on dendritic shafts of unlabeled neurons. Axosomatic contacts were rarely encountered, and we found no examples of labeled terminals contacting labeled somata or dendrites.

submetidos ao anticorpo secundário e outra série de fotografias era feita, finalmente as duas séries de fotografias eram comparadas, para então chegarmos à conclusão de quantas e quais células co-expressavam tanto o MCH quanto o NEI.

4.3 - Imunohistoquímica simultânea

Em virtude da existência de outros sistemas neuroquimicamente já definidos e também localizados nas mesmas áreas do MCH e NEI, resolvemos utilizar um "cocktail" de anticorpos, para determinarmos se haveria ou não co-localização; primeiramente foi usada uma combinação de anti-rMCH com anti-serotonina, logo após uma outra combinação de anti-rMCH e anti-colina-acetiltransferase (CAT, um marcador para neurônios colinérgicos), e também combinações com seus anticorpos secundários para testar reação cruzada entre o anticorpo primário impróprio. Entretanto, nada encontramos que pudéssemos considerar como resposta positiva.

4.4 - Imunohistoquímica ultra-estrutural

Para este procedimento baseamo-nos no método usado por Pickel et al. (1986) para perfusão-fixação. Os ratos foram profundamente anestesiados com hidrato de cloral a 35% (1ml/rato, via intraperitoneal) e perfundidos, primeiramente, através da aorta ascendente com 50-75 ml de solução salina a 0,9%, a seguir, 100 ml de um fixador preparado com 3,75% de acroleína, 2% de paraformaldeído e 0,1-0,25% de glutaraldeído em tampão

Fig. 10. MCH-ir fibers in neocortex. Darkfield photomontage of immunoperoxidase-stained axons and varicosities in the second somatosensory area of parietal neocortex, a field found to contain a moderate MCH- and NEI-ir input. Note the preferential accumulations of stained fibers in laminae I and VI. Magnification $\times 100$.

Fig. 11. MCH- and NEI-ir terminals in neuroendocrine targets. Fluorescence photomicrographs to show the distribution of MCH-ir in the pituitary stalk at the level of the mammillary recess (A) and in the posterior lobe of the pituitary gland (B). In the stalk and median eminence, a moderate density of fibers stained for MCH- (or NEI-) ir is

seen in the internal lamina, while the external zone is relatively sparsely invested with stained terminals. Correspondingly, a moderate density of stained terminals is apparent in the posterior lobe (B). Both micrographs $\times 150$.

To provide a comparison with these intraparenchymal projections, the vesicular makeup and distribution of reaction product were also examined in MCH- and NEI-ir terminals in the external lamina of the median eminence. Terminals stained for either peptide contained both small and larger (dense-cored) vesicles of sizes comparable to those found in the hypothalamus (Fig. 13). Here, however, we noted no preferential association of reaction product with the larger vesicles; the electron density of these was indistinguishable from that of dense-cored vesicles in nearby unlabeled profiles. The "rimming" of small clear vesicles appeared the dominant site of reaction product deposition at this locus.

DISCUSSION

The present results serve to further define a chemically specified neuronal system in the mammalian CNS whose expansive terminal distribution rivals those of some the more broadly distributed monoaminergic and neuropeptidergic complexes. Figure 14 provides a schematic summary of the distribution of ppMCH-expressing cells and fibers in the rat brain. The availability of species-specific antisera directed against two peptides processed from the ppMCH precursor, and nucleic acid probes to the corresponding mRNA, has been exploited to provide strong converging evidence as to the validity of the localizations reported here, and has allowed a number of new details concerning the organization of the ppMCH system to be discerned. In general, the results support two major, and somewhat contradictory, overviews of the system. On one hand, delineation of the projection fields of the ppMCH system serves to convey a sense of the expansive array of targets of a large population of cells in the dorsolateral hypothalamic area, whose close association with the MFB has quite effectively stifled attempts to place them in anatomical or functional context (see, e.g., Swanson, '87). On the other hand, the results indicate that the ppMCH system is not an homogeneous one; expression of ppMCH-derived peptides and mRNA is not limited to the diencephalon, and, even within it, subsets of ppMCH expressing cells may discerned that are associated with cytoarchitecturally, connectionally, and functionally diverse systems.

