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ESTROGÉNIOS E PLASTICIDADE SINÁPTICA NO HIPOTÁLAMO DO RATO

Dissertação de candidatura ao grau de Doutor apresentada à Faculdade de Medicina da Universidade do Porto

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A candidata declara que teve uma contribuição determinante na realização do trabalho experimental (execução de protocolos e elaboração técnica), interpretação dos resultados e discussão dos mesmos. Além disso, contribuiu activamente para a redacção dos trabalhos apresentados.

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No início do meu trabalho no Instituto de Anatomia, aprendi diversas técnicas usadas na investigação em neurociências, nomeadamente técnicas de manuseamento animal, laboratoriais e de quantificação. Os meus estudos centraram-se em diferentes áreas do SNC, mas cedo me estabeleci no núcleo ventromedial do hipotálamo (VMN), particularmente na sua divisão ventrolateral, estudando a plasticidade sináptica dependente das hormonas sexuais no rato fêmea. A complexidade estrutural do VMN, o seu envolvimento em funções e comportamentos essenciais para a sobrevida da espécie e o facto de representar um excelente modelo de estudo das propriedades neurotróficas dos estrogénios, justificam a sua escolha para tema da minha dissertação de doutoramento.

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INTRODUÇÃO

Acção dos estrogénios no sistema nervoso central

Tem cinquenta e um anos o conceito de que os efeitos dos esteróides sexuais sobre o sistema nervoso central (SNC) durante o período pré-natal são profundos e determinam os comportamentos típicos dos machos e das fêmeas na vida adulta (Phoenix *et al.*, 1959). Este processo decorre durante um curto intervalo de tempo, o período crítico da diferenciação sexual do cérebro, que nos primatas é essencialmente pré-natal e nos roedores se prolonga até ao 10º dia pós-natal (para revisão, ver Döhler *et al*., 1984; Wallen, 2005). Durante esta janela temporal, a exposição de regiões do SNC possuidoras de receptores de estrogénios a elevados níveis de estradiol proveniente da aromatização da testosterona (Lieberburg e McEwen, 1977; McEwen *et al*., 1977) leva à masculinização e desfeminização do SNC nos machos, ou seja, ao desenvolvimento de características morfológicas, funcionais e comportamentais tipicamente masculinas e à supressão das características femininas, que, na ausência de estradiol, se desenvolveriam. Apesar da sensibilidade do SNC aos esteróides sexuais diminuir significativamente após este período, a acção *organizacional* destes esteróides, iniciada no período perinatal, parece só ter fim, tanto no Homem como em animais de experiência, no período peri-pubertal, altura em que o eixo hipotálamo-hipófise-gónadas é activado tanto nos machos como nas fêmeas (revisto em Schulz *et al.*, 2009).

No entanto, a acção dos esteróides sexuais não se faz sentir apenas no decurso do desenvolvimento. No adulto, as variações fisiológicas dos níveis de estrogénios que ocorrem durante o ciclo ovárico ou as induzidas por esteróides sexuais de origem exógena induzem respostas fisiológicas e comportamentais, bem como fenómenos de plasticidade morfológica e neuroquímica, que tendem a atenuar-se ou anular-se quando os níveis hormonais decrescem (revisto em Madeira e Lieberman, 1995; Cooke e Woolley, 2005). Estes são os designados efeitos *activacionai*s dos esteróides sexuais (Phoenix *et al.*, 1959).

Durante muitas décadas pensou-se que estes efeitos se faziam sentir apenas em áreas do SNC responsáveis pelo controlo de funções e comportamentos indispensáveis à reprodução. Porém, é já hoje relativamente claro que os estrogénios influenciam a morfologia e/ou a actividade doutras áreas do SNC, como a formação do hipocampo, o prosencéfalo

basal, o caudado-putámen, a substância negra, o mesencéfalo e o cerebelo (revisto em McEwen e Alves, 1999). Com efeito, estudos realizados no Homem (Hampson, 1990a,b; Maki *et al.*, 2002; Gasbarri *et al.*, 2008; Hatta e Nagaya, 2009) e em animais de experiência (revisto em Luine, 1997; ver também Daniel *et al.*, 1997; Lacreuse *et al.*, 2001) mostram que a flutuação dos níveis hormonais ao longo do ciclo ovárico e a administração de estrogénios induzem alterações a nível cognitivo, na atenção, na memória, na orientação espacial, na coordenação motora fina e na fluência verbal. É hoje também claro que, em contrapartida, a redução dos níveis hormonais na menopausa se acompanha de deficiências cognitivas e mnésicas, que são revertidas pela administração de estrogénios (Resnick *et al.*, 1997; Jacobs *et al.*, 1998; Drake *et al.*, 2000). É ainda conhecido o papel protector do estradiol em doenças degenerativas do SNC como a esclerose múltipla (para revisão, ver Voskuhl, 2003; Offner, 2004), a doença de Alzheimer (Honjo *et al.*, 1995; Birge, 1996) e a doença de Parkinson (revisto em Saunders-Pullman, 2003), e em quadros complexos do foro psiquiátrico, como a depressão (Gath e Iles, 1990) e a esquizofrenia (Riecher-Rössler *et al.*, 1994; Huber *et al.*, 2001). Devido à sua acção anti-inflamatória e de regulação da morte neuronal (Lee e McEwen, 2001; Stein, 2001; Maggi *et al.*, 2004), os estrogénios também exercem influência protectora e até reorganizadora em lesões cerebrais de causa vascular ou traumática, reduzindo a extensão das áreas de necrose e promovendo recuperação morfológica e mnésica, tanto nos machos como nas fêmeas (Hall *et al.*, 1991; Alkayed *et al.*, 1998; Toung *et al.*, 1998).

A última década foi particularmente rica em estudos que mostram que os estrogénios regulam o funcionamento do cérebro adulto normal e, pela sua acção neuroprotectora, limitam os fenómenos de degenerescência neuronal que acompanham uma variedade de doenças do SNC. Este facto é, ainda, de maior relevância se tivermos em linha de conta o aumento significativo da sobrevida das mulheres, levando a que um terço da sua vida decorra após a menopausa, ou seja, em situação de carência relativa de esteróides sexuais. Acresce que os moduladores específicos dos receptores de estrogénios representam uma nova classe de agentes terapêuticos que, pelas suas propriedades anti-estrogénicas em órgãos periféricos, se encontram no grupo dos fármacos anti-neoplásicos mais prescritos em todo o mundo. Compreende-se, portanto, que os estudos que têm como objectivo contribuir para o conhecimento aprofundado dos efeitos e dos mecanismos de acção dos estrogénios no SNC continuem a ser da maior importância e actualidade.

O núcleo ventromedial do hipotálamo

Uma das áreas do SNC que mais tem contribuído para a compreensão dos mecanismos de acção dos esteróides sexuais é o núcleo ventromedial do hipotálamo (VMN). Este núcleo, fundamental para a iniciação de comportamentos motivados necessários à conservação das espécies, como o comportamento sexual e alimentar e a agressividade (Simerly, 1995), está localizado na zona medial da região tuberal do hipotálamo. É de forma oval e está rodeado por uma zona rica em neuritos e relativamente pobre em células, a cápsula do VMN (Heimer e Nauta, 1969; Millhouse, 1973), que o separa dos núcleos hipotalâmicos adjacentes, nomeadamente do núcleo dorsomedial, dorsalmente, do núcleo arqueado, ventromedialmente e da área hipotalâmica lateral, lateralmente (Broadwell e Bleier, 1976; Simerly, 1995). É composto essencialmente por dois agrupamentos celulares, a divisão dorsomedial e a ventrolateral, que estão separados ao longo de quase toda a extensão do núcleo por uma zona esparsa em células, a divisão central, e que rostralmente convergem para formar a divisão anterior do VMN (Gurdjian, 1927; Simerly, 1995). As duas principais divisões do VMN têm diferente citoarquitectura, características neuroquímicas e conectividade, e estão envolvidas na modulação de comportamentos distintos.

A divisão dorsomedial tem elevada densidade celular e é formada por neurónios relativamente pequenos, com corpos celulares arredondados ou ovais (Van Houten e Brawer, 1978; Madeira *et al.*, 2001) e que expressam, de forma marcada, receptores para peptídeos orexigénicos, designadamente para a leptina e neuropeptídeo Y (revisto em Kalra *et al.*, 1999). É-lhe reconhecido papel importante na regulação da homeostasia energética e do comportamento alimentar, particularmente na indução da saciedade, através das conexões que estabelece com a área hipotalâmica lateral e com os núcleos arqueado, paraventricular e dorsomedial do hipotálamo (Saper *et al.*, 1976; Luiten *et al.*, 1987; Canteras *et al.*, 1994, Sternson *et al.*, 2005). Quanto ao seu envolvimento na produção energética é de realçar a importância das suas conexões com a substância cinzenta periaqueductal e a formação reticular do mesencéfalo, através das quais activa os neurónios da coluna intermediolateral da medula espinhal e os componentes periféricos do sistema nervoso simpático, assim modulando a termogénese no tecido adiposo castanho (revisto em Saito *et al.*, 1989; Yoshimatsu *et al.*, 1993; King, 2006). É-lhe ainda atribuído papel de relevo quer na sincronização dos ritmos biológicos pela ingestão alimentar, função que depende das suas conexões com os núcleos pré-óptico medial e dorsomedial e com a zona sub-paraventricular

do hipotálamo (Choi *et al.*, 1998; Ribeiro *et al.*, 2007), quer na estimulação da actividade antecipadora da ingestão alimentar, acção esta mediada por projecções glutamatérgicas que sinalizam os neurónios orexigénicos da área hipotalâmica lateral, da área pré-óptica e da formação reticular do mesencéfalo (Ribeiro *et al.*, 2009).

Localizada inferolateralmente a este componente está a divisão ventrolateral do VMN (VMNvl). Também de elevada densidade celular, é formada sobretudo por neurónios cujos corpos celulares têm orientação axial mediolateral, são de maiores dimensões que os da vizinha divisão dorsomedial, possuem núcleos ovais e grandes nucléolos e estão, a níveis mais caudais, intercalados com neurónios redondos e de menores dimensões (Van Houten e Brawer, 1978). As suas árvores dendríticas são relativamente pobres e pouco ramificadas, e possuem poucas ou nenhumas espinhas (Millhouse, 1973). Ao contrário da divisão dorsomedial, os seus neurónios caracterizam-se por expressarem, entre outros, receptores para os estrogénios (Pfaff e Keiner, 1973; Simerly *et al.*, 1990; Shughrue *et al.* 1997; Shughrue e Merchanthaler, 2001), para a progesterona (Brown *et al.* 1987; Blaustein *et al.*, 1988) e para a oxitocina (Johnson *et al.*, 1989; Tribollet *et al.*, 1990; Bale *et al*., 1995). Funcionalmente, esta divisão está envolvida na regulação do comportamento sexual feminino, sobretudo da sua fase copulatória (Blaustein e Erskine, 2002) ou receptiva (Beach, 1976), que se caracteriza pelo desenvolvimento do reflexo de lordose em fêmeas que, após exposição sequencial a estrogénios e progesterona, são estimuladas sensorialmente. Trata-se de um movimento estereotipado, que consiste na dorsiflexão da coluna vertebral, e consequente elevação da cabeça e da região do quadril, acompanhada de ligeira extensão dos membros anteriores (Pfaff e Lewis, 1974), postura que facilita a intromissão no acto da cópula, sendo, portanto, vital para a reprodução.

A lordose é um arco reflexo espinho-bulbo-espinhal. O seu ramo aferente conduz os estímulos somatossensoriais, recebidos pelos nervos pudendos e pélvicos, para os neurónios da região lombar da medula espinhal localizados, sobretudo, nas lâminas IV a VIII, a partir de onde são veiculados pela coluna anterolateral da medula espinhal até à formação reticular do bolbo, especialmente o núcleo gigantocelular, e daqui para a substância cinzenta periaquedutal (Kow e Pfaff, 1998). É na substância cinzenta periaquedutal que se inicia o ramo eferente do arco reflexo, que envia informação para o núcleo gigantocelular e daqui, através do feixe reticulo-espinhal, para a região lombar da medula espinhal, sobretudo para as lâminas V-VII, onde estão localizados os neurónios motores dos músculos epaxiais que, ao contrair-se, provocam lordose (Kow e Pfaff, 1998). O VMNvl controla o reflexo da lordose através das projecções que envia pelo fascículo prosencefálico medial, comissura supraóptica

ventral e sistema mesencefálico de fibras periventriculares (Canteras *et al.*, 1994) para o elo comum o ramo aferente e o ramo eferente do arco reflexo espinho-bulbo-espinhal, ou seja, para a substância cinzenta periaquedutal. A activação pelos estrogénios dos neurónios do VMNvl induz a libertação tónica de neurotransmissores na substância cinzenta periaquedutal, o que, por sua vez, modula a actividade do ramo eferente do arco reflexo e, consequentemente, o reflexo de lordose (Kow e Pfaff, 1998).

Esta função do VMNvl depende, não só da sensibilidade dos seus neurónios aos esteróides sexuais, mas também das conexões recíprocas que estabelece com outros núcleos/regiões do SNC que, tal como o VMNvl, possuem receptores para os estrogénios. É o caso do núcleo medial da amígdala (Krettek e Price, 1978; Blaustein *et al.*, 1994; Petrovich *et al.*, 1996), *bed nucleus* da estria terminal (Heimer e Nauta, 1969; Blaustein *et al.*, 1994), subículo ventral (Canteras e Swanson, 1992), núcleos septais laterais (Saper *et al.*, 1976; Canteras *et al.*, 1994), núcleo pré-óptico medial (Saper *et al.*, 1976; Simerly e Swanson, 1988; Canteras *et al.*, 1994), área hipotalâmica anterior (Saper *et al.*, 1978; Risold *et al.*, 1994) e tronco cerebral, particularmente a substância cinzenta periaqueductal, o núcleo parabraquial e a área peripeduncular (Krieger *et al.*, 1979; Fulwiler e Saper, 1984; Arnault e Roger, 1987). A conjugação da influência hormonal e da informação que chega ao VMNvl veiculada através destas aferências, permite-lhe, ainda, integrar o reflexo de lordose no decorrer do comportamento sexual feminino e fazê-lo em perfeita sincronia com a activação do eixo neuroendócrino responsável pela ovulação, tornando assim possível que da cópula resulte fecundação.

Efeitos dos estrogénios no VMN

O VMN é um alvo dos esteróides sexuais tanto no decurso do desenvolvimento como no adulto. Os seus neurónios expressam receptores para os androgénios em todas as divisões citoarquitectónicas do núcleo (Simerly *et al.*, 1990), e receptores para os estrogénios apenas no VMNvl (Pffaff e Keiner, 1973; Simerly *et al.*, 1990; Shughrue *et al.,* 1997; Shughrue e Merchanthaler, 2001). Ao contrário dos receptores para os androgénios, que sob o ponto de vista quantitativo não parecem diferir entre machos e fêmeas (Simerly *et al.*, 1990), os receptores para os estrogénios são no adulto, mas não no período neonatal, mais abundantes nas fêmeas (Yokosuka *et al.*, 1997).

Durante o desenvolvimento, a acção *organizacional* dos esteróides sexuais é responsável pelo estabelecimento de diferenças sexuais estruturais e funcionais que persistem no adulto independentemente dos níveis circulantes de esteróides sexuais, e que apenas podem ser revertidas ou anuladas por manipulação hormonal no período perinatal. Do ponto de vista morfológico, destacam-se as diferenças sexuais no volume do VMN (Matsumoto e Arai, 1983; Madeira *et al.*, 2001) e do seu neurópilo (Madeira *et al.*, 2001), maior nos machos que nas fêmeas, e nas densidades de superfície (Matsumoto e Arai, 1986a,b) e de volume (Miller e Aoki, 1991) das sinapses axodendríticas e axospinhosas, que têm sido referidas como sendo também maiores nos machos que nas fêmeas. Pelo contrário, a densidade das espinhas dendríticas dos neurónios do VMN é maior nas fêmeas que nos machos (Segarra e McEwen, 1991; Madeira *et al.*, 2001). Do ponto de vista funcional é de referir a acção organizacional dos esteróides sexuais no reflexo da lordose já que, em contraste com as fêmeas, os machos castrados apenas desenvolvem este reflexo após exposição prolongada a doses elevadas de estrogénios, não tendo a progesterona qualquer efeito facilitador (Davidson, 1969; Harlan *et al.*, 1984).

Ao contrário do que acontece nos machos, nas fêmeas adultas os níveis de esteróides sexuais flutuam ciclicamente e, assim, fazem variar as características morfológicas e funcionais dos neurónios do VMNvl ao longo do ciclo ovárico. Com efeito, o reflexo de lordose é apenas desencadeado quando os níveis circulantes de esteróides sexuais são elevados, estando inibido em animais ovariectomizados (Boling e Blandau, 1939; Pfaff e Sakuma, 1979; Rubin e Barfield, 1980). Este efeito comportamental é acompanhado de um vasto conjunto de variações morfológicas e das propriedades neuroquímicas dos neurónios do VMNvl que reflectem a acção *organizacional* dos estrogénios sobre este núcleo e que se descrevem, de modo sucinto, em seguida.

Nas fêmeas adultas, a administração de estradiol a ratos ovariectomizados estimula a actividade metabólica dos neurónios do VMNvl, como o demonstram o aumento dos níveis de rRNA (Jones *et al.*, 1986) e de transcrição génica (Meisel e Pfaff, 1984; Yahr e Ulibarri, 1986), a hipertrofia do retículo endoplasmático rugoso, dos corpos de Nissl e do aparelho de Golgi, e o aumento da área dos nucléolos, dos núcleos e dos somas dos neurónios do VMNvl (Cohen e Pfaff, 1981; Carrer e Aoki, 1982; Cohen *et al.*, 1984; Jones *et al.*, 1985; Meisel e Pfaff, 1988). A administração de estrogénios a fêmeas adultas altera também o padrão de conectividade dos neurónios do VMNvl, através do aumento que induz na densidade das espinhas dendríticas (Frankfurt *et al.*, 1990; McEwen e Wooley, 1994; Calizo e Flanagan-Cato, 2000) e das sinapses axodendríticas (Nishizuka e Pfaff, 1989; Frankfurt e McEwen,

1991). São, no entanto, menos claros os efeitos dos estrogénios sobre as arborizações dendríticas dos neurónios do VMNvl já que, embora os resultados de alguns trabalhos mostrem que os estrogénios aumentam o comprimento das árvores dendríticas (Madeira *et al.*, 2001), os de outros apontam para um efeito oposto (Griffin e Flanagan-Cato, 2008).

No VMNvl de fêmeas adultas, os estrogénios também induzem variações na expressão e libertação de substâncias neuroactivas, como a colecistoquinina (Micevych *et al.*, 1988), o GABA e o glutamato (Luine et al., 1997), a serotonina (Lu *et al.*, 1998), o neuropeptídeo Y (Sahu *et al.*, 1992) e a encefalina (Romano *et al.*, 1988), e na expressão e/ou actividade dos receptores de estrogénios (Simerly e Young, 1991; Lauber *et al.*, 1991a), progesterona (Parsons *et al.*, 1982; Brown *et al.*, 1987; Lauber *et al.*, 1991b), colecistoquinina (Akesson *et al.*, 1987), vasopressina (Dubois-Dauphin *et al.*, 1991), oxitocina (Johnson *et al.*, 1989; Tribollet *et al.*, 1990), muscarínicos (Rainbow *et al.*, 1980) e $GABA_A$ (Schumacher *et al.*, 1989).

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Do exposto, pode concluir-se que os numerosos trabalhos realizados no VMN produziram dados que indiscutivelmente demonstram, por um lado, a importância da acção dos esteróides sexuais na modulação do comportamento sexual feminino e, por outro, que a influência dos estrogénios está associada à presença de variações morfológicas e funcionais dos neurónios do VMNvl. Na vertente morfológica, tinha sido também demonstrado que as diferenças sexuais na morfologia do VMNvl ou se tornam aparentes ou são mais distintas, e atenuam-se ou desaparecem, em função dos níveis circulantes de esteróides sexuais (Madeira *et al.*, 2001).

Assim, no **TRABALHO I** teve-se como objectivo analisar se as flutuações dos níveis circulantes de estrogénios e de progesterona ao longo do ciclo ovárico se repercutiam nas dimensões dos organelos citoplasmáticos envolvidos na síntese proteica, e.g., retículo endoplasmático rugoso e aparelho de Golgi, e no número de poros nucleares, que se sabe mediarem a translocação activa e selectiva de ribonucleoproteinas do núcleo para o

citoplasma (Watson, 1959). A comparação destes dados com os existentes na literatura sobre os efeitos da administração de estradiol a ratos ovariectomizados permitiu também avaliar se os sinais morfológicos de activação celular presentes na fase de proestro do ciclo ovárico, quando os níveis circulantes de estrogénios e progesterona são elevados, eram, ou não, idênticos aos induzidos apenas pelos estrogénios exógenos. Com esta finalidade, aplicaram-se métodos estereológicos a fotografias de microscopia electrónica obtidas de neurónios do VMNvl de ratos fêmeas em fase de proestro ou de diestro 1, quando os níveis circulantes de estrogénios e progesterona são baixos, para determinar as dimensões do retículo endoplasmático rugoso e do aparelho de Golgi e o número de poros nucleares. Com o objectivo de avaliar se estes parâmetros eram sexualmente dimórficos, o trabalho foi realizado também em ratos machos.

No **TRABALHO II** teve-se como objectivo estudar as variações ao longo do ciclo ovárico do número total e da dimensão dos contactos sinápticos estabelecidos sobre os corpos celulares, os dendritos e as espinhas dendríticas dos neurónios do VMNvl. Utilizaram-se, para o efeito, técnicas de microscopia electrónica e métodos estereológicos *unbiased* que permitiram estimar o número total de sinapses por neurónio e a área das densidades pós--sinápticas em ratos em fase de proestro e de diestro 1 do ciclo ovárico. Com o objectivo de avaliar se estes parâmetros eram sexualmente dimórficos, o trabalho foi realizado também em ratos machos.

Os resultados obtidos nos TRABALHOS I e II não permitiram destrinçar a acções dos estrogénios e da progesterona dado que os níveis plasmáticos destas hormonas estão ambos em fase de pico no proestro e deprimidos no diestro 1. Assim, no **TRABALHO III** teve-se como objectivo avaliar se a progesterona contribui, ou não, para a exuberante plasticidade estrutural patenteada pelos neurónios do VMNvl ao longo do ciclo ovárico. Com esta finalidade, aplicaram-se os métodos utilizados no TRABALHO II para determinar o número de sinapses axodendríticas e axospinhosas em ratos que, após ovariectomia, foram tratados apenas com benzoato de estradiol, ou com benzoato de estradiol seguido de progesterona ou *mifepristone* (RU486), o antagonista selectivo dos receptores da progesterona. Teve-se também como objectivo neste TRABALHO avaliar se o aumento do número de sinapses induzido pelos estrogénios nos neurónios do VMNvl era mediado pela activação do receptor do estrogénio do tipo α (ERα), do tipo β (ERβ) ou de ambos. Para o efeito, aplicaram-se os métodos utilizados no TRABALHO II para determinar o número de sinapses axospinhosas e axodendríticas em ratos fêmeas que, após ovariectomia, foram tratados com o agonista específico do ERα, *propyl-pyrazole-triol* (PPT) ou do ERβ, *diarylpropionitrile* (DPN). Com

o objectivo de aquilatar se as doses das hormonas administradas eram eficazes do ponto de vista fisiológico, avaliou-se também nestes animais o reflexo de lordose induzido por estimulação cérvico-vaginal.

No **TRABALHO IV** teve-se como objectivo averiguar qual a influência das aferências nervosas na mediação dos efeitos dos estrogénios na plasticidade sináptica dos neurónios do VMNvl. Para a concretização deste objectivo determinou-se o número de sinapses axodendríticas e axospinhosas por neurónio em ratos ovariectomizados que, após serem submetidos a deaferenciação cirúrgica do VMN, foram tratados com benzoato de estradiol ou com veículo. Uma vez que, a par da plasticidade estrutural sináptica, a indução de receptores para a progesterona é um dos sinais mais exuberantes da acção dos estrogénios no VMNvl, estimou-se também o número total de neurónios imunorreactivos para estes receptores em ratos submetidos aos mesmos tratamentos.