Cells of Origin

Within the diencephalon, the loci of ppMCH-derived peptide and gene expression described here agree quite well with previous immunocytochemical analyses that made use of antisera directed against sMCH (Skofitsch et al., '85). In addition, the results are compatible with delineations of the extra-arcuate, or " α -2", system of α -MSH-ir cells (e.g., Khachaturian et al., '85; Köhler et al., '84; Saper et al., '86). The degree of correspondence with other immunoreactivities (Kawano et al., '88; Merchenthaler et al., '88) reported to be co-contained within these diencephalic loci also appears great, though the fact that these latter peptide systems include multiple sites of expression within the diencephalon prohibits detailed comparisons. Our direct assessments of the extent to which MCH- and NEI-ir may be colocalized in individual diencephalic neurons suggests the degree of correspondence to be essentially complete.

It is noteworthy that, in contrast to previous work with antisera against teleost MCH (Skofitsch et al., '85), pretreatment with colchicine was not required to visualize labeled perikarya, which speaks to the heightened immunohistochemical sensitivity achievable with antisera raised against species-specific peptide sequences. This, coupled with the robust signals for ppMCH mRNA that were evident in hybridization histochemical material, which in our experience are exceeded in the hypothalamus only by the oxytocin and vasopressin mRNAs, suggests indirectly that MCH and NEI are expressed in high abundance in the diencephalon, and likely function as important, if not dominant, transmitter/modulator substances of these neurons. As noted previously, cross-reactivity with epitopes encoded by ppMCH can account for staining of this cell group with antisera raised against α -MSH, rCRF, and hGRF (Nahon et al., '89a,b). Peptides derived from the MCH precursor at present comprise the dominant neurochemical signatures of this incerto-hypothalamic cell group. Thus far, it has been established that peptides chromatographically indistinguishable from synthetic rMCH and rNEI are released from primary cultures of rat hypothalami both basally and in response to activation of several second messenger systems (Parkes et al., '91), suggesting strongly that both are normally processed from ppMCH. Whether a third peptide,

Fig. 12. Fine structure of MCH- and NEI-ir perikarya in the hypothalamus. Low-magnification electron micrographs to show the typical appearance of NEI- (A) and MCH- (B) stained cell bodies. Reaction product in NEI-ir cells is concentrated near the periphery of the cytoplasm, often in association with rough endoplasmic reticulum (rer); the Golgi apparatus (Go) is typically unstained. MCH-ir cells display a similar, though less marked, accumulation of reaction product

associated with rer, and a very prominent labeling within the Golgi saccules. Note also that both cell types display invaginated nuclei, a moderate number of dense-cored vesicles in the cytoplasm, and close membrane appositions with nearby (unlabeled) dendrites (uD; top left of panel A), neurons (bottom right of B), and glia (bottom left of B). Both micrographs $\times 7,000$.

Fig. 13. Additional fine structural features of MCH- and NEI-ir in the hypothalamus. **A, B:** Typical appearance of MCH- (A) and NEI-ir (B) in the Golgi complex. MCH-stained cells show dense reaction product deposition in 1–4 saccules situated near the trans face of the Golgi apparatus, while the organelle is typically unstained in NEI-ir neurons. **C, D:** Examples of NEI-ir terminals in the lateral hypothalamic area (C) and median eminence (D). Panel C shows an intraparenchymal NEI-ir axon terminal (AT) that terminates (black arrows) upon

an unlabeled dendrite (D); unlabeled terminal contacts upon the same dendrite are indicated (open arrows) for contrast. Reaction product in both NEI- and MCH-ir terminals is distributed diffusely around small clear vesicles and is especially prominent over larger, dense-cored, ones. In the median eminence (D), however, no clear deposition of reaction product over dense-cored vesicles is evident. A and B, $\times 37,500$; C, $\times 11,500$; D, $\times 22,500$.

NGE (see introduction and Nahon et al., '89a,b), may be generated from the precursor in physiologically significant quantities remains to be determined.

It must be emphasized that despite their contiguity and neurochemical commonalities, diencephalic ppMCH-expressing neurons do not comprise an homogeneous population. Apart from the obvious fact that MCH- and NEI-ir cells are distributed principally in two cell groups, the zona incerta and lateral hypothalamic area, that are cytoarchitectonically and connectionally distinct (e.g., Ricardo, '81; Saper et al., '79), even within the hypothalamus, ppMCH

expressing cells are localized within three distinct territories of the LHA (see Saper et al., '86), and smaller clusters are associated with the posterior hypothalamic area (Skofitsch et al., '85), the anterior periventricular nucleus, and the tuberal magnocellular nucleus of Bleier et al. ('79), a component of the tuberomammillary complex (see, e.g., Köhler et al., '85; Staines et al., '87). The extent to which ppMCH-expressing cells in any or all of these may give rise to distinctive projection fields remains to be determined. Based solely on the breadth of the MCH- and NEI-ir projection fields, some considerable degree of collateraliza-

Fig. 14. Schematic drawing of a sagittal section through the rat brain to summarize the organization of the ppMCH neuronal system. Dots indicate the relative location and abundance of ppMCH-expressing cell bodies, and arrows point out some of the more prominent terminal fields.

tion of individual axons would be expected, and experimental evidence exists to support this, in the regions of the lateral hypothalamic area that contain ppMCH-expressing cells (Veening et al., '87), as well as in incerto-hypothalamic α -MSH-ir neurons (Saper et al., '86). As discussed below, the present results, viewed in the context of previous studies of the connectivity of the regions listed above, support the notion that distinct subsets of ppMCH-expressing neurons may be discerned.

In addition to the generally predictable diencephalic sites of expression, we found converging evidence for MCH and NEI peptide and ppMCH gene expression all to be distributed similarly in two additional regions, one centered in the olfactory tubercle, and the second occupying a discrete, but cytoarchitectonically nondescript, region of the paramedian pontine reticular formation. To our knowledge, neither of these localizations had been reported previously with antisera against sMCH, α -MSH, or any of the other peptides found previously to be co-localized with them in the lateral hypothalamic area and zona incerta. This may be explained, in part, by indirect evidence suggesting the levels of gene and peptide expression in these extra-diencephalic regions to be relatively low. This evidence included the requirements for colchicine pretreatment and for longer autoradiographic exposure times to optimally detect positively immunostained and hybridized cells, respectively. The relatively diffuse localization of ppMCH expressing cells in the basal forebrain and the lack of any clear sense of the connectivity of the region of the pons in which positively immunostained and hybridized cells were found precludes any decisive statement as to how peptidergic axons arising from these regions might be distributed. In view of the collectively small number of cells involved, and ostensibly low levels of relevant gene and peptide expression (compared to the diencephalic population), we would expect their overall contribution to the MCH- and NEI-ir projection fields to be minor, though focally dense individual sites of termination cannot be ruled out.

Projections

As an almost universal rule, the axonal trajectories and apparent terminal distributions of MCH- and NEI-ir were found to be similar. Whether some perceived minor differences in the apparent density of these (Table 2) might be attributable to differential processing of ppMCH in subsets of expressing neurons seems unlikely, but remains to be evaluated rigorously. The single overt exception to this generality occurred in the interanterodorsal nucleus of the thalamus, which contained a moderately dense terminal field stained for MCH-ir, but not for NEI-ir. In view of the apparent identity of MCH- and NEI-expressing cells in the diencephalon, it would seem unlikely that this might be indicative of the existence of a subpopulation of neurons that preferentially expresses MCH. Cross-reactivity of our MCH antisera with an unknown epitope contained within fibers in the interanterodorsal nucleus cannot be ruled out. The only chemically specified input of particular prominence to the interanterodorsal nucleus of which we are aware is one that stains with antisera to enkephalin-like sequences (L.W. Swanson and P.E. Sawchenko, unpublished observations). This is intriguing in view of the fact that immunoreactive metorphamide, a prepro-enkephalin-derived octapeptide, has been shown to coexist with α -MSH in incerto-hypothalamic neurons (Merchenthaier et al., '88). Like α -MSH, metorphamide possesses an amidated valine residue at its carboxy terminus, which might explain the apparent association of enkephalin-derived peptides with the ppMCH system, but not the disparity in the MCH- and NEI-stained terminal fields in the thalamus.

The distribution of MCH- and NEI-ir fibers described here generally encompasses and extends the observations of Skofitsch et al. ('85), who provided the only available detailed overview of the distribution of sMCH-ir in the rat brain. Many qualitative and quantitative differences between this and the present analysis are evident, and are doubtless attributable to the sensitivity afforded by the availability of antisera to rat-specific peptide sequences. For

example, some of the more prominent terminal fields evident in our material (e.g., in the medial septal and mammillary nuclei), were unremarkable in this earlier study, and a number of other moderately dense terminal fields noted here (e.g., in the pretectal region and superior colliculus) apparently contained no detectable sMCH-ir inputs.