Em resumo, com o conjunto de trabalhos incluídos na presente dissertação pretendeu-se avaliar se:

1. A hipertrofia neuronal que se observa ciclicamente no VMNvl na presença de elevadas concentrações plasmáticas de esteróides sexuais reflecte activação neuronal e aumento da complexidade do padrão de organização sináptica dos seus neurónios.

2. Tais alterações resultam exclusivamente da acção dos estrogénios ou da acção conjunta dos estrogénios e da progesterona.

3. A acção trófica dos estrogénios é mediada pela activação dos receptores α ou β, ou pela acção combinada de ambos.

4. A influência dos estrogénios na plasticidade estrutural e neuroquímica do VMNvl é exercida localmente ou é indirecta e mediada pelas aferências neuronais.

TRABALHOS

I.

Neuronal organelles and nuclear pores of hypothalamic ventromedial neurons are sexually dimorphic and change during the estrus cycle in the rat

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NEURONAL ORGANELLES AND NUCLEAR PORES OF HYPOTHALAMIC VENTROMEDIAL NEURONS ARE SEXUALLY DIMORPHIC AND CHANGE DURING THE ESTRUS CYCLE IN THE RAT

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Abstract—Neurons in the ventrolateral division of the hypothalamic ventromedial nucleus (VMNvl) become hypertrophied when exposed to high estrogen levels, an effect that has been observed after estrogen treatment of ovariectomized rats as well as during the proestrus stage of the ovarian cycle. In an attempt to examine whether the neuronal hypertrophy noticed in these conditions reflects metabolic activation of the neurons we have examined, using quantitative methods, the cytoplasmic organelles involved in protein synthesis and the nuclear pores of VMNvl neurons from females on proestrus, when estrogen levels are high, and on diestrus, when estrogen levels are low. Because VMNvl neurons are sexually dimorphic with respect to their size we have performed, in parallel, similar analyses in neurons from age-matched male rats. Our results show that the volume and the surface area of the rough endoplasmic reticulum (RER) and Golgi apparatus are increased at proestrus. They also show that the density of nuclear pores is greater in males than in females whereas the volume and the surface area of the RER and Golgi apparatus are sexually dimorphic only at specific phases of the ovarian cycle: the male–female differences are notorious in the RER when females are on diestrus and in the Golgi apparatus when they are on proestrus. Given that the size of the RER and of the Golgi apparatus correlates with the level of neuronal protein synthesis, data obtained in this study suggest that the sex-related differences and the estrus cycle variations in neuronal size reflect corresponding differences and fluctuations in the metabolic activity of VMNvl neurons.

Key words: hypothalamus, sex differences, estrus, rough endoplasmic reticulum, Golgi apparatus, stereology.

The ventromedial nucleus of the hypothalamus (VMN) is widely recognized as a sexually dimorphic cell group of the brain that has been implicated in the modulation of a variety of physiological mechanisms and behaviors, such as the female reproductive behavior (Pfaff, 1980). The sex differences noticed in its anatomy are particularly evident in the ventrolateral division (VMNvl), where numerous neurons that express receptors for gonadal steroids are concentrated (Pfaff and Keiner, 1973; Simerly et al., 1990; Shughrue et al., 1997). Neurons located in the VMNvl display male–female differences in their volume (Matsumoto and Arai, 1983; Madeira et al., 2001), length of dendritic trees, dendritic spine density (Madeira et al., 2001), neurochemistry (reviewed in Madeira and Lieberman, 1995) as well as in the density (Matsumoto and Arai, 1986; Miller and Aoki, 1991) and total number (Sá and Madeira, in press) of the synapses established upon their dendritic shafts and spines. It is also known that some of these sex differences, namely those noticed in the volume of the neuronal cell bodies and in the length of the dendritic trees, are not constant across the ovarian cycle, being evident when females are in diestrus and disappearing when they are in proestrus (Madeira et al., 2001).

In contrast to the wealth of information regarding the influence of sex steroids in shaping the cytoarchitecture of the VMNvl, relatively little is known about their effects on the size of the cytoplasmic organelles of its constituent neurons. Earlier studies have shown that the administration of estrogen to ovariectomized rats leads to hypertrophy of neuronal perikarya, condensation of nucleolar material, increased stacking of the rough endoplasmic reticulum, enlargement of the Nissl substance and Golgi complexes, and emergence of pleomorphic mitochondria in VMNvl neurons (Cohen and Pfaff, 1981; Carrer and Aoki, 1982; Cohen et al., 1984; Meisel and Pfaff, 1988). Even though these data suggest that exogenous estrogen modifies the metabolic activity of VMNvl neurons, it is not known whether identical changes occur in response to the fluctuation of estrogen levels across the estrus cycle. In addition, to our knowledge only one study has addressed the possibility that the cytoplasmic organelles of VMNvl neurons might display sexual dimorphic features (Ishunina et al., 2001). This investigation, performed in human material, showed that the size of the Golgi apparatus relative to cell size is larger in young women than in young men.

In this study we sought to quantitatively analyze whether the morphology of the cytoplasmic organelles of VMNvl neurons differs between the sexes and, also, if in females the size of these organelles varies over the estrus cycle. To accomplish this, we have used electron microscopy and unbiased stereological techniques to examine the volume and the surface area of the rough endoplasmic reticulum (RER) and of the Golgi apparatus of VMNvl neurons in males and in females on proestrus, when the circulating levels of estrogen are high, and on diestrus day 1, when the circulating levels of estrogen are low. Nuclear pores are known to mediate the selective and energy-

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Abbreviations: ANOVA, one-way analysis of variance; RER, rough endoplasmic reticulum; S.D., standard deviation; S.E.M., standard error of the mean; Sv, surface density; VMN, ventromedial nucleus; VMNvl, ventrolateral division of the ventromedial nucleus; Vv, volume density.

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dependent translocation of ribonucleoprotein particles from the nucleus to the cytoplasm (Watson, 1959). Since VMNvl neurons seem to be activated in response to estrogen, we have additionally analyzed the density of nuclear pores in the same groups of animals.

EXPERIMENTAL PROCEDURES

Animals

Young-adult male and female Wistar rats were maintained under standard laboratory conditions (12-h light/dark cycle and ambient temperature of 22 °C) and had free access to food and water. The group of males consisted of six animals whereas the group of females was composed of six rats in proestrus and six rats in diestrus day 1 (diestrus). The estrus cycle was monitored by daily inspection of vaginal cytology for at least 3 weeks before killing. Only regularly cycling rats were used in the present study. Animals were killed at 3 months of age between 14 and 16 h. All studies were performed in accordance with the European Communities Council of 24 November 1986 (86/609/EEC) and Portuguese Act no. 129/92. All efforts were made to minimize the number of animals used and their suffering.

Hormonal determinations

Prior to perfusion, 500 μl of blood were taken directly from the heart into Eppendorf tubes. The serum was separated by centrifugation and stored at −20 °C until the time of assay. The concentrations of 17 β-estradiol were determined by radioimmunoassay techniques (MP Biomedicals, Irvine, CA, USA).

Tissue preparation

Animals were anesthetized with 3 ml/kg of a solution containing 10 mg/ml of sodium pentobarbital and 40 mg/ml of chloral hydrate given i.p. and killed by transcardiac perfusion of a fixative solution containing 1% paraformaldehyde and 1% gluteraldehyde in 0.12 M phosphate buffer, pH 7.2. After removal from the skulls, the brains were weighed and post-fixed for 1 h in fresh fixative. They were then cut longitudinally in the mid-sagittal plane to separate the cerebral hemispheres, which were transected in the coronal plane through the posterior border of the optic chiasm. After removal of the occipital poles, the cerebral hemispheres were mounted on a vibratome with the rostral surface up and serially sectioned in the coronal plane at 40 μm. When the VMN was first identified as being formed by two well-defined clusters of cells, the dorsomedial division and the VMNvl, separated by a cell-poor central zone (Bleier et al., 1979; Simerly, 1995; Madeira et al., 2001), one 500 μm-thick coronal section containing the VMN was collected, followed by a sequence of alternate 40 and 500 μm-thick sections. The 40 μm-thick sections were mounted and stained with Thionin for precise identification of the boundaries of the VMN and of its dorsomedial division and VMNvl and used as reference for the micro-dissection of the VMNvl in the adjacent 500 μm-thick sections.

The four blocks of tissue containing the VMNvl that were collected per animal were postfixed for 2 h in a 2% solution of osmium tetroxide in 0.12 M phosphate buffer, dehydrated in graded ethanols and embedded in Epon according to the isector method (Nyengaard and Gundersen, 1992) to obtain isotropic, uniform random sections. From each block, eight serial 2 μm-thick sections of the VMNvl were cut, which provided a total of 32 serial semithin sections per animal. These semithin sections were stained with Toluidine Blue and coverslipped with Entellan. Pyramids were trimmed on each of the blocks and series of 10–12 ultrathin sections were cut, collected on Formvar-coated grids and double stained with uranyl acetate and lead citrate.

Quantifications were made in neurons photographed from sections obtained from all blocks containing the VMNvl. From each block, alternated sections were sampled and in each of these sections only one neuron was photographed, which provided an average of 20 neurons per animal. The presence of the nucleus and of a complete nucleolus was considered the only requisite for sampling neurons. These neurons were photographed at primary magnification of 5400× and enlarged photographically to a final magnification of 16,200×.

Stereological analyses

In VMNvl neurons, RER is often seen as being formed by short, independent segments and the ribosomes occur mainly as unbound rosettes scattered throughout the cytoplasm (Fig. 1). Nissl bodies are rare and, when present, they are usually small and located in the periphery of the soma (Millhouse, 1978). Conversely, the Golgi apparatus (Fig. 1) is seen in almost every region of the cell body and is arranged in several distinct patches, either stretched out along the perimeter of the nucleus or modulated in curvilinear arrays (Millhouse, 1978). The volume of these organelles was determined on the basis of their volume density (Vv) and on the volume of the cytoplasm of the neuronal somata. The Vv was estimated by point counting using an appropriate test point system (Jensen and Gundersen, 1982). Points that fell on the channel system and on the surrounding cytosol (Fig. 1) were counted as points on the RER, and those that fell on the field

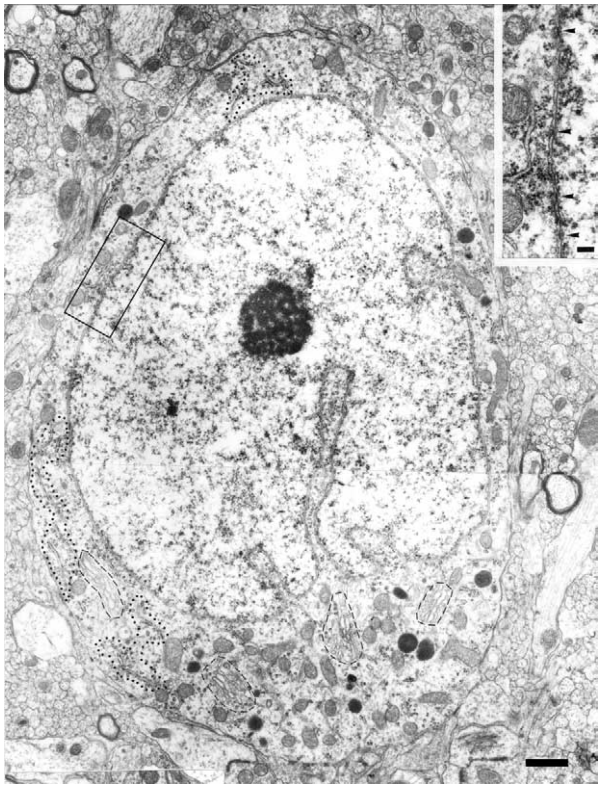

Fig. 1. Electron micrograph of a VMNvl neuronal cell body. The electron-dense cytoplasm contains relatively few short segments of RER, which are outlined by dotted lines, and numerous groups of polyribosomes and patches of Golgi complex, which are outlined by dashed lines. The box delineates the part of the cell nuclear envelope that is shown at higher magnification in the inset. Inset. Note that the membrane of the nuclear envelope is perpendicularly sectioned. Four nuclear pores (arrowheads) can be seen. Scale bars=1.0 μm; inset=0.2 μm.

enclosed by the Golgi complex (Fig. 1) were counted as points on the Golgi apparatus (Picard et al., 1972; Peters et al., 1976). The volume of the cytoplasm was calculated by subtracting the volume of the neuronal nuclei from the volume of the neuronal cell bodies. These volumes were estimated with the nucleator method implemented in isotropic, uniform random (Gundersen, 1988) semithin sections of the VMNvl. Neurons were selected for measurements with physical disectors using the nucleolus as the sampling unit. In each sampled neuron, the distance from the nucleolus to the nucleus boundary and the distance from the nucleolus to the cell boundary were measured in four different directions. The average number of neurons measured per animal was 70.

The surface area of the cisternae of the RER and of the Golgi apparatus was estimated, as described by Baddeley et al. (1986), by multiplying the surface density (Sv) of the RER and Golgi apparatus by the volume of the respective organelle. The Sv was estimated with the help of a 'staggered' cycloid test system (Baddeley et al., 1986; Cruz-Orive and Hunziker, 1986) by counting the number of intersections of the cycloid arcs with the membranes of the cisternae of the RER, and of the Golgi complex and Golgi vesicles.

The number of nuclear pores per unit length of the nuclear envelope was estimated by dividing the number of nuclear pores visible along the nuclear membrane by the length of the nuclear envelope, which was measured by using a MOP-videoplan. The nuclear pore complexes are protein assemblies that form channels through the double nuclear membrane at points where the inner and outer membranes are fused (Franke et al., 1981; Featherstone et al., 1988). Thus, quantifications were all made in perpendicularly cut nuclear profiles, in which the inner and outer membranes appear as two parallel lines with easily recognized pores (Fig. 1; Andrade et al., 1988).

Statistical analysis

Differences among groups were assessed by one-way analysis of variance (ANOVA). Whenever significant results were found from the overall ANOVA, pair-wise comparisons were subsequently made with the post hoc Tukey honest significant difference test. Differences were considered significant if $P<0.05$. Throughout the text, data are presented as means with their coefficients of variation (CV=S.D./mean), and hormonal concentrations are shown as means and S.E.M.

RESULTS

Hormone levels

The plasma concentration of estradiol was 176 (6) pg/ml by 16 h on proestrus day, and 100 (4) pg/ml by the same time on diestrus day. As expected, proestrus females had higher estradiol levels than diestrus females.

Cytoplasmic and nuclear volumes

The volume of the cytoplasm of VMNvl neurons was 1774 (0.10) μm^3 in male rats, 1751 (0.08) μm^3 in proestrus rats and 1267 (0.10) μm^3 in diestrus rats. ANOVA showed that the variations observed in these volumes were related to sex and estrus cycle phase ($F=21.88$, $P<0.0005$). The volume of the cytoplasm of VMNvl cell bodies was higher in proestrus females ($P<0.0005$) and in males ($P<0.0005$) than in diestrus females, and did not significantly differ between males and proestrus rats.

The volume of the nuclei of VMNvl neurons was 658 (0.06) μm^3 in male rats, 678 (0.10) μm^3 in proestrus rats and 488 (0.10) μm^3 in diestrus rats. ANOVA showed that these variations were dependent on the sex of the animals and on the phase of the estrus cycle ($F=16.15$, $P<0.0005$). The nuclear volume was larger in males ($P=0.001$) and in proestrus females ($P=0.0005$) than in diestrus rats. No differences were found between males and proestrus rats.

RER

ANOVA showed that the variations in the Vv and in the volume of the RER were related to the phase of the estrus cycle and to the sex of the animals ($F=6.83$, $P=0.006$ and $F=36.25$, $P<0.0005$, respectively). The Vv was higher in proestrus than in diestrus rats (Fig. 2A) and did not differ between males and females. In addition, the volume of the RER was larger in proestrus females and in males than in diestrus females and did not differ between males and proestrus females (Fig. 2A).

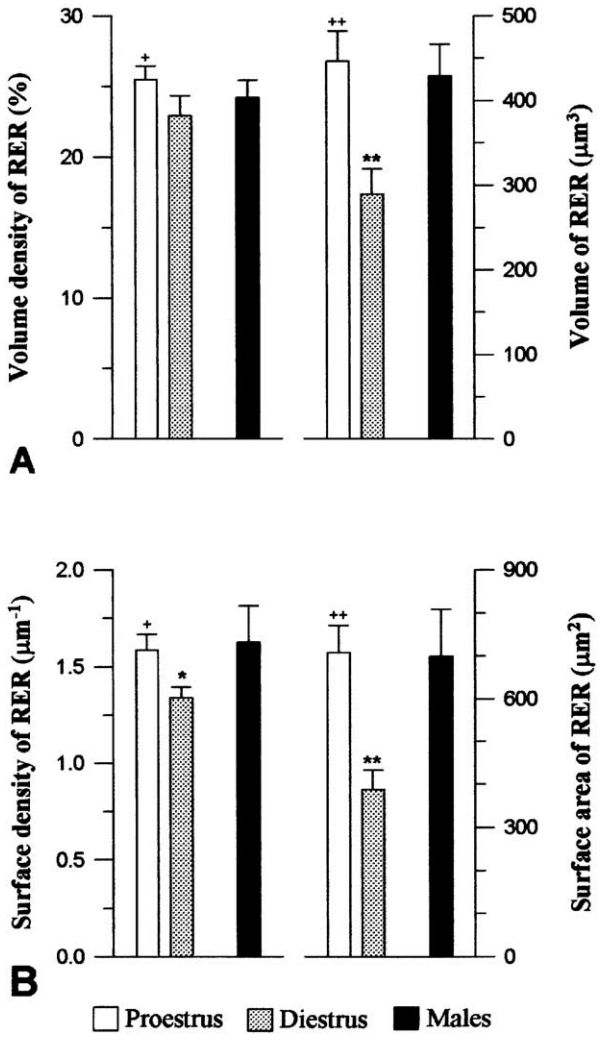

Fig. 2. Graphic representation of the morphometric data obtained from the RER of VMNvl neurons of male rats, and of female rats on diestrus and proestrus. Columns represent means and vertical bars 1 S.D. (A) Vv and volume of the RER. (B) Sv and surface area of the RER. Tukey's post hoc tests: * $P<0.005$, ** $P<0.0005$, compared with male rats; $^{+}$ $P<0.01$, $^{++}$ $P<0.0005$, compared with diestrus rats.

The Sv of the RER was also influenced by the phase of the estrus cycle and by the sex of the animals ($F=9.60$, $P=0.002$), similarly to what happened with the surface area of this organelle ($F=32.51$, $P<0.0005$). The Sv and the surface area were higher in males and in proestrus rats than in diestrus rats (Fig. 2B). No differences were found in these parameters between males and proestrus females.

Golgi apparatus

As shown by ANOVA, the Vv of the Golgi apparatus varied as a function of the sex of the rats and of the estrus cycle phase ($F=14.1$, $P<0.0005$), similarly to what occurred with its volume ($F=17.02$, $P=0.0005$). Specifically, the Vv and the volume of the Golgi apparatus were higher in proestrus females than in males and diestrus females (Fig. 3A). No differences were noticed between males and diestrus females (Fig. 3A).

The Sv and the surface area of the Golgi apparatus were also dependent on the sex of the animals and on the phase of the estrus cycle ($F=6.23$, $P=0.01$ and $F=23.21$, $P<0.0005$, respectively). The Sv and the surface area were higher in proestrus females than in males and diestrus females (Fig. 3B). No differences were found in these parameters between male rats and diestrus females.

Nuclear pores

ANOVA showed that the variations in the number of pores per unit length of nuclear membrane were dependent on the sex of the animals and on the phase of the estrus cycle ($F=14.3$, $P<0.0005$). The density of nuclear pores was higher in males than in females, and did not differ between females at different phases of the estrus cycle (Fig. 4).

DISCUSSION

Earlier studies have revealed that estrogen, when administered to ovariectomized rats, augments the protein synthesizing machinery in VMNvl neurons (Cohen and Pfaff, 1981; Carrer and Aoki, 1982; Jones et al., 1985; Erskine and Miller, 1995). Data obtained in the present study lend support and extend these observations by showing that the size of the RER fluctuates across the ovarian cycle in parallel with the variations in the levels of endogenous estrogen. Specifically, the volume and the surface area of the RER increase from diestrus to proestrus, while estrogen levels rise, and then decrease until the next diestrus, as estrogen levels decline. Our results also show that the Vv of the RER is greater in proestrus than in diestrus rats, which indicates that at proestrus there is a proportionally larger increase in the volume of the RER than in the volume of the cytoplasm. The observation that the surface area and the Sv of the RER undergo a similar estrus-related variation denotes that the larger volume of the RER in proestrus relative to diestrus rats is attributable not to dilation of the existing cisternae, but to an effective increase in the extent of the RER membrane and/or in the number of RER cisternae. Since the ribosomes connected to the RER membrane are involved in protein synthesis (Droz, 1973) there are reasons to assume that VMNvl

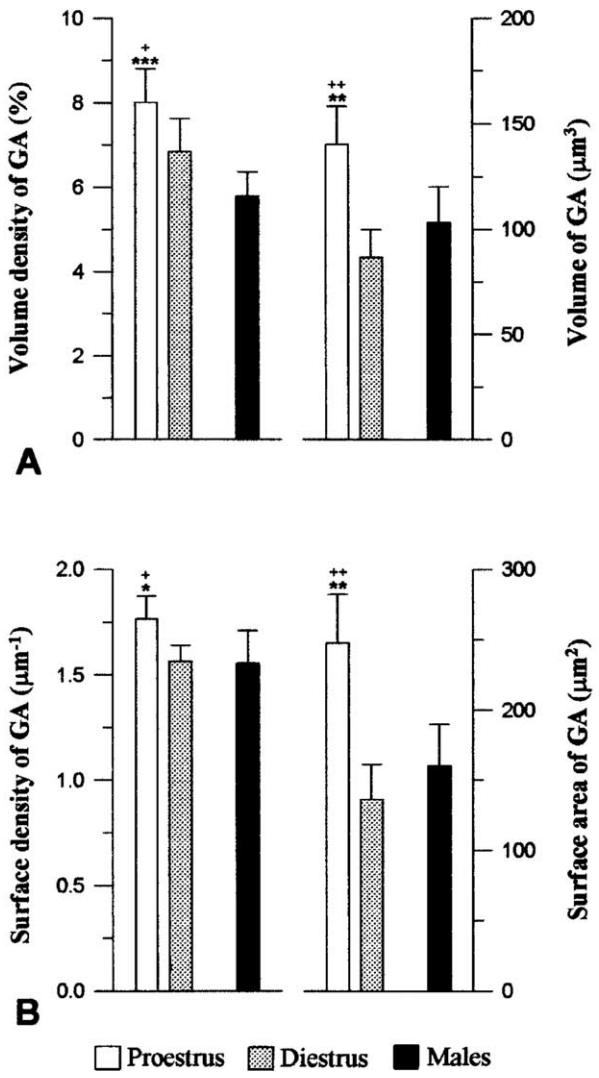

Fig. 3. Graphic representation of the morphometric data obtained from the Golgi apparatus of VMNvl neurons of male rats, and of female rats on diestrus and proestrus. Columns represent means and vertical bars 1 S.D. (A) Vv and volume. (B) Sv and surface area. Tukey's post hoc tests: * $P<0.05$, ** $P<0.005$, *** $P<0.0005$, compared with male rats; $^{+}$ $P<0.05$, $^{++}$ $P<0.0005$, compared with diestrus rats.

neurons are metabolically more active in proestrus than in diestrus rats. As a result of the gonadal steroid-induced changes in the morphology of the RER, the sex differences in the size of this organelle are not constant across the estrus cycle. Specifically, although the volume and the surface area of the RER were 1.5 and 1.8 times larger, respectively, in males than in diestrus females, no sex-related differences could be noticed when females were in proestrus.

In contrast to the RER, the sex differences in the volume and in the surface area of the Golgi apparatus were noticeable only when females were in proestrus. In particular, the volume and the surface area of this organelle were approximately 1.5 times larger in proestrus females than in males, which is in agreement with data obtained by Ishunina and coworkers (2001) in the human

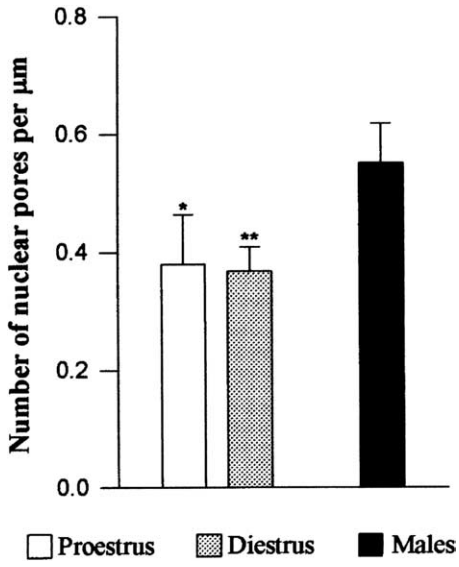

Fig. 4. Graphic representation of the number of pores per unit length of nuclear membrane in VMNvl neurons of male rats, and female rats in diestrus and proestrus. Columns represent means and vertical bars 1 S.D. Tukey's post hoc tests: * $P<0.005$, ** $P<0.001$, compared with male rats.

VMN. Yet, this finding cannot be considered as evocative of the sexual dimorphic features of the Golgi apparatus because opposite sex differences have been noticed in its volume in other hypothalamic neurons, namely in the magnocellular neurons of the supraoptic nucleus (Paula-Barbosa et al., 1993). According to our results, diestrus females are similar to males in what concerns the complexity of the organization of the Golgi apparatus. Actually, no differences were noticed between these groups in what concerns the Vv of the Golgi apparatus and the Sv of its membranes. Conversely, the Vv of the Golgi apparatus was increased in proestrus relative to diestrus rats, which indicates that gonadal hormones provoke a proportionally greater enlargement of this organelle than of the cytoplasm of VMNvl neurons. Moreover, the observation that the Sv and the surface area of the Golgi membranes were both greater in proestrus than in diestrus rats shows that the hormone-induced hypertrophy of the Golgi apparatus is accounted for by an enlargement of the cisternal membranes and/or by an increase in number of cisternae and Golgi vesicles. These effects are similar to those observed in the RER and conform to the earlier descriptions of enlargement of the Golgi apparatus in the VMNvl neurons of ovariectomized rats exposed to exogenous estrogen (Cohen and Pfaff, 1981; Carrer and Aoki, 1982; Cohen et al., 1984).

Although the variations we have described in the RER and Golgi apparatus are likely to occur in response to fluctuations in endogenous estrogen, as they mimic the changes induced by the administration of estrogen to ovariectomized rats, it is possible that they might also be related to the actions of progesterone and/or testosterone. As a matter of fact, the levels of these steroids vary during the estrus cycle (Rush and Blake, 1982; Scharfman et al., 2003) and there are studies showing that progesterone can affect the morphology of ventromedial neurons in estrogen-treated rats (Meisel and Pfaff, 1988; McEwen and Woolley, 1994) and that testosterone can modify the synapse density in the hippocampus of ovariectomized rats (Leranth et al., 2004; MacLusky et al., 2004).

Our results show that there are no changes in the number of pores per unit length of nuclear envelope along the estrus cycle. Despite this fact, we have reasons to assume that the total number of nuclear pores is higher in proestrus than in diestrus rats, i.e. that the number of nuclear pores correlates positively with estrogen levels, because the nuclear volume, and thus the size the nuclear membrane, is larger in proestrus than in diestrus rats. Actually, variations of the same type have been observed in the arcuate nucleus in response either to physiological variations of estrogen levels (Garcia-Segura et al., 1987) or to exogenous estrogen (Perez et al., 1991). In addition, our data also show that the density of nuclear pores is 1.5 times higher in males than in females, regardless the phase of the estrus cycle. Therefore, it is very probable that the total number of nuclear pores might be also sexually dimorphic and greater in males than in females because the volume of the VMNvl neuronal nuclei, and thus the area of the nuclear membrane, is similar in males and in proestrus females. Although the mechanisms of pore formation and turnover are not well understood, it is known that nuclear pore density is associated with nuclear transcriptional activity (Maul et al., 1980). Therefore, the existence of more nuclear pores in proestrus than in diestrus rats indicates that the nuclear transcriptional activity of VMNvl neurons is enhanced at high estrogen levels.

In summary, in this study we present evidence that the size of the protein synthesizing machinery of VMNvl neurons displays sex dimorphic features and that, due to its dependency on gonadal steroid levels, the male–female differences are apparent only at specific phases of the ovarian cycle. Conversely, the density of nuclear pores does not change as a function of estrogen levels, and is smaller in females than in males.

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II.

Estrogen modulates the sexually dimorphic synaptic connectivity of the ventromedial nucleus

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Estrogen Modulates the Sexually Dimorphic Synaptic Connectivity of the Ventromedial Nucleus

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ABSTRACT

Neurons in the ventrolateral division of the hypothalamic ventromedial nucleus (VMNvl) display a remarkable estrogen-dependent functional and structural plasticity, which is likely to be mediated, in part at least, by neuronal afferents. The present study was designed to determine whether the number of synapses per neuron and the size of individual synapses in the VMNvl vary across the estrus cycle and, also, whether they differ between the sexes. To accomplish this, the VMNvl of adult female rats at proestrus or diestrus day 1 and of age-matched male rats was analyzed using electron microscopy. We found that a single VMNvl neuron receives around 7,000 synapses during diestrus and ~10,000 during proestrus. This estrus cycle-related variation is accounted for by increases in the number of all types of synapses. In males, the number of synapses received by each VMNvl neuron is similar to that of diestrus rats (~7,500). However, in males the number of axodendritic and axospinous synapses is smaller than in proestrus rats, whereas the number of axosomatic synapses is higher than in diestrus rats. In addition, we found that the size of the postsynaptic densities of axospinous and axosomatic synapses is consistently larger in males than in females. Our results show that the synaptic organization of the VMNvl is sexually dimorphic, with females having more dendritic synapses and males more somatic synapses. They also show that the synaptic plasticity induced by estrogen in the VMNvl is characterized by changes in the number, but not the size, of the synapses. J. Comp. Neurol. 484:68–79, 2005.

Indexing terms: VMN; synaptic plasticity; sex differences; estrus cycle; stereology; hypothalamus

The ventromedial nucleus (VMN) of the hypothalamus is a key region in the regulation of the hormone-dependent expression of the lordosis reflex, the sexual response in female rodents. This functional attribute has been mainly ascribed to its ventrolateral division (VMNvl), a major target for gonadal steroids (Pfaff and Keiner, 1973; Simerly et al., 1990; Shughrue et al., 1997) that has strong connections with other limbic-hypothalamic nuclei containing estrogen-sensitive neurons (for reviews, see Madeira and Lieberman, 1995; Simerly, 2002; see also Canteras et al., 1994). One particularly interesting feature of the VMNvl is that its neurons display a remarkable estrogen-dependent functional and structural plasticity. Estrogen implants in the VMNvl restore lordosis behavior in female rats made nonreceptive by ovariectomy (Rubin and Barfield, 1980) and estrogen treatment of ovariectomized rats increases the size of neuronal cell bodies and organelles involved in protein synthesis (Cohen and Pfaff, 1981; Carrer and Aoki, 1982; Cohen et al., 1984; Jones et al., 1985), the number of dendritic spines (Frankfurt and McEwen, 1991a; McEwen and Wooley, 1994; Calizo and Flanagan-Cato, 2000), and the density of terminals (Carrer and Aoki, 1982) and synapses (Carrer and Aoki, 1982; Nishizuka and Pfaff, 1989; Frankfurt and McEwen, 1991b; McEwen and Wooley, 1994).

There is also evidence that, during the female reproductive cycle, physiological levels of gonadal steroids influence the morphology of VMNvl neurons. Neuronal cell bodies enlarge (Madeira et al., 2001), dendrites elongate (Madeira et al., 2001), and dendritic spines increase in

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number (Frankfurt et al., 1990; Madeira et al., 2001) in response to high levels of estrogen, and these changes occur reversibly every 4–5 days. To modulate neuronal plasticity in the VMNvl of the female rat, estrogen might act through classic nuclear receptors and/or via non-genomic mechanisms. Actually, it is known that 30% of the neurons in the VMNvl concentrate estrogen (Morrell and Pfaff, 1983) and that some of the effects of this hormone, namely, those noticed in dendritic spine density, are not confined to neurons that express the estrogen receptor (Calizo and Flanagan-Cato, 2000). The finding that some of the effects of estrogen are blocked by surgical deafferentation of the VMN (Nishizuka and Pfaff, 1989) suggests that they are mediated by VMN afferents, inasmuch as most of them express estrogen receptors (Heimer and Nauta, 1969; Saper et al., 1978; Simerly and Swanson, 1988; Canteras and Swanson, 1992; Canteras et al., 1992, 1995). This possibility led us to examine whether the pattern of connectivity of VMNvl neurons changes over the estrus cycle. Thus, we estimated the number and the size of the synaptic contacts established upon the perikarya and the dendritic trees, including their spines, of VMNvl neurons in proestrus and in diestrus rats, i.e., at phases of the estrus cycle that typically show opposite hormonal profiles.

The VMN has long been thought of as a sexually dimorphic nucleus, and there are descriptions of male–female differences in its volume (Matsumoto and Arai, 1983; Madeira et al., 2001), neurochemistry (reviewed in Madeira and Lieberman, 1995), spine density (Madeira et al., 2001), and density of axodendritic spine and shaft synapses (Matsumoto and Arai, 1986b; Miller and Aoki, 1991). There is evidence that the sex-related differences in the density of axodendritic synapses result from the organizational effects of sex steroid hormones during development (Matsumoto and Arai, 1986a; Miller and Aoki, 1991), and that the adult steroid environment in females is a determining factor of the magnitude of the morphological sex differences in the VMN (Madeira et al., 2001). In fact, the sex differences in the volume of the VMN and in dendritic spine density are maximal when females are in diestrus and in proestrus, respectively, whereas the sex-related differences in neuronal size, namely, in the length of the terminal dendritic branches, become apparent only at certain phases of the estrus cycle. Thus, even though male–female differences have been noticed in the numerical and areal density of synapses, parameters which are sensitive to variations in the volume of the reference space, it still remains unknown whether or not the absolute number of synapses in the VMNvl is sexually dimorphic. The present work was also designed to evaluate this issue by estimating in parallel the number and the size of synaptic contacts in the VMNvl of male and female rats.

MATERIALS AND METHODS

Animals

Young-adult male and female Wistar rats were maintained on a 12-hour light/dark cycle (lights on at 0700) and ambient temperature of 22°C, with food and water available ad libitum. The estrus cycle was monitored by daily inspection of vaginal cytology for at least 3 weeks before killing. Only regularly cycling rats were used in the present study. Animals were killed at 3 months of age between 1400 and 1600 hours. The group of males consisted of six animals, and the group of females of six rats in proestrus and six rats in diestrus day 1 (diestrus). All studies were performed in accordance with the European Communities Council Directives of 24 November 1986 (86/609/EEC) and Portuguese Act №129/92.

Hormonal determinations

Prior to perfusion, blood samples (500 μl) were taken directly from the heart into Eppendorf tubes. The serum was separated by centrifugation and stored at –20°C until the time of assay. The concentrations of 17 β-estradiol were determined by radioimmunoassay techniques using kits that were purchased from MP Biomedicals (Irvine, CA).

Tissue preparation

Animals were anesthetized with 3 ml/kg of a solution containing sodium pentobarbital (10 mg/ml) and chloral hydrate (40 mg/ml) given i.p. and killed by transcardiac perfusion of a fixative solution containing 1% paraformaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer, pH 7.2. The brains were removed from the skulls, weighed, and immersed in the same fixative solution for 1 hour. After postfixation they were bisected sagittally through the midline.

The right and left hemispheres were transected in the coronal plane through the posterior border of the optic chiasm. After removal of the occipital poles, they were mounted on a Vibratome with the rostral surface up. Forty-μm-thick coronal sections were cut through the tuberal region of the hypothalamus, mounted on slides, stained with thionin, and observed under the optic microscope. When the VMN was first identified as being formed by two well-defined clusters of cells, the dorsomedial and the ventrolateral (VMNvl) divisions, separated by a cell-poor central zone (Bleier et al., 1979; Simerly, 1995; Madeira et al., 2001), Vibratome sections of alternate thickness (40 μm and 500 μm) were obtained. The 40-μm-thick sections were mounted on slides and stained with thionin for precise identification of the boundaries of the VMN and of its dorsomedial and ventrolateral divisions (Fig. 1). Based on tissue landmarks visible in these sections, the adjacent 500-μm-thick sections were dissected under microscope observation to isolate the VMNvl (Fig. 1C,D). The blocks of tissue containing the VMNvl were then processed for electron microscopy. For this purpose, they were postfixed for 2 hours in a 2% solution of osmium tetroxide in 0.12 M phosphate buffer, rinsed in 25% ethanol, and dehydrated through graded series of ethanol solutions (90%, 95%, and 100%). The blocks were then stained in 1% uranyl acetate for 1 hour at room temperature at the 70% alcohol stage. After passage through propylene oxide, the blocks were embedded in Epon according to the isector method (Nyengaard and Gundersen, 1992). Accordingly, embedding took place in molds containing spherical cavities; the resulting spherical embedded blocks were rolled and thereafter reembedded in Epon.

From each of the four blocks obtained per animal, eight serial 2-μm-thick sections of the VMNvl were cut, which provided a total of 32 serial semithin sections per animal. Each semithin section was placed on a drop of distilled water on a gelatin-coated microscope slide, dried on a slide-warming plate at 60°C, stained with Toluidine blue,

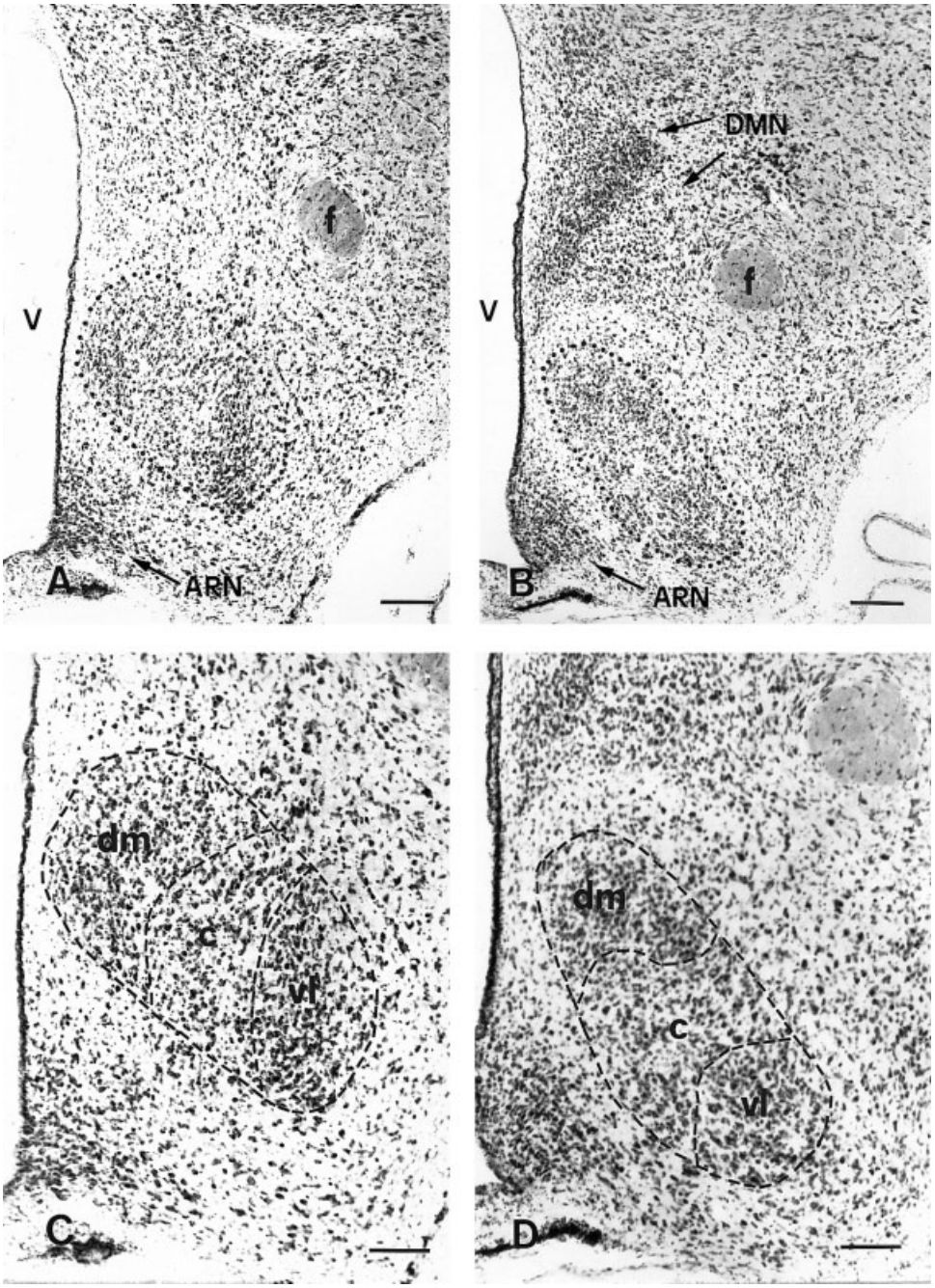

Fig. 1. Photomicrographs of 40-μm-thick coronal sections of the hypothalamus taken at two different rostrocaudal levels of the ventromedial nucleus (VMN). **A:** Section through the rostral part of the VMN at the level where the dorsomedial, the ventrolateral, and the central divisions can be easily identified for the first time. **B:** Section taken at approximately the midlevel of the rostrocaudal extent of the VMN. At both levels, the VMN has an elliptic shape and is entirely surrounded by a lightly staining zone. This zone corresponds to a cell-poor area that clearly demarcates the cellular core of the VMN (delineated by a dashed line) from the rest of the hypothalamus. The arcuate nucleus (ARN) and the fornix (f) can be seen at both levels, whereas the dorsomedial nucleus (DMN) can be visualized only in B. **C,D:** Higher magnification of the photomicrographs shown in A and B to illustrate the cytoarchitectonic features of the dorsomedial (dm), central (c), and ventrolateral (vl) divisions of the VMN. The dashed lines indicate the border of the cellular core of the VMN and the limits between its constituent divisions. V, third ventricle. Scales bars = 200 μm in A,B; 150 μm in C,D.

rinsed with distilled water, dried again, and coverslipped with Entellan. Pyramids were then trimmed on each of the four blocks. From each block, 8–10 serial ultrathin sections were cut, collected on Formvar-coated grids, and double-stained with uranyl acetate and lead citrate.

Stereological analyses

Synapse density. The number of dendritic synapses per unit volume of neuropil (numerical density, N_v) was estimated by using the physical disector method (Sterio,

1984; Madeira and Paula-Barbosa, 1993). For this purpose, electron micrographs of corresponding fields of the neuropil were taken at a primary magnification of 5,400× and enlarged photographically to a final magnification of 16,200×. Two separate counting fields were photographed per block. Areas of the neuropil occupied by potentially interfering structures, such as large blood vessels, glial cells, and myelin, were intentionally avoided. Disectors were made from micrographs obtained from pairs of adjacent sections (Figs. 2, 3). Because each section was used in turn as the reference section, nine disectors were made per block, which provided a total of 36 disectors per animal. A transparency with an unbiased counting frame was superimposed onto the reference section micrograph. A synapse was counted whenever its postsynaptic density (the counting unit) was seen in the reference section, but not in the look-up section, entirely or partly within the counting frame without intersecting the forbidden lines and their extensions. Synapses were identified by the presence of synaptic densities, at least three synaptic vesicles at the presynaptic site and a synaptic cleft (Gray and Guillery, 1966; Colonnier, 1968). Because the number of symmetrical synapses received by the dendritic trees of VMNvl neurons is very low (Nishizuka and Pfaff, 1989), for the purpose of the estimations herein performed no distinction was made between symmetrical and asymmetrical synapses. However, the estimates were performed independently for axospinous and axodendritic synapses (Figs. 2, 3). Dendritic shafts were identified by the presence of characteristic organelles, such as mitochondria and microtubules (Fig. 2), and dendritic spines by the absence of these organelles and/or by the presence of spine cysternal structures (Fig. 3). When two or more postsynaptic densities were visible on the same spine they were considered a single synaptic junction. The mean thickness of the ultrathin sections, estimated using the minimal fold technique (Small, 1968), was 70 nm. On average, 100 axospinous and 130 axodendritic synapses were counted per animal; the coefficient of error of the estimates was 0.050 and 0.045, respectively.

The numerical density (N_v) of axosomatic synapses (Figs. 2, 4) was estimated as the number of synapses per unit volume of neuronal perikaryon. For this purpose, four perikaryal profiles and the surrounding neuropil, where the terminals establishing synapses with the cell bodies are located, were photographed per block. Thus, a total of 16 neuronal cell bodies, photographed from four alternate ultrathin sections, that is, 140 nm apart, were analyzed per animal. The photographs of each profile, taken at primary magnification of 5,400× and enlarged to a final magnification of 16,200×, were used to estimate the N_v of the somatic synapses by applying the physical disector method (Sterio, 1984; Madeira and Paula-Barbosa, 1993). The reference volume, i.e., the volume of the disector, was the area of the neuronal perikarya multiplied by the height of the disector; that is, the distance between the upper surface of the reference and look-up sections. The area of the neuronal perikarya was estimated by point-counting techniques by using an appropriate system of test points (Gundersen and Jensen, 1987). Although both symmetrical (Fig. 4) and asymmetrical (Fig. 2) synapses occur on the soma of VMNvl neurons (Milhouse, 1978; Nishizuka and Pfaff, 1989) the former predominate, and thus in this study we did not subdivide axosomatic synapses according to the morphology of the synaptic junction and synaptic vesicles.

Neuronal density and neuronal volume. The numerical density (N_v) of the neurons located in the VMNvl was estimated from series of semithin sections, obtained as described above, by applying the physical disector method (Sterio, 1984; Madeira and Paula-Barbosa, 1993). Sets of four alternate semithin sections were selected per block, which provided a total of 16 semithin sections per animal. Because each section was used in turn as the reference section, 24 disectors were performed on average per animal. The sections were analyzed using a modified Olympus BH-2 microscope interfaced with a color video camera and equipped with a Heidenhain ND 281 microcator (Traunreut, Germany), a computerized stage, and an object rotator (Olympus, Albertslund, Denmark). A computer fitted with a framegrabber (Screen Machine II, FAST Multimedia, Germany) was connected to the monitor. By using the C.A.S.T. – Grid system software (Olympus), two counting frames equivalent in shape and with an area of 1580 μm^2 each were superimposed onto the tissue images on the screen. One of the images was frozen on the left half of the screen (the look-up section). In the right half of the screen, the software displayed a live video image of the same area of the VMNvl, but obtained from the next sampled section (the reference section). Neurons were counted at a final magnification of 800×, when their nuclei (the counting unit) were visible in the reference section (the live image), but not in the look-up section (the frozen image), within the counting frame without being intersected by the exclusion edges or their extensions. Cells that were obviously microglia or oligodendrocytes (Ling et al., 1973) were not included in the estimations. On average, 150 neurons were counted per animal; the coefficient of error of the estimates was 0.055.

Estimates of perikaryon volumes were obtained with the nucleator method implemented in isotropic, uniform random sections (Gundersen, 1988). Neurons were selected for measurements with physical disectors using the nucleolus as the sampling unit. Then the distance from the nucleolus to the cell boundary was measured in four different directions. The average number of neurons measured per animal was 70.

Number of synapses per neuron. The number of axospinous and axodendritic synapses per neuron was estimated by dividing the numerical density of each type of synaptic contact by the numerical density of VMNvl neurons. The number of axosomatic synapses per neuron was estimated by multiplying the numerical density of these synapses by the volume of the neuronal cell bodies.

Surface area of synapses. The surface area (S_A) of the postsynaptic densities of individual axodendritic and axospinous synapses was determined by dividing the surface density (S_v) of the postsynaptic densities by the numerical density (N_v) of the respective synapses. The S_v was estimated, in all photographs used for the estimation of the numerical density of the synapses, by counting the total number of intersections of the cycloid arcs of a "staggered" cycloid test system (Baddeley et al., 1986) with the postsynaptic densities. The total surface area per neuron of the postsynaptic densities was estimated by dividing the respective S_v by the neuronal numerical density.