In general, and as indicated in Table 2, the distribution of MCH- and NEI-ir fibers may be viewed as very largely representing an amalgam of projections that have been described as arising from the lateral hypothalamic area (e.g., Berk and Finkelstein, '82; Hosoya and Matsushita, '81; Saper, '85; Saper et al., '79; Villalobos and Ferassiwi, '87a,b) and/or the zona incerta (Ricardo, '81). Certainly we have detected immunoreactive fibers in some sites not previously recognized as projection fields of these two regions, and the relative densities of MCH- and NEI-ir axons in any given cell group do not necessarily conform to those that may be inferred from the literature as being attributable to the lateral hypothalamic area and/or zona incerta, collectively. Some of these apparent discrepancies may be attributed to differences in the sensitivity and resolving power of the different methods employed (i.e., immunohistochemical localization of relatively high abundance antigens versus autoradiographic tract tracing), differences in the aims and/or scope of the individual studies being compared, differences in anatomical nomenclature employed, and the fact that the axonal transport data must be assumed to sample projections of cell types in addition to those that express ppMCH-derived peptides. A comparison of the organization of cortical projections arising from the lateral hypothalamic area with those expressing ppMCH-derived peptides may serve to illustrate some of these issues.

Previous descriptions of the α -MSH (Khachaturian et al., '85; Köhler et al., '84) and sMCH (Skofitsch et al., '85) immunostained projections to the cortical mantle described these as being generally sparse and diffusely organized. Köhler and colleagues ('84) did, however, note preferential accumulations of α -MSH-stained fibers in the deeper layers of entorhinal cortex and in the stratum oriens of Ammon's horn, observations fully in accord with those reported here. We found, in addition, a relatively high density of rMCH- and rNEI-ir fibers not only in layer VI, but in the superficial laminae (principally layer I) of most neocortical fields. We are inclined to attribute our ability to resolve these superficial fibers to the fact that our best preparations involved the use of very short, if any, postfixation of tissue blocks prior to sectioning (see Sawchenko et al., '90b).

While the laminar and areal distributions of MCH- and NEI-ir fibers outlined here contrast to some degree with those described by Saper ('85) as arising from the posterolateral region of the hypothalamus as whole, it must be emphasized that apparently not all cells in the lateral hypothalamic area that give rise to cortical projections express ppMCH-related antigens (Saper et al., '86), and that hypothalamic cell groups clearly distinct from the foci of ppMCH expression contribute to the cortical innervation. These include the tuberomammillary nucleus, proper, and the supramammillary nucleus, both of which contribute ostensibly widespread projections to cortex, but whose terminal distributions have not been thoroughly characterized (e.g., Köhler et al., '85; Saper, '85; Staines et al., '87). The supramammillary nucleus is, however, known to provide a distinctive projection to the stratum granulosum

and immediately adjoining portion of the molecular layer of the dentate gyrus (e.g., Wyss et al., '79) which, predictably, did not stain for ppMCH-derived peptides.

Fine Structure

With two notable exceptions, the appearance and subcellular distributions of MCH- and NEI-ir in diencephalic perikarya and terminals were similar, and conformed with patterns typical of peptidergic neurons in general (see, e.g., Pickel, '85). These results are compatible with other, more limited, ultrastructural observations made in rat and human brain with antisera raised against sMCH (Naito et al., '88; Pelletier et al., '87).

One distinctive and unusual feature noted here concerned the perikaryal distributions of MCH- and NEI-ir, particularly their associations with the Golgi apparatus. At the ultrastructural level, NEI-ir was distributed densely and quite diffusely through the cytoplasm, primarily in the vicinity of the rough endoplasmic reticulum. Staining of the Golgi apparatus proper was rare and, where encountered, weak. By contrast, deposition of reaction product in a subset of Golgi saccules was consistently seen to be the most salient feature of perikaryal MCH immunostaining; other cytoplasmic sites were invariably far less intensely labeled. Labeling appeared to be associated with the middle to inner saccules (trans face) of the Golgi, though precise localization will require direct comparisons with histochemical markers for various compartments of the organelle (e.g., Farquhar, '85). Overall, this disparity may reflect differences in the manner and/or extent to which the two peptides are normally processed from ppMCH, but several caveats make such an intimation quite preliminary. For example, we do not know the extent to which our MCH and NEI antisera might differentially bind their respective epitopes in processed (free peptide) versus unprocessed (prohormonal) forms. In addition, we have observed no corresponding discrepancy in the relative abundance or staining intensity of MCH- and NEI-ir terminals at the light or electron microscopic levels.

Along this same line, it is worthy of mention in the general context of protein trafficking that the primary and predicted secondary structures of the ppMCH mRNA show clear similarities to those of 7SL RNA (Nahon et al., '90), which functions as a component of the signal recognition particle (or SRP), a small ribonucleoprotein that is thought to play a role in targeting mRNAs encoding secretory proteins to endoplasmic reticulum-bound ribosomes (Walter and Lingappa, '86). It is certainly unusual that an mRNA which itself encodes secreted peptides should display such a relatedness to a nontranslated (7SL) RNA species involved generically in protein processing. Whether this may suggest a broader array of intracellular functions for ppMCH-derived peptides and/or mRNA is unclear.

A second unusual feature encountered at the electron microscopic level concerned the distribution of MCH- and NEI-ir in axons and terminals in the median eminence. Here, labeling for both peptides appeared to be preferentially associated with small, electron-lucent vesicles, and not the larger, dense-cored ones that represent not only the dominant sites of MCH and NEI reaction product deposition in the hypothalamic parenchyma, but the dominant site of sequestration of hypothalamic hormones in axons in both laminae of the median eminence, as well (e.g., Page, '86). We are unaware of precedents for such a vesicular segregation of reaction product in any hypophysiotropic, or,

for that matter, non-hypophysiotropic, peptidergic projection that ends in or passes through the median eminence. Clearly, preembedding staining methods are generally poorly suited to make such discriminations, and this matter awaits confirmation with postembedding and/or cryofixation methods with greater resolving power.

Functional Considerations

While the present survey has provided a more complete anatomical context in which to view the ppMCH-derived peptide system, it remains fair to say that in the mammalian brain, at least, MCH, NEI, and any other moieties that may be processed from the precursor very much remain peptides in search of functions. In contrast to a number of other essentially hypothalamic neuropeptides, which were isolated and structurally characterized with the aid of bioassays with which to evaluate suspected functionality, the mammalian ppMCH system was characterized on the basis of structural similarity with a teleost peptide that plays a role in mediating adaptive changes in pigmentation, a function not shared with mammals (Baker, '88, '91; Eberle, '88). Before attempting to reconcile what is now known of the anatomy of the ppMCH system with functional data relevant to it, it must be emphasized that nearly all of the functional work has made use of MCH preparations. Both NEI and the putative NGE peptide remain to be investigated in any of the contexts touched upon below.

Neuroendocrine systems. Our initial interest in MCH was spawned by reports of the corticotropin-inhibiting activity of sMCH in teleost (see Baker, '88, '91; Eberle, '88) and rat (Baker et al., '85) pituitary. Subsequent studies have either failed to replicate (Eberle, '88) or described conflicting, if any, effects of rMCH on ACTH release in rats (Navarro et al., '90; Vaughan et al., '89). Similarly, several preliminary descriptions of effects of systemically or centrally administered sMCH on other tropic hormone systems of the anterior pituitary have been reviewed by Baker ('91). The present results suggest clearly that hypophysiotropic MCH- and NEI-containing projections to the median eminence are modest and almost certainly arise from a very small subset of ppMCH-expressing cells. Perseveration on anterior pituitary systems seems unlikely to provide any telling clues into more general aspects of ppMCH-derived peptide (or anterior pituitary) function, though the unusual distribution of MCH- and NEI-ir within terminals in the median eminence seems of some potential organizational interest.

A considerably stronger neuroendocrine representation of MCH- and NEI-ir is evident in the posterior pituitary. It may be inferred from past and present characterizations of the ppMCH system, and from the results of studies in which retrograde transport methods were combined with immunostaining for rCRF and α -MSH (Kawano et al., '88), that these likely arise from scattered accessory magnocellular neurosecretory neurons in the posterolateral hypothalamic area. This is quite unique, in that we are aware of no well-characterized peptide system that is expressed at all prominently in axons in the posterior lobe, and *not* in perikarya of the paraventricular and/or supraoptic nuclei, the principal cellular foci of the magnocellular system. Based on the levels of MCH-ir in the posterior pituitary, which are orders of magnitude lower than those of oxytocin and vasopressin (e.g., Zamir et al., '86), it would seem unlikely that either it or (presumably) NEI function as secreted hormones of any consequence. Rather, they may

be grouped with a large contingent of peptides that are co-localized within magnocellular neurosecretory neurons, and which are generally thought to exert local, autocrine or paracrine, effects on oxytocin and/or vasopressin secretion (see Brownstein and Mezey, '86).