The S_A of the plasmalemma of neuronal perikarya (see below) and of the postsynaptic densities of axosomatic synapses was estimated using the same method. The S_A of

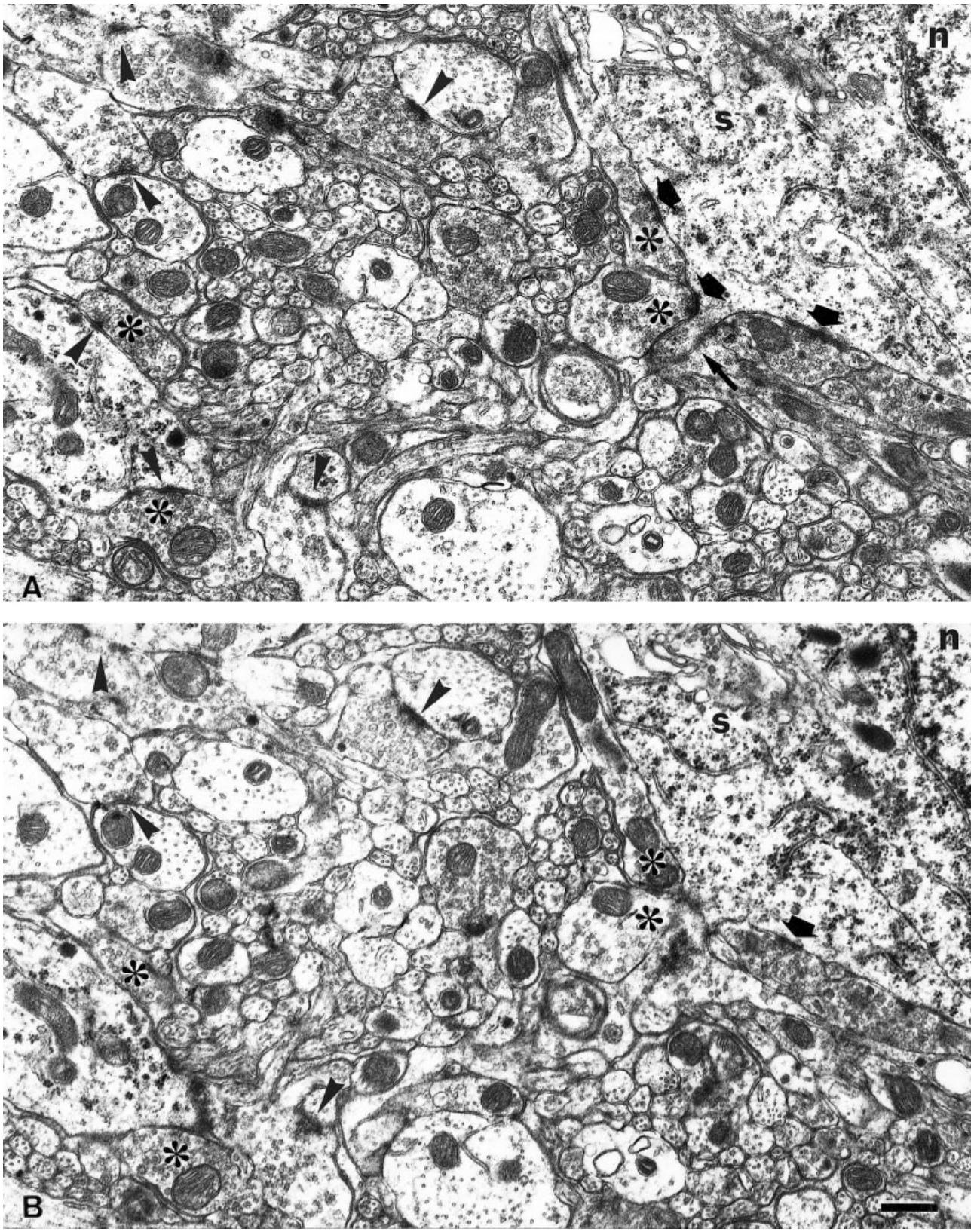

Fig. 2. Electron micrographs of two consecutive serial sections of the VMN, in which part of a neuronal cell body (s) with its nucleus (n) and the surrounding neuropil can be seen. The cell body is connected at asymmetrical synaptic contacts (arrows) by axon terminals. The terminals establishing synapses in **A**, but not in **B**, are indicated in both photographs by an asterisk. A spine or excrescence (long arrow) can be seen in continuity with the cell body. Most of the terminals contain clear round vesicles and make asymmetrical synapses (arrowheads) with dendrites. The terminals contacting dendrites at synaptic sites in A (the reference section), but not in B (look-up section), are indicated in both photographs by an asterisk. The synapses visible in A, but not in B, were those counted when performing the disector. Scale bar = 0.5 μm.

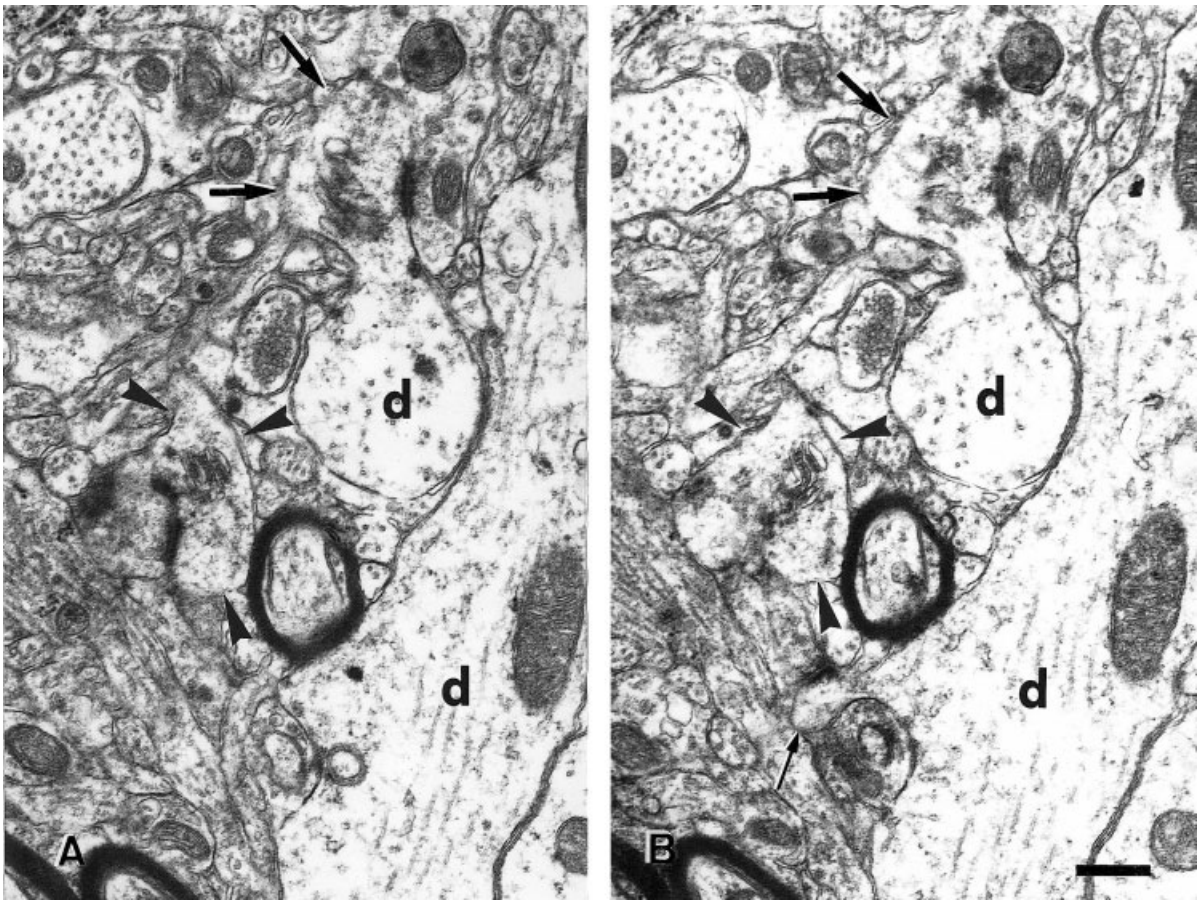

Fig. 3. Electron micrographs obtained from two consecutive serial sections of the VMN showing identical areas of the neuropil of its ventrolateral division. A dendritic shaft (d) bears a spine (thin arrow) that is particularly evident in B and makes an asymmetrical synaptic contact with an axon terminal. Two other dendritic spines can be seen. One (thick arrows) protrudes from a dendritic shaft (d) and is contacted at an asymmetrical synaptic thickening, which can be seen in **A** and in **B**, by an axon terminal. The remaining spine (arrowheads) receives an asymmetrical synapse that is visible only in A. Scale bar = 0.5 μm.

individual postsynaptic densities was determined by dividing the S_A of all synapses by the number of somatic synapses per neuron.

Percentage of plasmalemma occupied by postsynaptic densities. The perimeter of the profiles of the dendritic spines and the length of the postsynaptic densities were measured in every available photomicrograph using an MOP-Videoplan. The percentage of plasmalemma of dendritic spines occupied by postsynaptic densities was calculated by dividing the perimeter of the spine by the length of the postsynaptic densities. The percentage of the plasmalemma of the neuronal cell bodies occupied by postsynaptic densities was obtained by dividing the S_A of all somatic synapses received by each neuron by the S_A of the neuronal membrane.

Statistical analyses

Differences among groups were assessed by one-way analysis of variance (ANOVA). Whenever significant results were found from the overall ANOVA, pair-wise comparisons were subsequently made with the post-hoc Tukey HSD test. Throughout the text, data are presented as means with their coefficients of variation (CV = SD/mean), and hormonal concentrations are shown as means and SEM. Differences were considered to be significant if $P < 0.05$. The coefficient of error of the estimates of numerical densities was calculated on the basis of the mean number of synapses counted per counting field, the variance in the number of synapses among the counting fields, and the number of counting fields, as described by Geinisman et al. (1996) and Schmitz (1997).

RESULTS

Hormone levels

In keeping with earlier data (Butcher et al., 1974), plasma estradiol levels showed the expected high values by 1600 hours on proestrus and had returned to low levels by the same time on diestrus day 1. The values were 176 (6) pg/ml and 100 (4) pg/ml, respectively.

Neuronal densities and volumes

According to our estimates, the numerical density of VMNvl neurons, expressed as $n \times 10^{-4}/\mu m^3$ (CV), was 3,550 (0.09) for proestrus rats, 4,390 (0.10) for diestrus

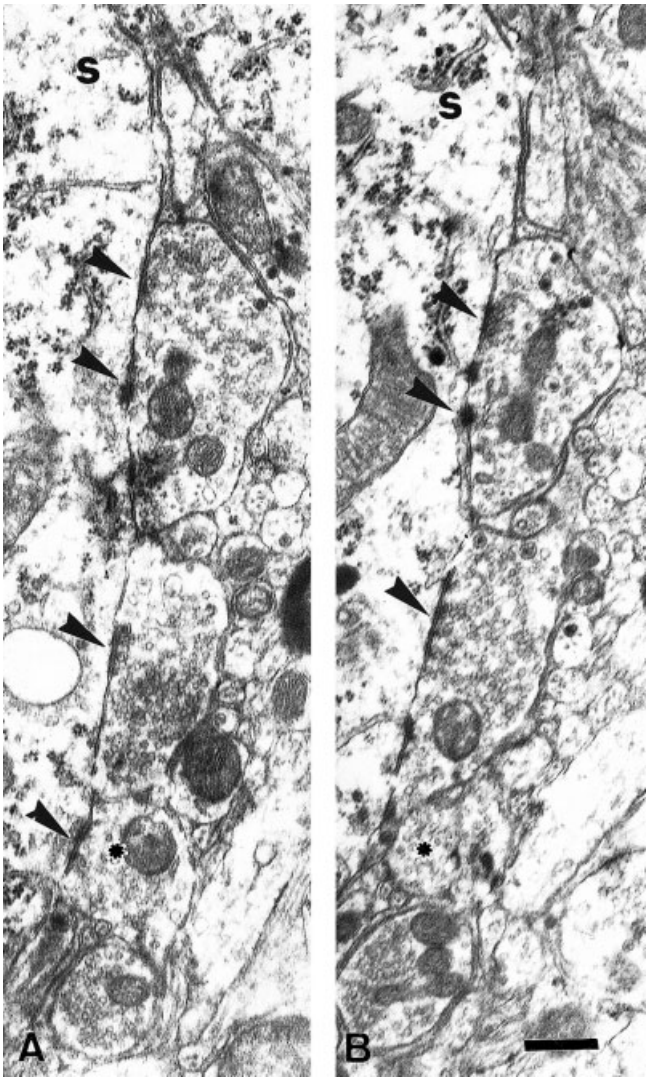

Fig. 4. Electron micrographs obtained from two consecutive serial sections of the VMN showing symmetrical synaptic contacts (arrowheads) between axon terminals and the soma (s) of a neuron of the ventrolateral division of the VMN. The terminal which makes a synaptic contact in **A** but not in **B** is indicated by an asterisk. Scale bar = 0.5 μm.

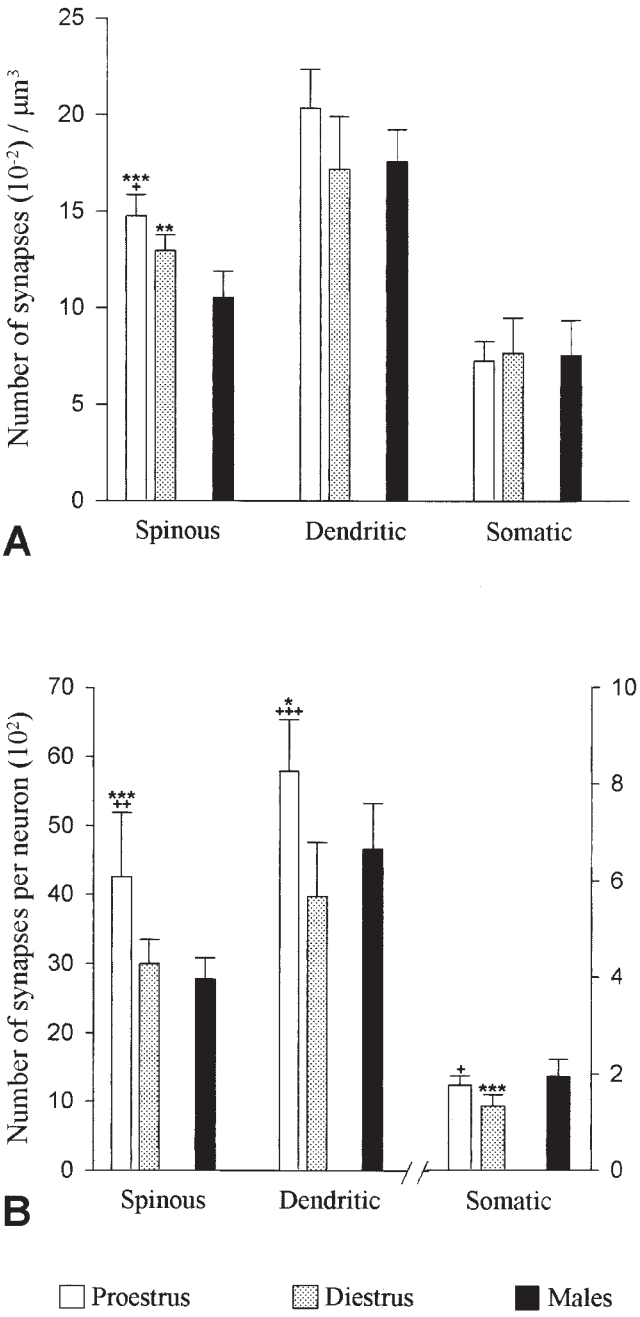

Fig. 5. Graphic representation of the morphometric data obtained from axospinous (spinous), axodendritic (dendritic), and axosomatic (somatic) synapses in the ventrolateral division of the VMN of male rats, and female rats in diestrus and proestrus. Columns represent means and vertical bars represent 1 SD. **A:** Numerical density of the synapses. **B:** Number of synapses per neuron. Tukey's post-hoc tests: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, compared with male rats; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.005$, compared with diestrus rats.

rats, and 3,800 (0.9) for male rats, and the mean somatic volume was 2,430 μm^3 (0.09), 1,755 μm^3 (0.09), and 2,432 μm^3 (0.07), respectively. No significant effects of sex and hormonal levels on the numerical density of VMNvl neurons were found ($F_{(2,15)} = 3.13$, $P = 0.073$). Conversely, the volume of the neuronal cell bodies differed among the groups analyzed ($F_{(2,15)} = 27.16$, $P < 0.0005$), with proestrus rats and males having larger neuronal cell bodies (38%, $P < 0.0005$) than diestrus rats.

Synapse densities

As shown in Figure 5A, the numerical density of axospinous synapses differs between the sexes, and between females at different stages of the estrus cycle ($F_{(2,15)} = 21.55$, $P < 0.0005$). The numerical density was significantly higher in proestrus than in diestrus rats, and in females than in males. Likewise, the numerical density of axodendritic synapses was influenced by sex and phase of the estrus cycle ($F_{(2,15)} = 3.70$, $P < 0.05$). However, when pairwise comparisons were performed, no significant differences between proestrus and diestrus rats or between

males and females were found. In addition, no differences between the sexes and no effect of the phase of the estrus cycle were noticed in the numerical density of axosomatic synapses ($F_{(2,15)} = 0.10$, $P = 0.905$).

Total number of synapses per neuron

According to our estimates, in a diestrus rat the number of synapses per each VMNvl neuron is ~7,000, of which 56% are located on dendritic trunks, 42% on dendritic spines, and 2% on the soma. In proestrus rats, the number of synapses per neuron rises to ~10,000, 56% of which are concentrated on dendritic trunks, 42% on dendritic spines, and 2% on neuronal cell bodies. In males, the number of synaptic contacts per neuron is similar to that observed in diestrus rats (~7,500), of which 61% are established on dendritic trunks, 36% on spines, and 3% on neuronal cell bodies.

As shown in Figure 5B, the total number of synaptic contacts per neuron located in the VMNvl was influenced by the sex of the animals and by the phase of the estrus cycle. These effects were significant for axospinous ($F_{(2,15)} = 10.73$, $P < 0.005$), axodendritic ($F_{(2,15)} = 9.36$, $P < 0.005$), and axosomatic ($F_{(2,15)} = 8.33$, $P < 0.005$) synapses. Proestrus rats had more axospinous and axodendritic synapses per neuron than diestrus rats (42% and 45%, respectively) and males (54% and 24%, respectively). There were no statistically significant differences in the total number of these synaptic contacts between diestrus rats and males.

Similar to axospinous and axodendritic synapses, the number of synapses received by each neuronal cell body was 32% higher in proestrus than in diestrus rats (Fig. 5B). In addition, and in contrast with the synapses established on the dendritic trees, the number of axosomatic synapses established per neuron was higher (46%) in males than in diestrus rats and did not differ between males and proestrus rats (Fig. 5B).

Size of postsynaptic densities

No variation across the estrus cycle and no sex-related differences were noticed in the area of the postsynaptic densities of individual axodendritic ($F_{(2,15)} = 1.35$, $P = 0.288$) synapses (Fig. 6A). Conversely, ANOVA showed that the sex of the animals and the phase of the estrus cycle significantly influenced the surface area of the individual postsynaptic densities of axospinous ($F_{(2,15)} = 9.62$, $P < 0.005$) and axosomatic ($F_{(2,15)} = 6.11$, $P = 0.011$) synapses. Specifically, the surface area of the postsynaptic densities of these synapses was 30% smaller in females than in males, irrespective of the phase of the estrus cycle (Fig. 6A).

However, as shown in Figure 6B, the surface area of all postsynaptic densities identifiable on each VMNvl neuron, which depends on the size of individual postsynaptic densities and on the total number of synapses established per neuron, was influenced by the sex of the animals and by the phase of the estrus cycle in axospinous ($F_{(2,15)} = 5.51$, $P = 0.016$), axodendritic ($F_{(2,15)} = 5.10$, $P = 0.020$), and axosomatic synapses ($F_{(2,15)} = 30.54$, $P < 0.005$). The total surface area of postsynaptic densities was significantly larger in proestrus than in diestrus rats in all types of synapses (Fig. 6B). In addition, the total surface area of the dendritic membrane occupied by postsynaptic densities was larger in males than in females, but only when females were in diestrus. In the case of axosomatic synapses, sex differences in the size of the postsynaptic densities were present when females were in diestrus as well as in proestrus.

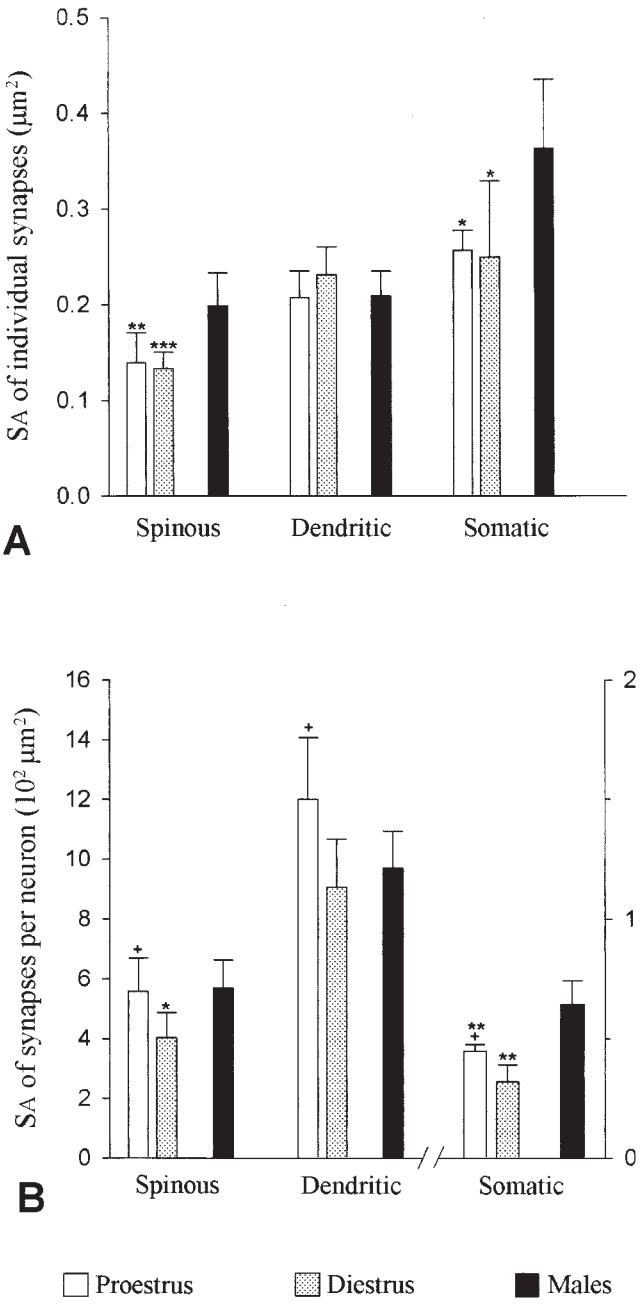

Fig. 6. Graphic representation of the morphometric data obtained from axospinous (spinous), axodendritic (dendritic) and axosomatic (somatic) synapses in the ventrolateral division of the VMN of male rats, and female rats in diestrus and proestrus. Columns represent means and vertical bars represent 1 SD. **A:** Surface area of individual postsynaptic densities. **B:** Surface area of the postsynaptic densities per neuron. Tukey's post-hoc tests: $^{*}P < 0.05$, $^{**}P < 0.005$, compared with male rats; $^{+}P < 0.05$, compared with diestrus rats.

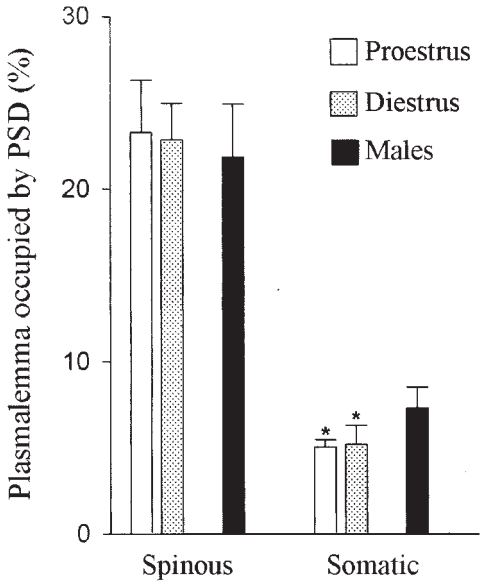

Fig. 7. Graphic representation of the estimates of the percentage of plasmalemma occupied by postsynaptic densities (PSD) of axospinous (spinous) and axosomatic (somatic) contacts in the ventrolateral division of the VMN of male rats, and female rats in diestrus and proestrus. Columns represent means and vertical bars represent 1 SD. Tukey's post-hoc tests: $^*P < 0.005$, compared with male rats.