Some data exist to support an involvement, or at least a responsivity, of this component of the ppMCH system to challenges that target neurohypophyseal mechanisms. Zamir et al. ('86) reported a marked increase in the MCH content of the neurointermediate lobe and lateral hypothalamic area in response to hyperosmotic challenges. We have recently obtained evidence for a widespread down-regulation of ppMCH mRNA in response to acute footshock, a generalized systemic stressor (F. Presse, G. Heruieu, T. Imkai, P.E. Sawchenko, W. Vale, and J.-L. Nahon, unpublished observations). Clearly, these stimuli affect mRNA expression in a much larger portion of the entire diencephalic ppMCH-expressing population than that which projects to the posterior lobe. Therefore, the extent to which the neurohypophyseal component of the system may be specifically responsive to osmotic, or any other, challenge remains to be determined.

Intra-CNS systems. Though neuroendocrine systems are attractive in terms of their offering of easily assayable end points, they clearly represent a minor offshoot of the greater ppMCH system. The present results also have implications for imposing a functional context upon the broader, intracerebral, network.

The most striking global feature of the axonal and terminal distributions of MCH- and NEI-ir axons described here is its essentially interneuronal character. Cell groups in receipt of primary sensory information, and many cell groups proximal to them along sensory information processing pathways, are generally sparsely innervated. Of all the cell groups along the primary auditory path, for example, only the inferior colliculus receives MCH- and NEI-ir inputs of any prominence. Similarly, only sparse projections are evident in the motor nuclei of the cranial nerves, magno- and parvocellular neurosecretory cell groups, and autonomic preganglionic nuclei. It thus seems clear that the ppMCH system is not apt to play a major direct role in modulating autonomic functions ostensibly influenced by lateral hypothalamic area neurons, though indirect access to preganglionic neuron pools could be achieved via projections to the central gray and cell groups embedded in the ventrolateral reticular formation of the pons and medulla (see, e.g., Hosoya and Matsushita, '83; Luiten et al., '86).

Of the functional contexts suggested for the lateral hypothalamic area, and to a lesser extent, the zona incerta, those associated with sensorimotor integration and generalized arousal seem most compatible with the overview of the ppMCH system described here. Neurons within the lateral hypothalamus are privy to multiple, and often converging, modalities of somatic, visceral, and special sensory information, and motor impairment and inattention to sensory stimuli are common sequelae of lateral hypothalamic damage (reviewed by Swanson, '87). While these lesion effects may, in large measure, be attributed to nonspecific damage to axons-of-passage, particularly dopaminergic ones, coursing through the MFB (Stricker and Zigmond, '76), low level electrical stimulation of the LHA does provoke motor activity and potentiate the activity of spinal motoneurons to stimulation of the corresponding dorsal roots (Valenstein et al., '69). Even the association of the LHA with specific goal-directed behaviors, particularly consummatory ones,

could be viewed as resulting from an activation or sensitization by salient sensory cues of a system with widespread access to somatomotor and diffuse activational (arousal) functions.

The broad associations of MCH- and NEI-ir terminal fields with cell groups comprising the extrapyramidal motor system, vast expanses of the mesencephalic, pontine, and medullary reticular formations, precerebellar relays, and the spinal cord (including the ventral horn) are all consistent with this view. Moreover, the laminar distribution of the widespread MCH- and NEI-ir projections to the neocortex is characteristic of the so-called nonspecific cortical afferents (Lorente de Nó, '43), which are generally thought to provide a basis for generalized cortical arousal (see discussion in Saper, '85). In addition, the substantia innominata and parabrachial nuclei, cell groups whose cortical projections conform to the classical description of nonspecific cortical afferents (Saper, '82, '84), also represent prominent targets of ppMCH-expressing neurons.

Finally, it may be noted that the principal path of ascending ppMCH-expressing fibers through the basal forebrain, hypothalamus, and rostral midbrain is virtually superimposable upon a map of the sites of increased metabolic activity (i.e., glucose utilization) generated by rewarding electrical stimulation of the posterolateral hypothalamic area (Gallistel et al., '85). While this correspondence may be of value in further characterizing the reinforcing effects of electrical stimulation of the brain, the extent to which such a "reward system" may fall within the purview of the general arousal and integrative functions considered above remains moot.

Although the morphologic data presented here themselves provide no obvious or telling insight as to how such imprecisely defined functions such as arousal or sensorimotor integration or reward may be effected, they do serve to better delineate a neuronal system and candidate transmitter substances that should be of value in approaching these questions experimentally.

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