Percentage of plasmalemma occupied by PSD

As shown in Figure 7, no male–female differences and no influence of the phase of the estrus cycle was observed in the percentage of plasmalemma of dendritic spines occupied by postsynaptic densities ($F_{(2,15)} = 0.43$, $P = 0.658$), as opposed to what occurred with the percentage of the plasmalemma of neuronal cell bodies occupied by postsynaptic densities ($F_{(2,15)} = 10.33$, $P < 0.005$), which was greater in males than in females.

DISCUSSION

Methodological considerations

In the present study we used the number of synapses per neuron and the size of individual synapses as the main estimators of the estrogen effects on synaptic plasticity in the VMNvl and of the sex-related differences in the synaptic organization of this nucleus. For the purpose of establishing comparisons between males and females and between proestrus and diestrus rats, the choice of these estimators was suitable because the total number of VMNvl neurons is similar in males and in females and, as expected, does not vary across the estrus cycle (Madeira et al., 2001). Therefore, any variation over the estrus cycle or any sex-related difference in the number of synaptic contacts per neuron is a sensitive measure of the changes in the number of synapses within the VMNvl. In addition, the use of an estimator such as the number of synapses per neuron was convenient because it enabled us to overcome the bias introduced by differential shrinkage due to tissue processing (for a review, see Oorschot, 1994). In fact, all estimations were performed using serial semithin and ultrathin sections, and thus histological sections embedded in the same medium. That would not be possible if we had chosen to estimate the total number of synapses in the VMNvl because that would require thick sections, and thus sections embedded in a different medium, to determine the volume of the VMNvl.

The use of the physical disector, as opposed to techniques based on single sections, to estimate numerical densities was also appropriate because this method is independent of the size of the particles under study (Sterio, 1984). Particle size can introduce a bias in estimates based on single sections because objects have more chances of being counted when they are relatively large than when they are small in size. This is a critical issue in the case of the VMNvl because the neuronal volume and the area of the postsynaptic densities were not constant in the groups analyzed. However, and contrary to what might be considered appropriate, we did not apply the double disector technique (Braendgaard and Gundersen, 1986; Gundersen, 1986), which allows the direct estimation of the ratio of synapses to postsynaptic neurons, because it implies extensive ultramicrotomy to obtain an adequate sample of neurons. Since the density of VMNvl neurons is low, as they occupy only about 20% of the volume of the VMNvl (Madeira et al., 2001), we chose not to apply this method. Instead, we estimated the numerical densities of the neurons and of the synapses in parallel on the assumption that, although thickness might potentially bias synaptic measures, it is unlikely that it might bias the experimental comparisons given that care was taken to ensure the equivalency of section thickness between the groups.

Estrus phase influences on the synaptic organization of the VMNvl

We have shown that, in female rats, the physiological fluctuations in hormonal levels are associated with parallel variations in the number of dendritic synapses. In effect, from diestrus to proestrus, the number of axospinous synapses increased by 42% and the number of axodendritic synapses by 45%. These results were not totally unexpected because in a previous study we have found that the density of dendritic spines and the length of dendritic trees of VMNvl neurons augment by 30% and 20%, respectively, from diestrus to proestrus (Madeira et al., 2001), and similar data has been also reported by other authors (Frankfurt et al., 1990). Taken together, our observations indicate that VMNvl neurons have the capability of cyclically changing their size and connectivity in such a way that the greater availability of postsynaptic membrane corresponds to the presence of more synaptic contacts, whereas the opposite occurs when there is synapse elimination.

It might be expected that estrogen would influence preferentially the synapses established upon dendritic spines, as these structures are exceedingly plastic and can be generated and eliminated in short periods (Hering and Sheng, 2001; Segal, 2001). However, such was not the case and, actually, we have found a parallel variation in the number of axospinous and axodendritic synapses, which represent about 40% and 60%, respectively, of the synapses received by the dendritic trees of VMNvl neurons during proestrus as well as during diestrus. Therefore, it appears that in parallel with the fluctuation in hormonal levels there is a fluctuation in the number of axodendritic

and axospinous synapses rather than a shift in synapse location between dendritic shafts and dendritic spines. In addition, and in line with observations carried out in other regions of the brain, such as the hippocampal formation (Woolley and McEwen, 1992) and the cerebral cortex (Trachtenberg et al., 2002), our results show that in the VMNvl of the adult rat changes in dendritic spine density correlate positively with changes in the number of axospinous synapses.

Data obtained in this study also show that estrogen does not interfere with the size of individual synaptic contacts. In fact, the surface area of the individual postsynaptic densities of axodendritic and axospinous synapses did not differ between proestrus and diestrus rats, a finding that is in agreement with the lack of variations in the length of the synaptic contacts in the VMNvl after the administration of estrogen to ovariectomized rats (Carrer and Aoki, 1982). However, because the number of axodendritic and axospinous synapses is higher in proestrus than in diestrus rats, the total area of dendritic membrane occupied by synaptic contacts is significantly increased when estrogen levels are high. The finding that the percentage of plasmalemma of dendritic spines occupied by postsynaptic densities does not vary across the estrus cycle indicates that spines that are newly formed in each proestrus, as hormone levels rise, do not differ with respect to their size from the spines that persist during diestrus, when hormone levels decline. There is increasing evidence that spines with large heads are stable and contribute to strong synaptic connections, contrary to spines with small heads that are motile and unstable, and contribute to weak or silent synaptic connections (Kasai et al., 2003). Therefore, our data indicate that the synapses that are added to, and removed from, VMNvl neurons during each estrus cycle do not differ with respect to their stability and function from the pool of synapses received by these neurons at low estrogen levels.

We have also found that sex steroids influence not only the dendritic synapses, but also the smaller population of synapses established upon the cell bodies of VMNvl neurons. In effect, although somatic synapses did not change in size over the estrus cycle, they were more numerous at high estrogen levels, that is, during proestrus. This observation lends support to the view that the plastic changes displayed by hypothalamic synapses over the estrus cycle are region-specific. Actually, in the nearby located arcuate nucleus, the number of axosomatic synapses does not increase in response to estrogen, as happens in the VMNvl, but declines when estrogen levels are low, i.e., during estrus (Olmos et al., 1989), as opposed to what occurs in the anteroventral periventricular nucleus, where low estrogen levels lead to increases in the number of axosomatic synapses (Langub et al., 1994). It is known that somatic synapses are mostly symmetrical and that most of these synapses are inhibitory (Uchizono, 1965; Colonnier, 1968), contrary to asymmetrical synapses, which are presumed excitatory (Westrum and Blackstad, 1962; Harris and Stevens, 1989). Our data indicate that, in each proestrus, individual VMNvl neurons receive more than ~3,000 excitatory synapses and more than 40 inhibitory synapses than in the preceding or following diestrus, which suggests that estrogen enhances the activity of VMNvl neurons by promoting a proportionally greater increase in the excitatory drive than in the inhibitory inputs they receive.

Sex differences in the synaptic organization of the VMN

The VMN has been classically regarded as a sexually dimorphic nucleus, a concept corroborated by data obtained in this study. Actually, we have found that females in proestrus have more synapses than males, a sex-related difference no longer apparent when females are in diestrus. The existence of more dendritic synapses in proestrus rats than in males was accounted for by sex differences in the number of axospinous (54%) and axodendritic (24%) synapses that favor females. Interestingly, opposite sex differences were noticed in the number of somatic synapses, which were 46% more numerous in males than in diestrus rats. These data show that, by comparison with males, VMNvl neurons from proestrus females receive more excitatory inputs and the same amount of inhibitory inputs, whereas neurons from diestrus females receive a similar amount of excitatory, but a smaller amount of inhibitory inputs. This sexual dimorphic pattern appears to be specific to the VMNvl, as it does not mirror the sex differences in the synaptic organization of other steroid-sensitive regions of the brain. For example, in the arcuate nucleus, which, similar to the VMNvl, displays sex differences in its anatomy (Leal et al., 1998) and exhibits phasic synaptic changes across the estrus cycle (Olmos et al., 1989), the density of axodendritic synapses does not differ between the sexes contrary to the density of axospinous and axosomatic synapses, which is greater in females (Matsumoto and Arai, 1980; Pérez et al., 1990).

Interestingly, the sole parameter that consistently showed sex-related differences was the surface area of the postsynaptic densities of individual axospinous and axosomatic synapses, which was ~30% smaller in females than in males. It seems clear that the size of postsynaptic densities is determined by the organizational effects of sex steroids during the perinatal period and is not sensitive to the activational effects of these hormones during adulthood, as no correlation with hormonal levels was noticed in females. Because the postsynaptic densities of axospinous synapses are larger in males than in females and the percentage of plasmalemma occupied by postsynaptic densities does not differ between the sexes, there are reasons to assume that the volume of dendritic spines is greater in males than in females. This was not an unexpected finding, because earlier studies have shown that the size of the postsynaptic densities positively correlates with the size of spines (Harris and Stevens, 1989; Sorra and Harris, 2000; Kasai et al., 2003). Given that spines with larger heads and postsynaptic densities have greater sensitivity to glutamate (Harris and Stevens, 1989; Schikorski and Stevens, 1997; Kasai et al., 2003), there are reasons to assume that, in addition to differences in the number of synapses, males and females also differ with respect to the efficacy of synaptic transmission in the VMNvl.

Previous studies have shown that in the VMNvl there are sex-related differences that favor males in the density of axodendritic and axosomatic synapses (Matsumoto and Arai, 1986b; Miller and Aoki, 1991). Discrepancies between these and our own data are difficult to understand because in all cases, including the present study, analyses were centered on the VMNvl. However, in those studies the phase of the estrus cycle was not identified and, as already mentioned, the estimates report synapse densities

(Matsumoto and Arai, 1986b; Miller and Aoki, 1991), both factors that can interfere with the end results and, thus, with the conclusions regarding the existence of sex-related differences in the number of synapses. In fact, the volume of the VMNvl and of its neuropil is not constant at all phases of the estrus cycle (Madeira et al., 2001), and areal as well as numerical densities are influenced by variations in the reference volume (for a review, see Oorschot, 1994). Moreover, estimations of particle numbers from two-dimensional probes, that is, from single sections, are likely to be biased by variations in the size of the particle under study, and in the present case the postsynaptic densities of axospinous synapses are larger in males than in females. Finally, but not least important, is the fact that small or tangentially cut synapses cannot be reliably recognized in single sections (Curcio and Hinds, 1983; deToledo-Morrell et al., 1988) and, consequently, synaptic numerical densities estimated from single sections are systematically underestimated by as much as 20% (Curcio and Hinds, 1983). Since the size of the postsynaptic densities is smaller in females than in males, it is possible that the underestimation might be greater in females than in males, thus complicating the global detection of sex-related differences in the number of synapses.

The presence of sex differences that would favor females was somehow expected on the grounds of the existence of a higher spine density in females than in males (Madeira et al., 2001). In fact, it is generally held that spines develop as a response to a signal of the presynaptic component, thus indicating that the sprouting of a spine is associated with synapse formation and spine retraction with synapse elimination. Data obtained in other hypothalamic nuclei lend support to this statement. Specifically, in the medial preoptic area, spine density is greater in females than in males (Madeira et al., 1999) and sex differences in the density of spine synapses favor females (Raisman and Field, 1973), similar to what has been observed in the arcuate nucleus where male–female differences in the density of spine synapses (Matsumoto and Arai, 1980) reflect similar differences in the density of dendritic spines (Leal et al., 1998).

CONCLUSIONS

By using robust stereological approaches that generate numbers of synapses per neuron, we have demonstrated that the synaptic contacts established between VMNvl neurons and their afferents are not stable in number over the estrus cycle. These cyclical variations are due to the formation and withdrawal of axodendritic, axospinous, and axosomatic synapses, which are particularly numerous at high estradiol levels. Consequently, proestrus rats have approximately twice the number of dendritic synapses as male rats, whereas diestrus rats have the same number. Conversely, males have approximately twice as much axosomatic synapses as diestrus rats and the same number as proestrus rats. Finally, we have shown that the postsynaptic densities do not vary in size over the estrus cycle and that they are globally larger in males than in females.

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III.

Effects of estrogens and progesterone on the synaptic organization of the hypothalamic ventromedial nucleus

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EFFECTS OF ESTROGENS AND PROGESTERONE ON THE SYNAPTIC ORGANIZATION OF THE HYPOTHALAMIC VENTROMEDIAL NUCLEUS

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Abstract—The majority of the studies on the actions of estrogens in the ventrolateral part of the hypothalamic ventromedial nucleus (VMNvl) concern the factors that modulate the receptive component of the feminine sexual behavior and the expression of molecular markers of neuronal activation. To further our understanding of the factors that regulate synaptic plasticity in the female VMNvl, we have examined the effects of estradiol and progesterone, and of estrogen receptor (ER) subtype selective ligands on the number of dendritic and spine synapses established by individual VMNvl neurons and on sexual behavior. In contrast to earlier studies that analyzed synapse densities, our results show that exogenous estradiol increases the number of spine as well as of dendritic synapses, irrespective of the dose and regimen of administration. They also reveal that an effective dose of estradiol administered as one single pulse induces the formation of more synapses than the same dose administered as two pulses on consecutive days. Our results further show that both ER subtypes are involved in the mediation of the synaptogenic effects of estrogens on VMNvl neurons since the administration of the selective ERα, propyl-pyrazole-triol (PPT), and ERβ, diarylpropionitrile (DPN), agonists induced a significant increase in the number of synapses that, however, was more exuberant for PPT. Despite its relevant role in feminine sexual behavior, progesterone had no synaptogenic effect in the VMNvl as no changes in synapse numbers were noticed in rats treated with progesterone alone, with estradiol followed by progesterone or with the antiprogestin mifepristone (RU486). Except for the sequential administration of estradiol and progesterone, none of the regimens was associated with lordosis response to vaginocervical stimulation. Therefore, from the sex steroids that undergo cyclic variations over the estrous cycle, only estrogens, acting through both ERα and ERβ, play a key role in the activation of the neural circuits involving the ventromedial nucleus of the hypothalamus.

Key words: ventromedial nucleus, synapses, estrogen receptor, propyl-pyrazole-triol, diarylpropionitrile, mifepristone.

The ventromedial nucleus of the hypothalamus (VMN) has been implicated in a wide variety of functions, such as feeding and defensive behaviors, regulation of the autonomic responses and hormonal production by the anterior pituitary, antinociceptive mechanisms and somatomotor control (for a review see, Canteras et al., 1994). However, the VMN is particularly known for the important role it plays in the control of the feminine sexual behavior, namely the lordosis reflex (Pfaff and Sakuma, 1979a,b). In normally cycling rats, this behavior occurs typically at proestrus when females become sexually receptive as a response to the sequential secretion of estrogens and progesterone by the ovaries. Estrogens greatly increase the expression of progestin receptors (PR) in the VMN (MacLusky and McEwen, 1980; Parsons et al., 1982; Brown et al., 1987; Shughrue et al., 1997a), as they do in other regions of the brain (Gréco et al., 2001), and progesterone, by acting on estrogen-primed VMN neurons, facilitates the lordosis behavior (Rubin and Barfield, 1983a,b; Mani et al., 1994). Notably, estrogens appear to be essential for this response, as opposed to progesterone whose role can be fulfilled by other hormones, neurotransmitters and signaling molecules (Kow and Pfaff, 1998; Auger, 2001, 2004; Blaustein, 2003; Wu et al., 2006).

Cells in the ventrolateral division of the ventromedial nucleus of the hypothalamus (VMNvl) are essential for the development of the lordosis reflex. Neurons in this division express nuclear and extranuclear estrogen receptors (ERs; Pfaff and Keiner, 1973; Simerly et al., 1990; Milner et al., 2008) and PRs (MacLusky and McEwen, 1980; Parsons et al., 1982) and exhibit cyclic changes in their morphology over the estrous cycle in response to the natural fluctuations in the circulating levels of sex steroids (Madeira et al., 2001). Studies carried out in rats at different phases of the estrous cycle have shown that during proestrus VMNvl neurons have larger cell bodies, have longer dendritic trees with more dendritic spines, and establish more synapses than neurons from rats in diestrus (Frankfurt et al., 1990; Madeira et al., 2001; Sá and Madeira, 2005a,b). Because changes of the same type have been noticed in response to the administration of estradiol to ovariectomized rats (Carrer and Aoki, 1982; Jones et al., 1985; Frankfurt et al., 1990; Frankfurt and McEwen, 1991a,b; Calizo and Flanagan-Cato, 2000), the cyclic variations that VMNvl neurons undergo over the estrous cycle have been tentatively ascribed to the trophic actions of estrogens. However, it is not known if, and how, progesterone contributes to these alterations, namely at proestrus when the endogenous levels of estradiol and progesterone are both elevated (Butcher et al., 1974). In this study, we address this issue by analyzing the number of synapses established by each VMNvl neuron in rats treated either with

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Abbreviations: ANOVA, analysis of variance; DPN, diarylpropionitrile; EB, estradiol benzoate; ER, estrogen receptor; ERα, estrogen receptor-α; ERβ, estrogen receptor-β; N_V, numerical density; O, oil; P, progesterone; PPT, propyl-pyrazole-triol; PR, progestin receptors; RU486, mifepristone; VCS, vaginocervical stimulation; VMN, ventromedial nucleus of the hypothalamus; VMNvl, ventrolateral part of the ventromedial nucleus of the hypothalamus.

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progesterone or with estradiol followed by progesterone in a dose that is effective in inducing the lordosis reflex. To get a deeper insight into the possible influence of progesterone in the synaptic connectivity of VMNvl neurons, we extended our observations to rats treated with mifepristone, also known as RU486, a selective antagonist at PRs (Brown and Blaustein, 1984; Etgen and Barfield, 1986; Mani et al., 1994).

It is widely recognized that estrogens stimulate female sexual receptivity by priming VMNvl neurons to the action of progesterone through binding to specific receptors. In the VMNvl, estrogens may act through two types of receptors, the estrogen receptor-α (ERα) and the estrogen receptor-β (ERβ), as it contains neurons that express the ERα or the ERβ alone, and neurons that express both receptors (Shughrue et al., 1997b; Shughrue and Merchenthaler, 2001; Ikeda et al., 2003). However, whereas the ERα is expressed in cells throughout the VMNvl, neurons containing the ERβ are scattered in the rostral and middle parts of the nucleus and concentrate in its caudal part (Shughrue and Merchenthaler, 2001; Ikeda et al., 2003). Earlier studies using either knockout mouse models (Ogawa et al., 1998; Kudwa and Rissman, 2003) or ER agonists (Mazzucco et al., 2008) and antagonists (Walf et al., 2008) have shown that the ERα is necessary for female reproduction, namely for the induction of the estrogen-dependent lordosis reflex, a function that is not shared by the ERβ. However, the role played by these receptors in mediating the effects of estrogens in the morphological changes displayed by VMNvl neurons is unknown. Herein, we address this issue by investigating the effects of the ERα and ERβ specific ligands, propyl-pyrazole-triol (PPT) and diarylpropionitrile (DPN), respectively, upon the number of synapses established by individual VMNvl neurons. With the purpose of evaluating the functional repercussions of the regimens used, we have also examined the lordosis response to vaginocervical stimulation (VCS).

EXPERIMENTAL PROCEDURES

Animals

Female Wistar rats (n=48) were maintained on a 12-h light/dark cycle (lights on at 7:00 AM) and ambient temperature of 23 °C, with food and water continuously available. Throughout the experiments, the estrous cycles of females were monitored by daily vaginal lavage (Becker et al., 2005). Only females that exhibited two complete estrous cycles (4–5 days) were used. At 2.5 months of age, rats were ovariectomized bilaterally under deep anesthesia induced by xylazine (20 mg/ml) and ketamine (100 mg/ml) injected i.m. in a concentration of 0.132 ml/kg and 0.5 ml/kg b.w., respectively. Subsequent experimental manipulations were initiated 12 days after surgery. Studies were performed in accordance with the European Communities Council Directives of 24 November 1986 (86/609/EEC) and Portuguese Act no. 129/92. All efforts were made to minimize the number of animals used and their suffering.

Treatments

Estradiol benzoate (EB) and progesterone were purchased from Sigma-Aldrich Company Ltd. (Madrid, Spain), and PPT, DPN and RU486 from Tocris BioScience (Bristol, UK). After being dissolved in 0.1 ml of sesame oil (Sigma-Aldrich Company Ltd.), they were all injected s.c. Starting 12 days after ovariectomy, rats were separated in eight groups of six animals each and allotted to one of the following treatments: (1) 0.1 ml oil (O group); (2) 500 μg progesterone (P group); (3) two pulses of 10 μg EB 24 h apart (EB group; Frankfurt et al., 1990; Calizo and Flanagan-Cato, 2000, 2002); (4) one pulse of 20 μg EB (2 EB group; Carrer and Aoki, 1982; Brown et al., 1987); (5) two pulses of 10 μg EB 24 h apart followed, 48 h later, by 500 μg progesterone (EB+P group); (6) two pulses of 10 μg EB 24 h apart followed, 48 h later, by 5 mg RU486 (EB+RU group); (7) two pulses of 500 μg PPT 24 h apart (PPT group; Harris et al., 2002; Frasor et al., 2003; Mazzucco et al., 2008); and (8) two pulses of 500 μg DPN 24 h apart (DPN group; Harris et al., 2002; Frasor et al., 2003; Mazzucco et al., 2008).

In Experiment 1, we analyzed the O, P, EB, EB+P and EB+RU groups to evaluate the influence of estradiol and progesterone on synapse numbers, whereas in Experiment 2 we analyzed the O, EB, 2 EB, PPT and DPN groups to examine the influence of different patterns of estradiol administration and ER agonists on synapse numbers.

Behavioral studies

Behavioral testing was performed during the dark phase under dim red light. For P, EB+P and EB+RU groups, tests started 4 h after the last injection, whereas for the remaining groups they started 52 h after the last injection. Rats were submitted to experimenter-induced VCS and the response to this stimulation was measured as lordosis intensity (Lehmann and Erskine, 2004). A tool for manual VCS was manufactured using a plunger from a 1 cm^3 glass syringe (Super Eva Glass, Italy) and a plastic 1 cm^3 syringe barrel (Terumo, Leuven, Belgium), according to the method described by Crowley et al. (1976) and modified by Lehmann and Erskine (2004). After having the flanged proximal end removed with a saw, the plunger was inserted into a 1 cm^3 plastic syringe barrel into which two small metal springs had been placed. The polished distal end of the plunger, which extended 35 mm out of the syringe barrel, was used as a probe for stimulation. The force required for performing the simulation was measured by pressing the syringe plunger against the platform of a precision weighing scale. The device was calibrated to 200 g of force by noting the corresponding ml marking on the syringe barrel. Each VCS session consisted of five vaginal intromissions of 2 s each, 5 min apart. During stimulation, rats were suspended vertically by the operator's hand, with support given underneath the forearms. Each test was videotaped and the response to VCS was subsequently scored. The intensity of each lordosis was rated from 0 to 3, based on the level of spinal dorsiflexion and head extension resulting from contraction of the muscles of the back, as described by Lehmann and Erskine (2004): 0, no vertebral dorsiflexion; 1, slight extension of the head towards the vertical and minor dorsiflexion; 2, moderate dorsiflexion and further head extension; and 3, extreme dorsiflexion and complete extension of the head.

Hormonal determinations

Prior to perfusion, blood samples (2000 μl) were taken directly from the heart into Eppendorf tubes. After complete clot formation, each sample was centrifuged twice at 2000 rpm for 10 min. Serum was then removed, collected in aliquots and stored undiluted at −80 °C until further analysis. Estradiol and progesterone serum levels were assayed using a solid-phase competitive chemiluminescent enzyme immunoassay kit for IMMULITE 1 (Siemens Medical Solutions Diagnostics, Amadora, Portugal), with an analytical sensitivity of 15 pg/ml for estradiol and 0.1 ng/ml for progesterone.

Tissue preparation

Immediately after behavioral testing, rats were anesthetized with 3 ml/kg b.w. of a solution containing sodium pentobarbital (10 mg/

ml) and chloral hydrate (40 mg/ml) given i.p. and killed by intracardiac perfusion of a fixative solution containing 1% paraformaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer, pH 7.2. The brains were removed from the skulls, weighed and immersed in the same fixative solution for 1 h. The uteri were also removed, after being separated from the bladder, and weighed. After postfixation, the brains were bisected sagittally through the midline. The right and left hemispheres were transected in the coronal plane through the posterior border of the optic chiasm, rostrally, and the posterior limit of the mammillary bodies, caudally. The blocks of tissue thus obtained were mounted on a vibratome with the rostral surface up and sectioned at 40 μm until the rostral limit of the VMN was visualized. Then, alternate 40- and 500-μm-thick sections were obtained and collected. The 40-μm-thick sections were mounted on slides, stained with Thionin and used to identify the location and shape of the VMN and of its ventrolateral division. Based on this information, the VMNvl was isolated under microscope observation from the 500-μm-thick sections. The blocks of tissue containing the VMNvl were then processed for electron microscopy, as follows. They were post-fixed with a 2% solution of osmium tetroxide in 0.12 M phosphate buffer, dehydrated through graded series of ethanol solutions and stained in 1% uranyl acetate. After passage through propylene oxide, the blocks were embedded in Epon using moulds containing spherical cavities. The spherical embedded blocks thus obtained were rolled and re-embedded in Epon, according to the isector method (Nyengaard and Gundersen, 1992). From each of the four blocks obtained per animal, five serial 2-μm-thick sections were cut, which provided a total of 20 serial semithin sections per animal. Each semithin section was placed on a gelatin-coated microscope slide and stained with Toluidine Blue. Then, the tissue from each block was trimmed into a pyramidal shape and eight to 10 serial ultrathin sections were cut, collected on formvar-coated grids, and double-stained with uranyl acetate and lead citrate.

Stereological analyses

Number of synapses per neuron. Since the total number of neurons in the VMNvl does not vary as a function of hormonal levels (Madeira et al., 2001), the analysis of the effects of the different regimens used in Experiments 1 and 2 was done using, as main estimator, the number of synapses per neuron. The estimates were done independently for spine and dendritic synapses. The total number of synapses per neuron was estimated by dividing the numerical density (N_V) of each type of synapse by the N_V of VMNvl neurons.

Neuronal density. The N_V of VMNvl neurons was estimated from the series of semithin sections obtained as described above and by applying the physical disector method (Sterio, 1984; Madeira and Paula-Barbosa, 1993). Because the disector was made from pairs of alternate sections and each section was used in turn as the reference section or the look-up section, 22 disectors were performed on average per animal. The sections were analyzed using a modified Olympus BH-2 microscope interfaced with a color video camera and equipped with a Heidenhain ND 281 microcator (Traunreut, Germany), a computerized stage, and an object rotator (Olympus, Albertslund, Denmark). A computer fitted with a frame grabber (Screen Machine II, FAST Multimedia, Germany) was connected to the monitor. By using the CAST-Grid system software (Olympus), two counting frames equivalent in shape and with an area of 15,947 μm^2 each were superimposed onto the tissue images on the screen. The image of one section was frozen on the left half of the screen and compared with the image of the adjacent section displayed on the right side of the screen. Neurons were counted at a final magnification of 800×, when their nuclei (the counting unit) were visible in the reference section (the right sided image), but not in the look-up section (the left sided image), within the counting frame without being intersected by the exclusion edges or their extensions. On average, 90 neurons were counted per animal.

Synapse density. The number of synapses per unit volume of neuropil (N_V) was estimated by using the physical disector method (Sterio, 1984; Madeira and Paula-Barbosa, 1993; Sá and Madeira, 2005a). For this purpose, from each set of serial ultrathin sections, seven electron micrographs of corresponding fields of the neuropil were taken at primary magnification of 5400×. The micrographs were then enlarged photographically to a final magnification of 16,200×. Disectors were made from micrographs obtained from pairs of alternate sections (Fig. 1). Because each section was used in turn as the reference section, 40 disectors were made per animal. A transparency with an unbiased counting frame was superimposed onto the reference section micrograph. A synapse was counted whenever its postsynaptic density (the counting unit) was seen entirely or partly within the counting frame without intersecting the forbidden lines and their extensions in the reference section, but not in the look-up section. Synapses (Fig. 1) were identified by the presence of synaptic densities, at least three synaptic vesicles at the presynaptic site and a synaptic cleft (Gray and Guillery, 1966; Colonnier, 1968). Because the number of symmetrical synapses received by the dendritic trees of VMNvl neurons is very low (Nishizuka and Pfaff, 1989), for the purpose of the estimations herein performed no distinction was made between symmetrical and asymmetrical synapses. However, the estimates were performed independently for spine and dendritic synapses. The mean thickness of the ultrathin sections, estimated using the minimal fold technique (Small, 1968), was 70 nm. On average, 200 synapses were counted per animal.

Statistical analyses

In each experiment, the effect of the regimen used was assessed by one-way analysis of variance (ANOVA). Whenever significant results were found from the overall ANOVA, pair-wise comparisons were subsequently made using the post hoc Tukey's HSD test. Differences were considered significant if $P<0.05$.

RESULTS

Lordosis response

Results about the behavioral response to manual VCS are shown in Table 1. All females treated with estradiol followed by progesterone 48 h later (EB+P group) displayed moderate to maximal dorsiflexion of the back in response to VCS. In this group, lordosis intensity did not vary as a function of stimulus number (data not shown). Conversely, none of the rats in the remaining groups, even those primed with estradiol, displayed any degree of lordosis in response to VCS.

Hormone levels

The serum levels of estradiol and progesterone are shown in Table 1. ANOVA detected a significant effect of treatment on serum estradiol ($F_{7,40}=25.90$; $P<0.0005$) and progesterone ($F_{7,40}=56.47$; $P<0.0005$) levels. None of the administrations produced any increase in the serum levels of estradiol, except the administration of estradiol. In these groups, the levels of estradiol were significantly more elevated in rats treated with 20 μg of estradiol as a single pulse, than in rats receiving the same dose as two pulses on consecutive days. Progesterone administration (P and EB+P groups) significantly increased serum progesterone levels when compared to the remaining groups analyzed.

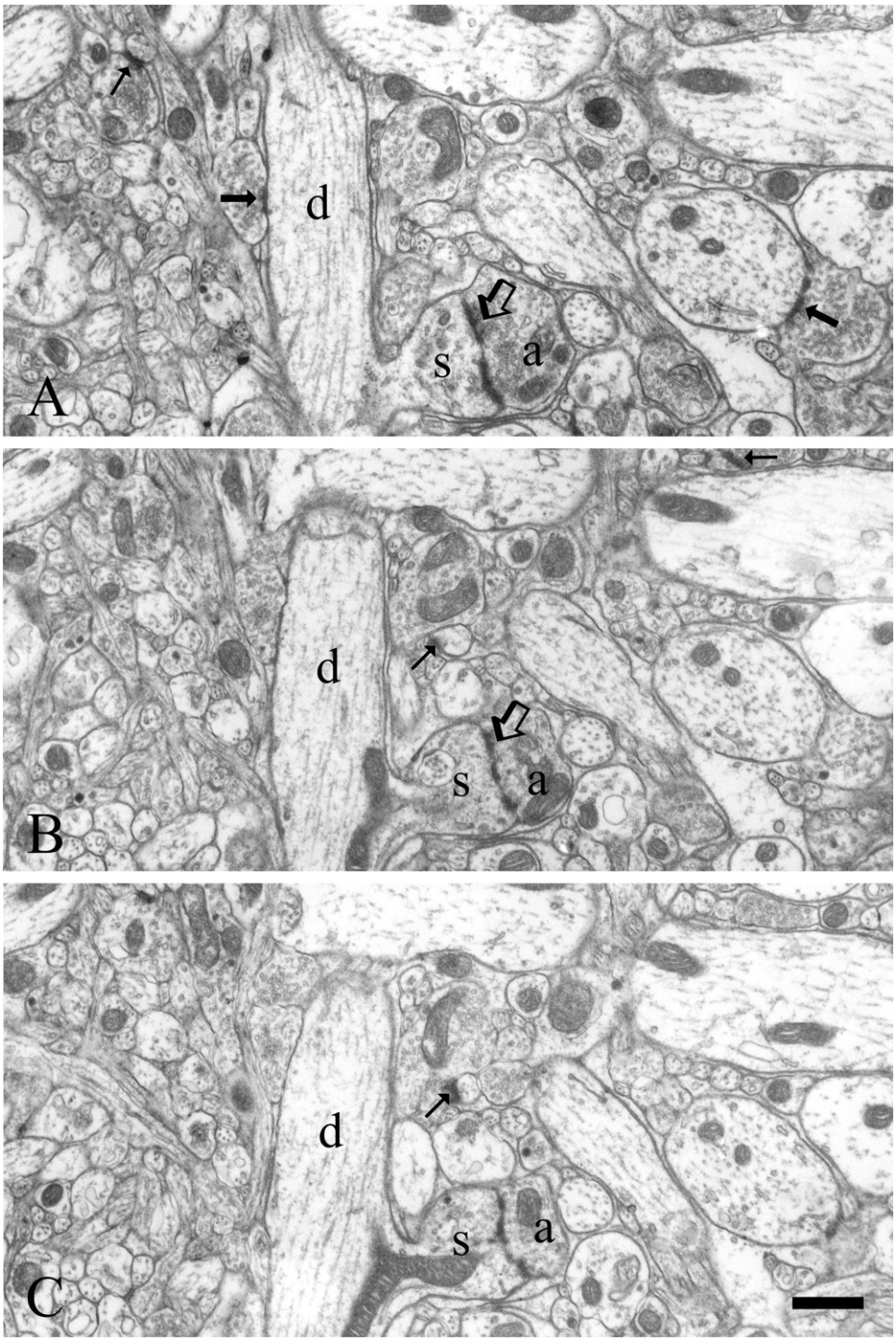

Fig. 1. Electron micrographs of three consecutive ultrathin sections (A–C) of the VMNvl showing identical areas of the neuropil. A dendritic shaft (d) bears a large spine (s) that makes an asymmetrical synaptic contact (transparent arrow) with an axon terminal (a). This synaptic contact can be seen only in A and B. Axon terminals contacting dendritic shafts at asymmetrical synapses (thick arrows) can be seen in A. Asymmetrical spine synapses that are visible in one micrograph but not in the adjacent micrographs are marked by thin arrows. Scale bar=0.74 μm.

Uterine weight

To confirm the efficacy of the estradiol injection, we measured the uterine weight of rats used in both experiments (Table 1). We found a significant overall effect on uterine weight across treatment groups ($F_{7,40}=19.74$; $P<0.0005$). Animals injected with EB, i.e. belonging to EB, 2 EB, EB+P and EB+RU groups had higher uterine weights than rats treated with either oil or progesterone alone. In females injected with the ERα agonist PPT the uterine

Table 1. Serum estradiol and progesterone levels, uterine weight and lordosis intensity in rats belonging to the different groups analyzed in Experiments 1 and 2

	Estradiol (pg/ml)	Progesterone (ng/ml)	Uterine weight (g)	Lordosis intensity
O group	33.17 (3.14)	4.02 (0.22)	0.25 (0.03)	0
P group	33.70 (±0.71)	14.52 (0.84)$^{+}$	0.24 (0.02)	0
EB+P group	59.55 (4.49)*	13.48 (0.59)$^{+}$	0.91 (0.09)$^{\#\#}$	2.5 (0.22)
EB+RU group	64.76 (9.14)**	4.69 (0.14)	1.10 (0.16)$^{\#\#}$	0
EB group	63.47 (5.87)*	5.82 (0.66)	0.89 (0.07)$^{\#\#}$	0
2 EB group	110.61 (7.09)^{xxx}	5.56 (0.64)	0.79 (0.05)$^{\#}$	0
PPT group	34.17 (4.09)	3.63 (0.54)	0.81 (0.08)$^{\#\#}$	0
DPN group	33.23 (3.90)	4.40 (0.60)	0.25 (0.21)	0

Values are expressed as means (SEM). * $P<0.05$; ** $P\leq0.005$, compared with groups that were not injected with estradiol; ^{xxx} $P<0.0005$ compared with all other groups; $^{+}$ $P<0.0005$ compared with groups that were not injected with progesterone; $^{\#}$ $P<0.005$, $^{\#\#}$ $P<0.0005$ compared with O-, P- and DPN-treated groups.

weight was similar to that of estradiol-treated rats and, accordingly, was significantly higher than in oil-treated rats. Rats treated with DPN had uterine weights similar to those of rats treated with oil or progesterone alone. Progesterone administration had no statistically significant effect ($P>0.05$) on uterine weight compared to oil-treated rats.

Numerical density of synapses

In Experiment 1 (Fig. 2), in which O, P, EB, EB+P and EB+RU groups were studied, we found no significant influence of treatment in the N_V of spine ($F_{4,25}=2.85$; $P=0.05$) and dendritic ($F_{4,25}=1.75$; $P=0.17$) synapses. In contrast, in Experiment 2 (Fig. 3), in which O, EB, 2 EB, PPT and DPN groups were analyzed, ANOVA revealed a significant effect of treatment on the N_V of both types of synapses ($F_{4,25}=19.90$; $P<0.0001$ for spine and $F_{4,25}=7.63$; $P<0.0004$ for dendritic synapses). With respect to spine synapses, the density was significantly higher in 2 EB- and PPT-treated rats than in the remaining groups. In dendritic synapses, this difference was only apparent for the PTT-treated group and no significant difference was found between 2 EB-treated rats and the remaining groups.

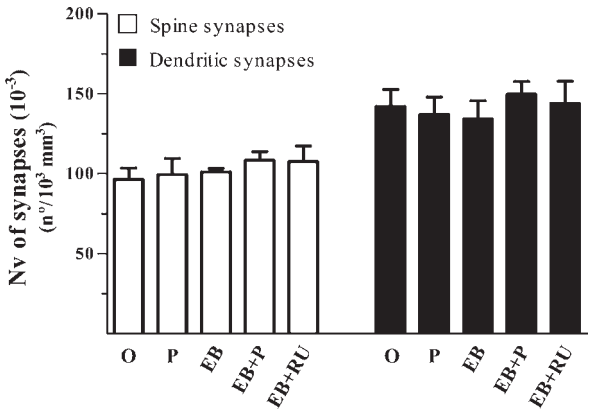

Fig. 2. Graphic representation of the N_V of spine (white columns) and dendritic (black columns) synapses in the VMNvl of rats studied in Experiment 1. The number of animals per group was six. On average, 200 synapses were counted per animal. Columns represent means and vertical bars 1 SD.

Neuronal numerical density

ANOVA revealed a significant influence of treatment in the N_V of VMNvl neurons in Experiment 1 ($F_{4,25}=5.70$; $P<0.005$) as well as in Experiment 2 ($F_{4,25}=7.46$; $P<0.0005$). In Experiment 1 (Fig. 4), rats injected with estradiol (EB, EB+P and EB+RU groups) had significantly fewer neurons per unit volume of the VMNvl than rats injected with oil or progesterone (O and P groups). In Experiment 2 (Fig. 4), rats treated with estradiol (EB and 2 EB groups) and rats receiving ER agonists (PPT and DPN groups) had significantly lower neuronal numerical densities than oil-treated rats. No significant differences were detected among EB, 2 EB, PPT and DPN groups.

Total number of synapses per neuron

The variations in synapse numbers induced by the administration of hormones, and receptor agonists and antagonists are shown in Figs. 5 and 6 and summarized in the diagram shown in Fig. 7. Treatments used in Experiment 1 significantly influenced the total number of spine ($F_{4,25}=9.57$; $P<0.0001$) and dendritic ($F_{4,25}=7.73$; $P<0.001$) synapses established by each VMNvl neuron (Fig. 5). The number of

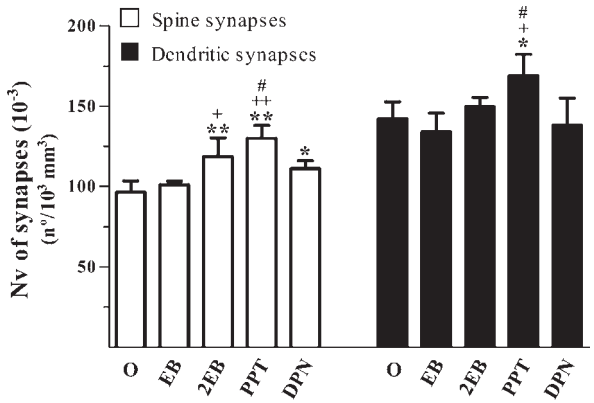

Fig. 3. Graphic representation of the N_V of spine (white columns) and dendritic (black columns) synapses in the VMNvl of rats studied in Experiment 2. The number of animals per group was six. On average, 200 synapses were counted per animal. Columns represent means and vertical bars 1 SD. Tukey's post hoc tests: * $P<0.05$, ** $P<0.0005$ compared with O-treated group; $^{+}$ $P<0.005$, $^{++}$ $P<0.0005$ compared with EB-treated group; $^{\#}$ $P<0.005$ compared with DPN-treated group.

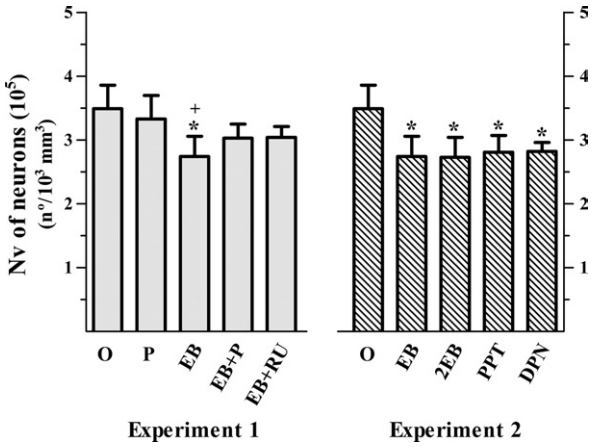

Fig. 4. Graphic representation of the N_V of VMNvl neurons in rats studied in Experiment 1 and in Experiment 2. The number of animals per group was six. On average, 90 neurons were counted per animal. Columns represent means and vertical bars 1 SD. Tukey's post hoc tests: * $P<0.005$ compared with O-treated group; $^{+}$ $P<0.05$ compared with P-treated group.

synapses did not differ between oil- and progesterone-treated rats, but was significantly higher in all groups injected with estradiol (EB, EB+P and EB+RU) than in O and P groups. No differences in the total number of spine and dendritic synapses were found among EB, EB+P and EB+RU groups.

In Experiment 2 (Fig. 6), we likewise found a significant effect of treatment on the total number of spine ($F_{5,30}=23.88$; $P<0.0001$) and dendritic ($F_{5,30}=15.45$; $P<0.0001$) synapses received by each VMNvl neuron. Rats injected with estradiol (EB and 2 EB groups) and with ER agonists (PPT and DPN groups) had significantly more spine and dendritic synapses than oil-treated rats. Among these groups, rats treated with 20 μg of estradiol as one single pulse (2 EB group) and rats treated with PPT had the highest number of spine and dendritic synapses, and these numbers were significantly higher than those from EB- and DPN-treated rats.

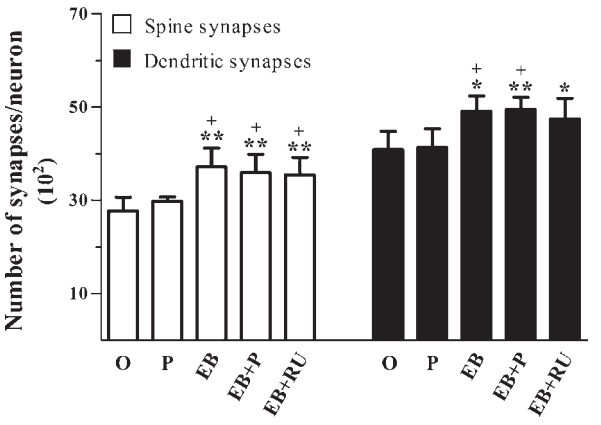

Fig. 5. Graphic representation of the number of spine (white columns) and dendritic (black columns) synapses received by each VMNvl neuron in rats studied in Experiment 1. The number of animals per group was six. Columns represent means and vertical bars 1 SD. Tukey's post hoc tests: * $P<0.05$, ** $P<0.005$ compared with O-treated group; $^{+}$ $P<0.05$ compared with P-treated group.

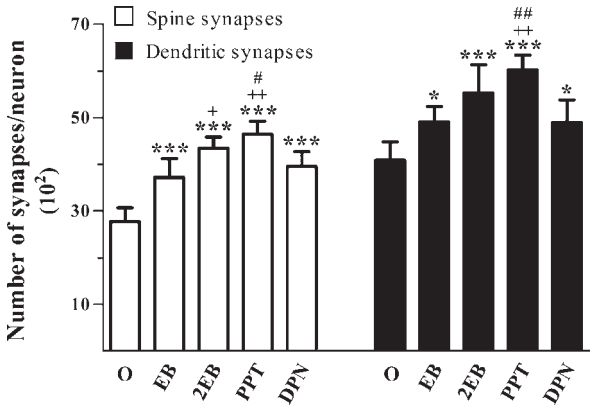

Fig. 6. Graphic representation of the number of spine (white columns) and dendritic (black columns) synapses received by each VMNvl neuron in rats studied in Experiment 2. The number of animals per group was six. Columns represent means and vertical bars 1 SD. Tukey's post hoc tests: * $P<0.05$, ** $P<0.005$, *** $P<0.0005$ compared with O-treated group; $^{+}$ $P<0.05$, $^{++}$ $P<0.005$ compared with EB-treated group; $^{\#}$ $P<0.05$, $^{\#\#}$ $P<0.005$ compared with DPN-treated group.

DISCUSSION

Influence of estrogens on the structural organization of the VMNvl

Earlier neuroanatomical studies on estrogen actions in the synaptic organization of the VMNvl have basically evaluated spine or synapse densities. With a single exception, in which a decrease in spine density was noticed in a subset of VMNvl neurons (Calizo and Flanagan-Cato, 2002), these investigations have shown that estrogens prompt the formation of new dendritic spines on VMNvl neurons

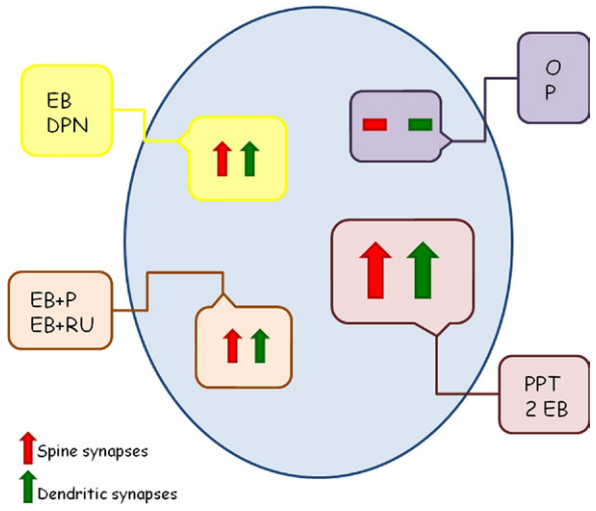

Fig. 7. Diagrammatic representation of the variations induced in synapse numbers by the administration of hormones, and receptor agonists and antagonists. Arrows indicate an increase in the number of synapses per neuron and dashes the absence of effect. Thick arrows denote large effects and thin arrows small effects.

(Frankfurt and McEwen, 1991a; Segarra and McEwen, 1991; Calizo and Flanagan-Cato, 2000) and increase the number of synapses received by these neurons (Carrer and Aoki, 1982; Nishizuka and Pfaff, 1989; Frankfurt and McEwen, 1991b). By measuring areal densities of synapses, i.e. numbers per unit surface area, the same authors have also shown that the effects of estrogens are apparent, both 2 days (Frankfurt and McEwen, 1991b) and 2 weeks (Nishizuka and Pfaff, 1989) after their administration, in dendritic but not in spine synapses.

Our results, which reflect variations in the total number of synapses established by each VMNvl neuron, are generally in agreement with data from these studies, but additionally reveal that estrogens act in a similar way upon spine and dendritic synapses. In fact, we found that the administration of behaviorally effective doses of estradiol increases the total number of dendritic and spine synapses established by each VMNvl neuron by approximately 30% and 20%, respectively. The possibility that estrogens might alter the number of both types of synapses had been suggested by an earlier study describing the variations over the estrous cycle in the total number of synapses received by individual VMNvl neurons (Sá and Madeira, 2005a).

The lack of concordance between our estimates and those reported in studies from other laboratories is most probably related to the estimator used, i.e. total numbers versus areal densities of synapses. In fact, data herein reported also show that estradiol-induced variations in the number of synapses established per neuron do not mirror the estradiol effects on the N_V of the same synapses. For example, EB-treated rats do not differ from oil-treated rats with respect to the N_V of spine and dendritic synapses, but their neurons establish significantly more spine and dendritic synapses than neurons from oil-treated rats, as revealed by the estimates of total numbers of synapses per neuron. Densities are not considered as strong estimators because they change in response to variations in the volume of reference. This is critical in the case of the VMN because the fluctuations in the circulating levels of estrogens that occur naturally over the estrous cycle (Madeira et al., 2001; Sá and Madeira, 2005a) or are provoked by exogenous estrogens (Carrer and Aoki, 1982; Jones et al., 1985) lead to marked changes in the volume of VMNvl neurons and neuropil. Lending further support to this view are our own results showing a significant decrease in the N_V of VMNvl neurons in response to the administration of estradiol to ovariectomized rats.

Estradiol and synaptic plasticity

Data from the present study also show that the number of dendritic and spine synapses in the VMNvl is influenced by the circulating levels of estrogens and by the pattern of estradiol administration. In fact, we found that the administration of 20 μg of estradiol as one single pulse 52 h before sacrifice (2 EB group) was associated to an increase of 56% and 35% in the total number of spine and dendritic synapses, respectively, relative to oil-treated rats. In 2 EB-treated rats, the serum levels of estradiol at the time of sacrifice were approximately twice those measured in rats that received the same dose of estradiol but as two pulses administered on consecutive days, the last of which also 52 h before sacrifice (EB group). Reflecting these differences, 2 EB-treated rats had significantly more (17%) spine synapses than EB-treated rats. We noticed a similar trend in dendritic synapses, but the increase (13%) observed did not reach statistical significance. In addition, only in the 2 EB-treated rats, but not in the EB group, there was a tendency towards an increase in the N_V of synapses that, in the case of spine synapses, was statistically significant.

It is probable that the differences between EB and 2 EB groups might rely on the different circulating levels of estradiol as they were significantly higher in 2 EB-treated rats. However, it is also possible that in EB-treated rats, the first pulse of estradiol might have downregulated the ERs expressed by VMNvl neurons or by estrogen-sensitive neurons that project to the VMN (Simerly and Swanson, 1988; Delville and Blaustein, 1993) leading to a lower activation of VMNvl neurons in response to the second pulse of estradiol 24 h later. Actually, there are reports showing that the ER mRNA levels are reduced in the VMNvl 24 h after estradiol administration to ovariectomized rats (Lauber et al., 1990; Simerly and Young, 1991) and during the estrus phase in normally cycling rats (Shughrue et al., 1992).

Progesterone and synaptic plasticity

In estrogen-primed rats, progesterone acts on VMNvl neurons to induce the proceptive component of the female sexual behavior and to generate the lordosis reflex (Rubin and Barfield, 1983a,b; Mani et al., 1994; Turcotte et al., 2005). Interestingly, the progesterone surge that occurs at late proestrus and is responsible for the induction of lordosis is not likely to be involved in the exuberant changes that occur, at that moment, in the connectivity pattern of VMNvl neurons. In fact, our results show that the administration of progesterone to estrogen-primed rats does not introduce further changes in the synaptic pattern of VMNvl neurons. Even though estrogen-primed rats treated with progesterone had a higher number of synapses than oil-treated rats, their number did not differ from that shown by rats treated only with estradiol. The dose of progesterone used is not a likely explanation for the lack of progesterone effects on synapse numbers because rats exhibited lordosis in response to VCS, which did not happen to rats treated only with estradiol.

Given that progesterone administered simultaneously or a few hours before estradiol inhibits the induction of sexual behavior (Blaustein and Wade, 1977), we also tested the unlikely possibility that progesterone might alter the synaptic pattern of VMNvl neurons when administered to rats not primed with estradiol. As expected, and probably because there are virtually no PRs in the VMNvl in the absence of estrogens (Blaustein and Turcotte, 1989; Romano et al., 1989; Lauber et al., 1991), the administration of progesterone to rats not primed with estradiol had no effect on synapse numbers, as it did not have on behavior. To investigate the effect of endogenous progesterone on

synapse formation we extended our observations to estrogen-primed rats treated with the PR antagonist RU486. Again, we found no differences in the number of either type of synapse relative to estrogen-primed rats treated or not with progesterone.

Thus, our results show that despite the recognized role of progesterone in the induction of behaviors that are regulated by the VMNvl, this hormone does not seem to be implicated in the changes of the connectivity pattern that its neurons undergo over the estrous cycle (Sá and Madeira, 2005a).

Role of ERs in synaptic plasticity

In an attempt to identify the ERs involved in mediating the synaptogenic effects of estrogens in the VMNvl, we have independently estimated the number of synapses per neuron in rats treated with either PPT, the agonist of the ERα, or DPN, the agonist of the ERβ. Our results revealed that estrogens promote synaptogenesis by activating the ERα and the ERβ, as both agonists significantly increased the number of spine and dendritic synapses received by each VMNvl neuron. In addition, they also significantly decreased the N_V of VMNvl neurons, which suggests that, similarly to estradiol (Carrer and Aoki, 1982; Jones et al., 1985), PPT and DPN lead to hypertrophy of the neuronal cell bodies and enlarge the neuropil in the VMNvl. The similarity of the effects of PPT and DPN in the VMNvl, where the expression of ERα clearly exceeds that of ERβ, strongly indicates that estrogens act upon VMNvl neurons not only by activating classical signaling pathways in ER-positive VMNvl neurons but also by enhancing the inputs conveyed by afferent fibers to the VMNvl. In fact, VMNvl neurons receive massive projections from brain regions whose neurons, although expressing both subtypes of ERs, are particularly rich in ERβ, as is the case of the preoptic area and bed nucleus of the stria terminalis (Shughrue et al., 1997b; Gréco et al., 2001; Shughrue and Merchenthaler, 2001). However, the administration of PPT induced the formation of significantly more spine and dendritic synapses than the administration of DPN.

Studies in the CNS and other non-neural organs have demonstrated that, depending on the tissue and variable analyzed, ERα and ERβ may mediate either divergent (Frasor et al., 2003; Gréco et al., 2003; Lund et al., 2005) or similar effects and, in this case, that ERβ activation has weaker effects than ERα activation (Frasor et al., 2003; Lindberg et al., 2003). There is also evidence indicating that when both receptors are activated, the activation of the ERβ reduces the magnitude of the stimulation induced by the ERα (Lindberg et al., 2003; Gonzales et al., 2008; Mazzucco et al., 2008). We cannot clearly conclude for the same type of interaction between ERα and ERβ in mediating estrogen effects on synaptic plasticity in the VMNvl because we did not include in our studies rats treated simultaneously with PPT and DPN. Still, our results show that the selective activation of ERα in the absence of ERβ activation did not consistently induce the formation of more synapses than estradiol alone. In fact, our results show that the number of synapses induced by PPT administration was higher than in EB-treated rats, but it did not differ from that observed in 2 EB-treated rats. In addition, even though there are reports showing dose-dependent effects of PPT, namely, in body and uterine weights (Harris et al., 2002; Frasor et al., 2003; Santollo et al., 2007), PR mRNA levels (Harris et al., 2002) and proceptive and receptive behaviors (Mazzucco et al., 2008), we have reasons to assume that the exuberant effects of PPT on synapse numbers are not attributable to the dosage used because the administration of PPT produced an increase in uterine weight that was similar to that provoked by estradiol.

CONCLUSION

Data presented herein reveal that despite the relevant physiological role of progesterone in the facilitation of female sexual behavior, this sex steroid plays no role in the determination of the synaptic pattern required for the activation of VMNvl neurons. In addition, they indicate that although the expression of sexual behavior requires the activation of the ERα, synaptogenesis in the VMNvl is mediated by activation of both subtypes of ERs.

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IV.

Role of neural afferents as mediators of estrogen effects on the hypothalamic ventromedial nucleus

Brain Research (Aceite para publicação)

Role of Neural Afferents as Mediators of Estrogen Effects on the Hypothalamic Ventromedial Nucleus

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Keywords:
Plasticity
Synapses
Neural afferents
Estrogens
Progesterone receptors
Ventromedial nucleus

ABSTRACT

The effects of estrogens on the ventrolateral division of the hypothalamic ventromedial nucleus (VMNvl) are essential for its role in the regulation of female sexual behavior. Enhanced synaptogenesis and induction of progesterone receptors (PRs) are hallmarks of the actions of estrogens on the VMNvl. To investigate the influence of neural afferents in mediating these effects, we estimated the number of spine and dendritic synapses per neuron and the total number of PR-immunoreactive neurons in ovariectomized rats treated with either estradiol benzoate or vehicle, after unilateral VMN deafferentation. The estimates were performed independently in the VMNvl of the deafferented and contralateral sides, and in the VMNvl of unoperated rats (controls). The administration of estradiol benzoate did not induce any increase in the number of synapses of the deafferented VMNvl. In the contralateral VMNvl, the synaptogenic effects of estrogen were apparent, but still reduced relative to the control VMNvl, where a 25% increase in the total number of synapses was observed after estrogenic stimulation. In the absence of estrogenic stimulation, i.e., in basal conditions, deafferentation reduced the number of dendritic and spine synapses, but particularly the latter. The reduction was also visible, but less marked, in the contralateral VMNvl. Contrary to synapses, the estrogen induction of PRs was unaffected by deafferentation, and the total number of PR-immunoreactive neurons was similar in the control, deafferented and contralateral VMNvl. The results show that estrogens enhance synaptogenesis in the VMNvl by acting through neural afferents and induce PR expression by acting directly upon VMN neurons.

1. Introduction

The ventrolateral division of the hypothalamic ventromedial nucleus (VMNvl) plays an essential role in the display of the female sexual behavior (Pfaff and Sakuma, 1979a,b). The onset and the duration of this behavior depend on the sequential exposure of specific regions of the brain, namely the VMNvl, to estrogens and progesterone. One of the major actions of estrogens in the VMNvl is the generation of new dendritic and spine synapses (Carrer and Aoki, 1982; Sá and Madeira, 2005a, Sá et al., 2009). Here, estrogens also lead to the sprouting of dendritic spines (Calizo and Flanagan-Cato, 2000; Madeira et al., 2001), elongation of dendritic trees (Madeira et al., 2001) and hypertrophy of neuronal cell bodies (Carrer and Aoki, 1982; Jones et al., 1985; Madeira et al., 2001). These changes are associated with other signs of activation of VMNvl neurons, including enhanced transcription of ribosomal RNA (Jones et al., 1986), increased number of nuclear pores (Sá and Madeira, 2005b), and enlarged nucleoli (Cohen et al., 1984), rough endoplasmic reticulum and Golgi complex (Cohen and Pfaff, 1981; Sá and Madeira, 2005b). Another major effect of estrogens in the VMNvl is the induction of progesterone receptors (PRs) in a subset of its neurons (Brown et al., 1987; McLusky and McEwen, 1980; Parsons et al., 1982). By acting through these receptors, progesterone amplifies the effects of estrogens on sexual behavior and facilitates lordosis behavior in estrogen-primed rats (Mani et al., 1994, Rubin and Barfield, 1983a,b).

The cellular basis for many of the actions of estrogens on the pattern of neuronal connectivity in the VMNvl and on the expression of PRs by its neurons is believed to involve the ability of estrogen receptors (ERs) to function as ligand-activated transcription factors. In a study of direct stimulation by ERα and ERβ agonists, it was shown that the synaptogenic effects of estrogens in the VMNvl are mediated by activation of both ER subtypes (Sá et al., 2009). Further, estrogens greatly increase PR expression in the VMNvl through a mechanism that, although still not well understood, seems to involve the ERα and, possibly, the ERβ (Harris et al., 2002; Kudwa and Rissman, 2003; Moffat et al., 1998; Musatov et al., 2006; Temple et al., 2001). The VMNvl contains neurons that express, or coexpress, both ER subtypes (Laflamme et al., 1998; Shughrue et al., 1997, 1998; Shughrue and Merchenthaler, 2001) and, therefore, the generation of new synapses and the induction of PRs can result from the direct action of estrogens on VMNvl neurons. However, estrogen effects can also be indirect and mediated by the inputs conveyed by VMN afferents. In fact, the VMN in general, and the VMNvl in particular, receive dense projections from brain regions that abundantly express the ERα and/or the ERβ (Gréco et al., 2001; Laflamme et al., 1998; Shughrue et al., 1997, 1998; Shughrue and Merchenthaler, 2001). This is the case of the medial amygdala (De Olmos and Ingram, 1972; Fahrbach et al.,

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1989; Krettek and Price, 1978), bed nuclei of the stria terminalis (Delville and Blaustein, 1993; Dong and Swanson, 2004; Gu et al., 2003), medial preoptic nucleus (Delville and Blaustein, 1993; Fahrbach et al., 1989; Simerly and Swanson, 1988), ventral subiculum (Canteras and Swanson, 1992; Fahrbach et al., 1989) and some hypothalamic (Canteras et al., 1992; Záborsky and Makara, 1979) and brainstem (Delville and Blaustein, 1993) nuclei.

The purpose of the present study was to examine if the estrogen effects on synapse formation and expression of PRs by VMNvl neurons are a consequence of local effects of estrogen, are mediated by trans-synaptic signaling mechanisms or are the result of convergent regulatory influences mediated by both. To examine these possibilities, we have surgically isolated the right VMN in ovariectomized rats. Rats were later treated with either vehicle or estradiol benzoate in a dose and schedule known to be behaviorally effective and to induce synaptogenesis in the VMNvl (Sá et al., 2009). The role played by VMN afferents in mediating the influence of estrogens in the VMNvl was evaluated by comparatively analyzing the number of spine and dendritic synapses established per neuron and the number of PR-immunoreactive neurons in the deafferented VMNvl, in the VMNvl of the contralateral hemisphere, and in the VMNvl of control rats.

2. Results

2.1. *Hormone levels and uterine weight*

The uterine weight, expressed in g (SD), was 0.252 (0.08) in oil-treated rats and 1.141 (0.25) in estradiol benzoate- (EB) treated

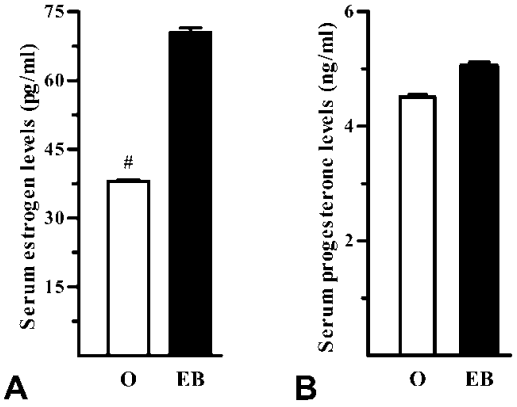

Fig. 1 - Serum hormone levels in oil- and EB-treated rats. (A) Estrogen levels. (B) Progesterone levels. Columns represent means±SEM. #p<0.0001 compared to EB-treated rats.

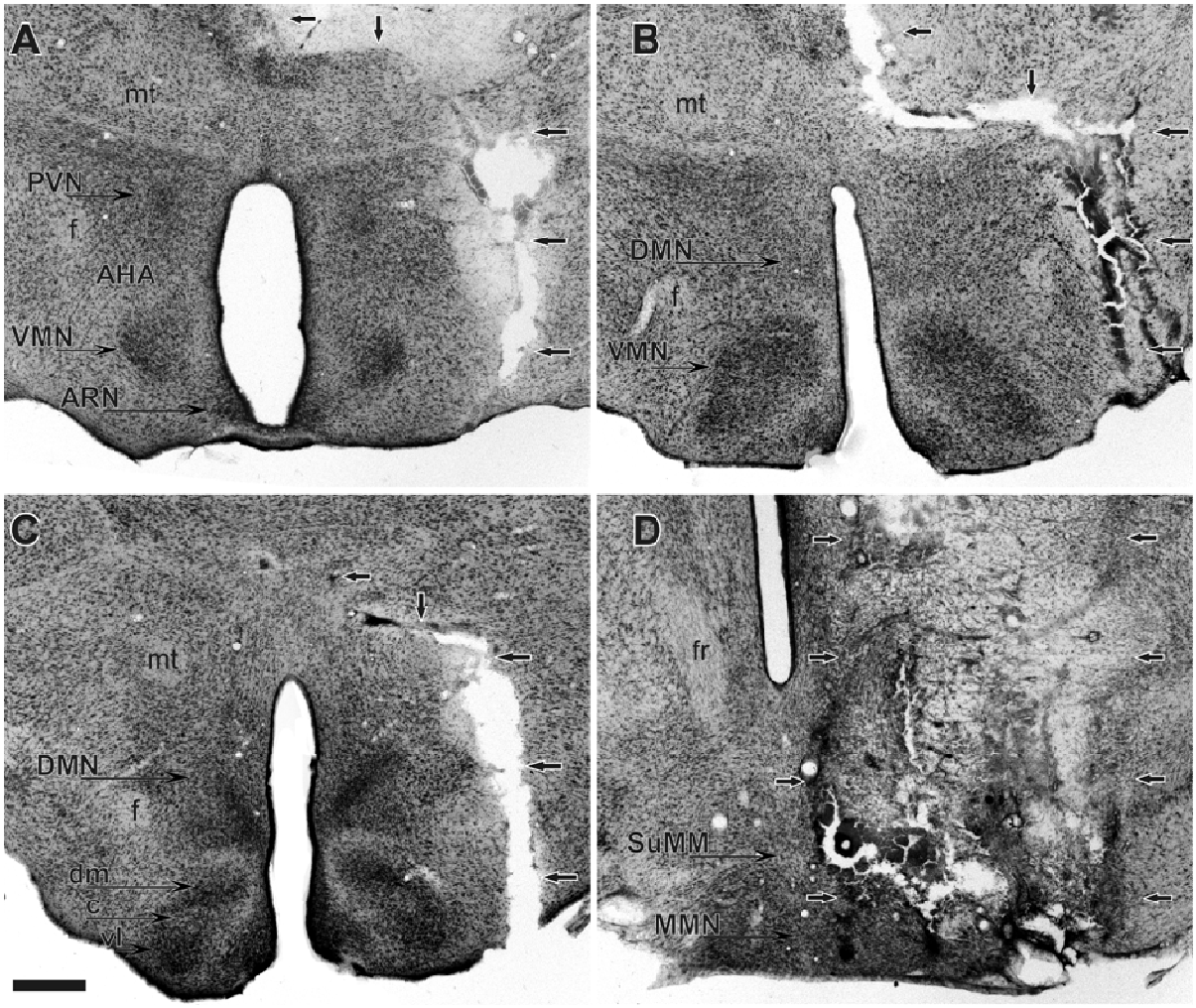

Fig. 2 - Lesions made by the knife cuts used to isolate VMN. The photomicrographs were obtained from Giemsa-stained 40 μm-thick coronal sections at four different rostrocaudal levels of the hypothalamus. The location and extent of the lesions are indicated by arrows. (A) Section through the anterior division of the VMN. The arcuate nucleus (ARN), the paraventricular nucleus (PVN) and the anterior hypothalamic area (AHA) can be seen at this level. (B) Section taken at approximately the mid-level of the VMN. The dorsomedial nucleus (DMN) is already present at this level. (C) Section taken at the mid-level of the compact division of the DMN. The dorsomedial (dm), central (c) and ventrolateral (vl) divisions of the VMN can be easily seen. (D) Section taken at the mid-level of the mammillary region of the hypothalamus showing, in its full extent and in a frontal view, the lesion provoked by the knife used to sever the posterior connections of the VMN. The medial mammillary nucleus (MMN) and the supramammillary nucleus (SuMM) can also been seen. Abbreviations: f, fornix; fr, fasciculus retroflexus; mt, mammillothalamic tract. Scale bar: 500 μm.

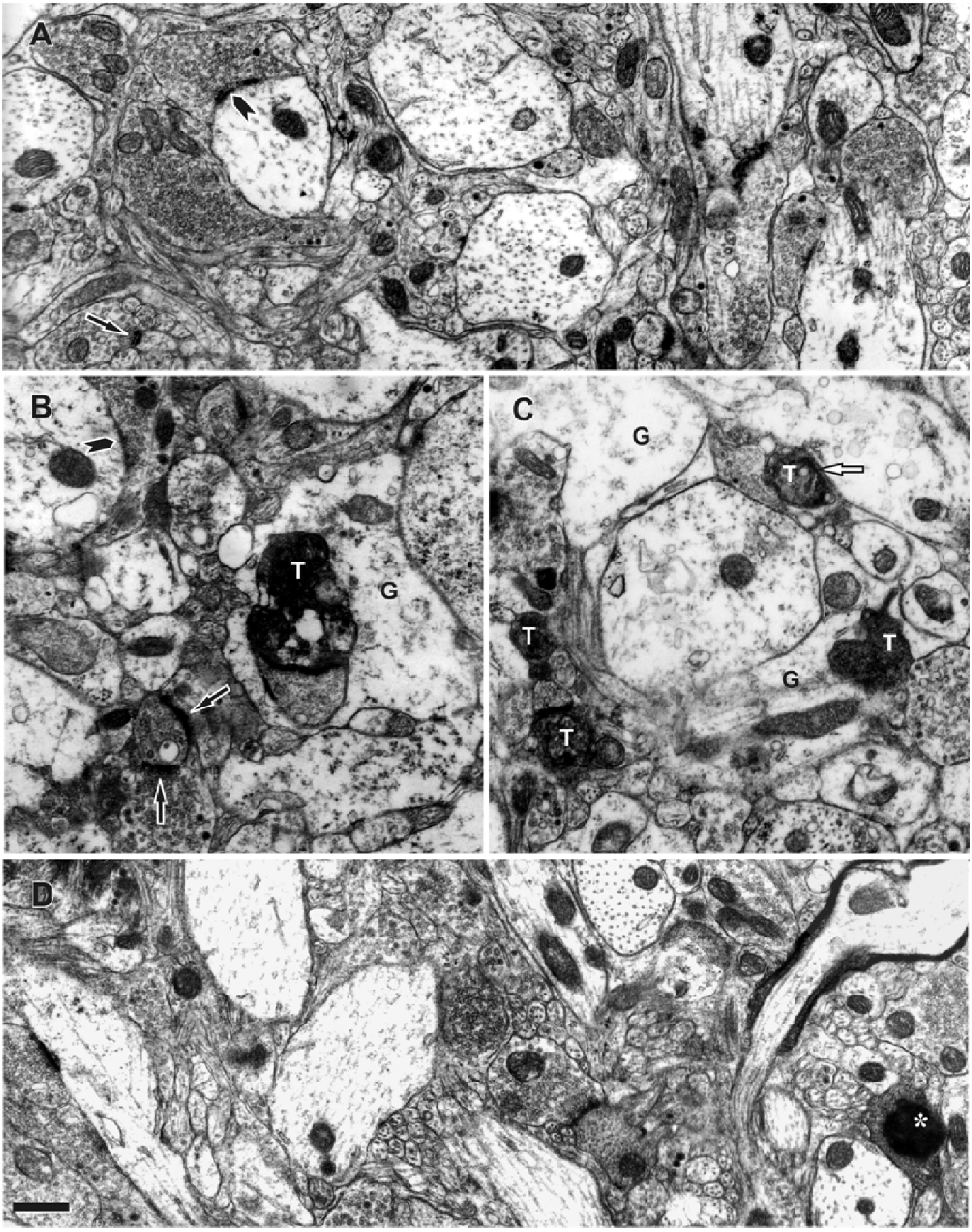

Fig. 3 - Ultrastructural features of the neuropil of the VMNvl. (A) A view of the neuropil of a control rat. Asymmetrical synaptic contacts on a dendritic shaft (arrowhead) and on a dendritic spine (arrow) are shown. (B and C) Neuropil of a deafferentated VMNvl. (B) A degenerating axonal terminal (T) containing disrupted organelles is completely engulfed by a glial process (G), in which glycogen granules and fibrils are recognized. Synaptic contacts on a single dendritic spine (arrows) and on a dendritic shaft (arrowhead) can been seen. (C) Four degenerating dark axonal terminals (T) are recognized in the proximity of extensive glial processes (G). A degenerating mitochondria is easily recognized in the terminal indicated by a white arrow. (D) Neuropil of a contralateral VMNvl. The overall view does not differ from that of the control VMNvl (see A). A dark terminal (white asterisk) is engulfed by a glial process. Scale bar: 0.62 µm.

rats. As expected, treatment of ovariectomized rats with EB significantly increased uterine weight (p<0.0001). In agreement with this finding, serum estradiol levels were significantly higher in EB-treated than in oil-treated rats (Fig. 1A). Conversely, the plasma levels of progesterone did not differ between EB- and vehicle-treated rats (Fig. 1B).

2.2. Histological identification of the knife cuts

The location and the extent of the surgical cuts used to isolate the right VMN are shown in Fig. 2. The lateral cuts passed adjacent to the lateral border of the VMN (Figs. 2A, B, C). The coronal cuts were done, rostrally, through the retrochiasmatic area and, caudally, through the mammillary region (Fig. 2D). In addition to the VMN, the isolated hypothalamic block contained parts of the arcuate, dorsomedial and paraventricular nuclei as well as the posterior part of the anterior hypothalamic area.

2.3. Ultrastructural features of the VMNvl neuropil

As can been seen in Fig. 3, the electron microscopic examination of the VMNvl neuropil revealed that there were clear signs of degenerative activity ongoing 3 days after surgery. Most of the degenerating axon terminals displayed typical features of electron-dense degeneration, including irregular outlines and darkening. Some of these terminals were surrounded, and others engulfed, by glial processes, in which great amounts of glycogen could be seen. Terminals undergoing degenerating neurofilamentous hypertrophy could also be seen, but they were less common. In both types of degeneration, axon terminals contained disrupted organelles, namely mitochondria. In the contralateral VMNvl, dark degenerating axon terminals were occasionally observed.

2.4. Number of synapses per neuron

ANOVA revealed a significant effect of deafferentation (F(2,30)=480.30, p<0.0001) and EB treatment (F(1,30)=107.45, p<0.0001) on the total number of synapses per neuron (Fig. 4A). EB treatment did not modify the total number of synapses in the deafferented VMNvl, but produced a significant, and similar, increase in the control (26%) and contralateral (24%) VMNvl. In addition, in oil-treated rats the number of synapses was reduced by 39% in the deafferented VMNvl and by 20% in the contralateral nucleus relative to the control VMNvl. After EB administration, the difference between the deafferented VMNvl and the control nucleus increased to 52% whereas the difference between the

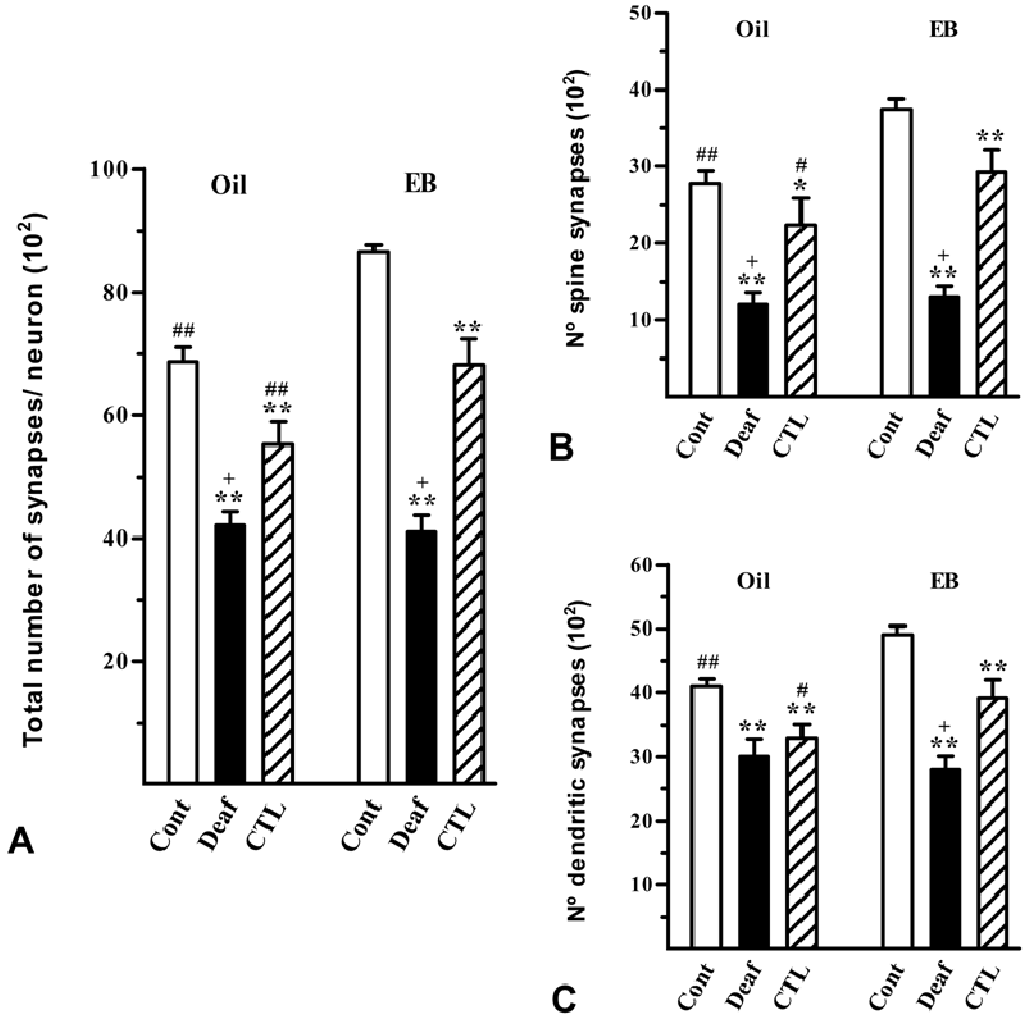

Fig. 4 - Influence of VMN deafferentation on the synaptogenic effects of estrogens in VMNvl. (A) Total number of synapses per VMNvl neuron. (B) Number of spine synapses per neuron. (C) Number of dendritic synapses per neuron. The estimates were done independently in the VMNvl of control rats (white columns), and in the deafferentated (black columns) and contralateral (dashed columns) VMNvl of rats that were treated with oil or estradiol benzoate (EB) after unilateral VMN deafferentation. Columns represent means±SD. Tukey's post hoc tests: *p<0.005, **p<0.0005, compared with the control VMNvl; +p<0.0005, compared with the contralateral VMNvl; #p<0.005, ##p<0.0005, compared with the respective EB-treated group.

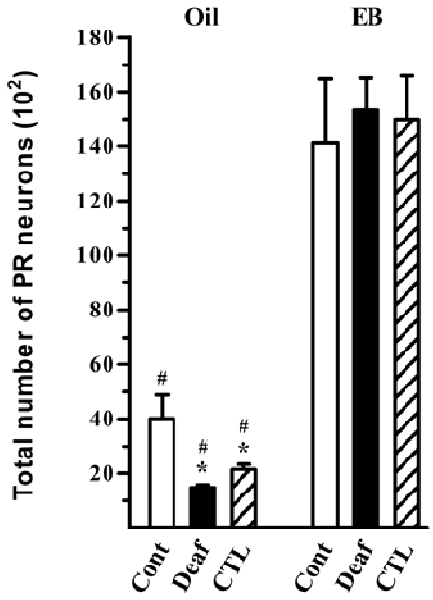

Fig. 5 - Influence of VMN deafferentation on the estrogen induction of progesterone receptors (PR) in VMNvl. The total number of PR-immunoreactive neurons was estimated independently in the VMNvl of control rats (white columns), and in the deafferentated (black columns) and contralateral (dashed columns) VMNvl of rats that were treated with oil or estradiol benzoate (EB) after unilateral VMN deafferentation. Columns represent means±SD. Tukey's post hoc tests: *p<0.0005 compared with the control VMNvl; #p<0.0005, compared with the respective EB-treated group.

contralateral and the control VMNvl remained proportionally at the same level as before EB administration.

The number of spine synapses per neuron (Fig. 4B) was significantly influenced by deafferentation (F(2,30)=246.37, p<0.0001) and EB administration (F(1,30)=59.96, p<0.0001); a significant interaction between these factors was also detected (F(2,30)=11.38, p<0.001). EB treatment produced a significant increase in the number of spine synapses in the control VMNvl (35%) as well as in the contralateral VMNvl (31%). In contrast, the same treatment did not modify the number of spine synapses in the deafferented VMNvl. In oil-treated rats, deafferentation was associated with a 57% reduction in the number of spine synapses; the number was also reduced in the contralateral VMNvl, but the difference relative to the control VMNvl was significantly smaller and did not exceed 20%. EB administration did not alter the relative difference between these groups, but increased by approximately 10% the differences between the deafferented VMNvl and the contralateral and control VMNvl.

The number of dendritic synapses (Fig. 4C) was also significantly influenced by deafferentation (F(2,30)=162.29, p<0.0001) and EB administration (F(1,30)=31.27, p<0.0001); a significant interaction between these factors was also detected (F(2,30)=18.40, p<0.0001). Similar to spine synapses, dendritic synapses did not change their number in the deafferented VMNvl in response to EB administration. In contrast, the contralateral and the control VMNvl increased their number by approximately 20% in response to the same treatment. Due to these differential effects of EB, the number of dendritic synapses was significantly smaller (30%) in the deafferented than in the contralateral VMNvl, as it was in these nuclei (43% and 20%, respectively) relative to the control VMNvl. In oil-treated rats, the number of dendritic synapses did not significantly differ between the deafferented and contralateral VMNvl, but it was smaller in these nuclei (by 27% and 20%, respectively) than in the VMNvl of control rats.

2.5. Number of PR-immunoreactive neurons

As can be seen in Fig. 5, the total number of PR-immunoreactive neurons in the VMNvl was strongly influenced by EB administration (F(1,30)=785.9, p<0.0001), but not by VMN deafferentation (F(2,30)=0.817, p=0.451). EB treatment resulted in a 3.5-fold increase in the number of PR-immunoreactive neurons in the control VMNvl, a 10-fold increase in the deafferented VMNvl and a 7-fold increase in the contralateral nucleus. ANOVA also revealed that the interaction between deafferentation and EB administration was statistically significant (F(2,30)=6.49, p<0.01). This was related to the presence, in oil-treated but not in EB-treated rats, of significantly less PR-immunoreactive neurons in the deafferented and contralateral VMNvl than in the control VMNvl. No such differences were detected among EB-treated rats.

3. Discussion

It has been often suggested, but never demonstrated, that the synaptogenic effects of estrogens in the VMNvl might be mediated by neural afferents instead of resulting from a direct action on the local ER-positive neurons. In this study, we provide solid evidence that this is indeed the case. In fact, the results show that the administration of EB in a schedule and dose that is behaviorally effective (Sá et al., 2009) and leads to a 25% increase in the total number of synapses in ovariectomized rats, does not modify the number of synapses received by individual VMNvl neurons in ovariectomized rats submitted to VMN deafferentation.

3.1. Deafferentation reduces synaptic connections in the absence of estrogenic stimulation

Deafferentation of the VMN led to a significant reduction in the number of synapses received by VMNvl neurons. Three days after deafferentation, the VMNvl contained 40% fewer synapses than the non-deafferented nuclei which indicates that approximately two thirds of the synaptic contacts received by VMNvl neurons are intrinsic to the nucleus. This proportion of intrinsic synaptic contacts does not seem to be influenced by the circulating levels of estrogens because our results, which were obtained in oil-treated ovariectomized rats, are in agreement with those obtained by Nishizuka and Pfaff (1989) in ovariectomized rats that were exposed to estradiol only before deafferentation. In fact, in the experimental model used by these authors the silastic capsules containing crystalline 17β-estradiol were removed immediately after deafferentation, in order to prevent phenomena based on

estrogen-induced synapse formation after deafferentation (Nishizuka and Pfaff, 1989).

From these intrinsic synapses, those established on dendritic shafts were largely predominant. Because the reduction induced by deafferentation was twice higher for spine synapses than for dendritic synapses (57% vs 27%), the deafferented VMNvl contained relatively more (10%) dendritic synapses than the control VMNvl. These finding indicates that although the axon terminals involved in the formation of dendritic and spine synapses have both intrinsic and extrinsic sources, the preferential targets of the extrinsic afferents are the dendritic spines. Taking into account that the great majority of spine synapses are asymmetrical, and thus presumably excitatory, and 15% of the dendritic synapses in the VMN are of the symmetrical type (Field, 1972; Nishizuka and Pfaff, 1989), and therefore inhibitory, our data indicate that the extrinsic afferents to VMNvl neurons play a key role in conveying excitatory inputs to VMNvl neurons. Those originating in the medial amygdala are strong candidates for exerting much of this action because, in addition to being mostly glutamatergic (Bian et al., 2008; Choi et al., 2005), they terminate preferentially on dendritic spines (Field, 1972) and participate in about 20% of the synapses in the VMNvl (Field, 1972), i.e., half of the synapses established by extrinsic afferents.

3.2. Deafferentation prevents estrogen-induced synaptogenesis

Estrogens prompt the cyclic increases in the number of synapses that occur in the VMNvl across the estrous cycle (Sá and Madeira, 2005a) and this effect is mediated by activation of both ER subtypes (Sá et al., 2009). It is not clear if, and how, the ERα and the ERβ interact to regulate the number of synapses in the VMNvl, but it is known that the independent activation of each receptor subtype by its specific agonist leads to an increase in the number of spine and dendritic synapses comparable to that resulting from EB administration (Sá et al., 2009).

The VMNvl contains a subset of neurons that express the ERα and/or the ERβ and, as shown by autoradiographic analysis of estradiol retention in ovariectomized rats, this population represents 40% of the VMNvl cells (Morrell et al., 1986). Therefore, by acting directly on these ER-positive neurons, estrogens might increase the number of synaptic contacts in the deafferented side, a hypothesis that the present results do not support. Consequently, the structural synaptic plasticity exhibited by VMNvl neurons in response to estrogens can only be mediated by inputs conveyed by afferents from brain regions that contain estrogen-responsive neurons. Although some of these afferents derive from cell groups that express either the ERα or the ERβ, the densest projections originate in nuclei/regions that abundantly express, or coexpress, both receptors (Shughrue et al., 1997, 1998; Shughrue and Merchenthaler, 2001). This is the case of the medial amygdala, medial preoptic area and bed nuclei of stria terminalis, all of them known to be activated by estrogens, as measured by changes in the electrical and transcriptional activity of their constituent neurons (e.g., Auger and Blaustein, 1995; Lehmann and Erskine, 2005; Wong and Moss, 1992). The lack of variations in the number of synapses in the deafferented VMNvl of EB-treated rats also shows that the adjacent hypothalamic nuclei that also contain ER-positive neurons, namely the arcuate, dorsomedial and paraventricular nuclei (Laflamme et al., 1998; Shughrue et al., 1997), are not involved in the mediation of the synaptogenic effects of estrogens in the VMNvl. This was not entirely unexpected because, although they were partially included in the hypothalamic region surgically isolated in this study, there is evidence that the projections they send to the VMN are sparse, e.g., the paraventricular and dorsomedial nuclei, or virtually none, e.g., the arcuate nucleus (Fahrbach et al., 1989).

3.3. Repercussions of VMN deafferentation on the contralateral nucleus

Earlier studies, using anterograde and retrograde tract-tracing techniques, have shown that the neural afferents to the VMN, although being largely ipsilateral, also derive from the contralateral hemisphere. The presence of decussating and/or commissural fibers has been noticed in the projections originating in the ventral subiculum (Canteras and Swanson, 1992), in the ventral premammillary nucleus (Canteras et al., 1992) and in the VMN itself (Canteras et al., 1994; Fahrbach et al., 1989; Ricciardi et al., 1996). These fibers cross the midline dorsal to the caudal part of the third ventricle, through the hippocampal, supraoptic or anterior commissures, or in the vicinity of the supramammillary decussation (Canteras and Swanson, 1992; Canteras et al., 1992; Ricciardi et al., 1996) and, thus, were severed by the surgical cuts done to isolate the VMN. The possibility of a partial deafferentation of the contralateral VMNvl is indeed suggested by the observation that, in the absence of estrogenic stimulation as well as after estrogen priming, the total number of synapses in the contralateral VMNvl was intermediate between that of the control and of the deafferented VMNvl, and the differences were, in all cases, statistically significant. Yet, despite the partial deafferentation, the contralateral VMNvl still displayed the ability to respond to estrogens by increasing the number of synaptic contacts received by its neurons. The relative increase was slightly, but significantly, smaller than that noticed in the control VMNvl, for dendritic as well as for spine synapses. Therefore, our results support the view that the VMN afferents that originate in the contralateral hemisphere are quantitatively important for maintaining the basal pattern of neuronal connectivity in the VMNvl, and suggest that the afferents mediating the synaptogenic influence of estrogens upon VMNvl neurons originate, for the most part, ipsilaterally.

3.4. Neural afferents and progesterone receptors

Neural afferents also seem to play a role in determining the basal number of PRs in the VMNvl. In fact, the results obtained in oil-treated rats show that the total number of PR-immunoreactive neurons is 1.8 and 2.7 times, respectively, higher in the control VMNvl than in the contralateral and deafferented VMNvl. Yet, these differences were overridden by estrogenic stimulation as shown by the similarity of the neuron numbers estimated in the control, deafferented and contralateral VMNvl of EB-treated rats. These findings clearly demonstrate that, in sharp contrast with the

synaptogenic effects of estrogens, the induction of PRs is not mediated by neural afferents, but instead results from the local action of estrogens. In the control VMNvl, EB administration induced a 3.5-fold increase in the number of PR-immunoreactive neurons, a variation that coincides with that reported for PR mRNA levels in estrogen-treated ovariectomized rats (Lauber et al., 1991), but is smaller than that noticed in the levels of progestin binding in VMN cytosols (Brown et al., 1987; Parsons et al., 1982). However, in the contralateral and in the deafferented VMNvl, EB treatment caused a larger increase (7 and 10 times, respectively) in the number of PR-immunoreactive neurons, thus annulling the differences noticed in the absence of estrogenic stimulation.

The comparison of the data obtained in this study and in a previous investigation in which the total number of VMNvl neurons was estimated using the same stereological method (Madeira et al., 2001), reveals that about 75% of the VMNvl neurons have the capability of expressing PRs after estrogen priming. The authors are not aware of any previous study reporting estimates of total numbers of PR-immunoreactive neurons in the VMNvl. As to the ER-positive neurons, it was shown that the total number of neurons immunoreactive for ERα is below 1,000 both in oil- and estrogen-treated rats (Chakraborty et al., 2003), a number that does not fit comfortably with the earlier observation that about 40% of the VMN cells concentrate estradiol (Morrell et al., 1986). Therefore, assuming that virtually all neurons expressing PRs also contain ERs, as previously reported in studies of the guinea pig brain (Blaustein and Turcotte, 1989; Warembourg et al., 1989), the number of PR-immunoreactive neurons found in the present study is surprisingly high. This finding might indicate that, in addition to the ERα other receptors might be involved in the estrogenic induction of PRs in the VMNvl (Harris et al., 2002; Kudwa and Rissman, 2003; Musatov et al., 2006), a hypothesis already rose by other authors after the observation of estrogen-induced PR immunoreactivity in the VMN of ERα knockout mice (Kudwa and Rissman, 2003; Moffat et al., 1998).

3.5. Conclusion

The results of the present investigation demonstrate that the two main fingerprints of estrogen action in the VMNvl, the generation of new synaptic contacts and the induction of PRs, are mediated by different mechanisms. The synaptic plasticity induced by estrogens is trans-synaptically mediated by inputs from the neural afferents to the VMN, whereas the induction of PR results from the local action of estrogens.

4. Experimental procedures

4.1. Animals and treatments

Female Wistar rats derived from the colony of rats maintained at the Institute of Molecular and Cell Biology, in Porto (Portugal) were maintained on a 12:12 hr light/dark cycle (lights on at 07:00 hr) and ambient temperature of 23 ºC, with free access to food and water. Estrous cycles were monitored daily by vaginal smear cytology; only females exhibiting at least two consecutive 4- to 5-day cycles were used. At 10 weeks of age, rats were ovariectomized bilaterally under deep anesthesia induced by subsequent injections of promethazine (0.4 ml/Kg body weight, subcutaneous), xylazine (0.132 ml/kg body weight, intramuscular) and ketamine (0.5 ml/kg body weight, intramuscular). Ten days later, half of the rats were anesthetized again and placed in a stereotaxic apparatus for unilateral VMN deafferentation. Six hours after surgery, rats were injected subcutaneously with either EB (10 μg dissolved in 0.1 ml sesame oil; all from Sigma-Aldrich Company Ltd., Madrid, Spain) or sesame oil (0.1 ml); these treatments were repeated 24 hr later. Age-matched ovariectomized rats that received the same EB or sesame oil injections, but were not submitted to VMN deafferentation, were used as controls. All groups consisted of 6 rats. Because in an early study (Nishizuka and Pfaff, 1989), it was shown that there is no further loss of synapses in the VMN 2 days after deafferentation, we performed all studies in rats that were killed 3 days after deafferentation.Therefore, 48 hr after the second EB or vehicle injection, rats were deeply anesthetized with 3 ml/kg body weight of a solution containing sodium pentobarbital (10 mg/ml) and chloral hydrate (40 mg/ml) given intraperitoneally and transcardially perfused with a fixative solution containing either 1% paraformaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer, pH 7.2 (brains processed for electron microscopy) or 4% paraformaldehyde in phosphate buffer, pH 7.6 (brains processed for immunocytochemistry). After removal of the brains, the uteri were surgically isolated and weighed.

All studies were performed in accordance with the European Communities Council Directives of 24 November 1986 (86/609/EEC) and Portuguese Act nº129/92.

4.2. Unilateral surgical deafferentation of the VMN

The method followed for VMN deafferentation was adapted from Halász and Pupp (1965), with some modifications aiming at preserving the integrity of the contralateral hemisphere. Accordingly, the surgical isolation of the right VMN was performed by using two types of knife: a Halász-type knife, with a 1.4 mm horizontal blade and a 2.8 mm vertical blade, and a smaller knife, with a 1.5 mm long blade, made from a razor and attached to a guide shaft. Rats were placed in a stereotaxic apparatus with bregma and lambda in the same horizontal plane. A midline incision was done to expose the calvaria, and a window was opened on the right side of the skull. The Halász-type knife was lowered vertically for 9.5 mm at the following coordinates (Paxinos and Watson, 1998): 3 mm posterior to the bregma and 1.7 mm lateral to the midline. The knife was then moved 2 mm caudally, returned on the same course to its initial position and, afterwards, moved 2 mm rostrally. After returning to its original position, the knife was slowly withdrawn. To sever the VMN neural connections in the coronal plane, the smaller knife was lowered for 9.5 mm at two different points, both located 0.2 mm lateral to the midline: the rostral surgical cut was done 1 mm posterior to the bregma and the caudal one 5 mm posterior to the bregma.

4.3. Hormonal determinations

Blood samples (2000 μl) were taken directly from the heart, prior to perfusion, into Eppendorf tubes. The samples were left at 4 ºC to allow the complete clot formation. Each sample was then centrifuged twice at 2000 rpm for 10 min. Serum was removed, collected in aliquots and stored undiluted at -80 ºC until further analysis. Estradiol and progesterone serum levels were assayed using a solid-phase competitive chemiluminescent enzyme immunoassay kit for IMMULITE 1 (Siemens Medical Solutions Diagnostics, Amadora, Portugal), with an analytical sensitivity of 15 pg/ml for estradiol and 0.1 ng/ml for progesterone.

4.4. Tissue processing for electron microscopy

The brains were removed from the skulls and immersed in the fixative solution for 1 hr. They were then transected in the coronal plane through the anterior border of the optic chiasm, rostrally, and the posterior limit of the mammillary bodies, caudally. The blocks of tissue containing the hypothalamus were included in a 1.5% agar solution in order to preserve the topographical relationship of the deafferented VMN with the adjacent brain tissue. Alternate 40 and 500 μm-thick sections were obtained in the coronal plane from these agar-embedded blocks. The 40 μm-thick sections were mounted on slides and stained with Giemsa. They were used for light microscopic identification of the location of the knife cuts and visualization of the precise location of the VMNvl. The brains in which the VMN was not intact or completely isolated were discharged. The VMNvl from the right (deafferented) and left (contralateral) hypothalami were isolated under microscope observation from the 500 μm-thick sections and processed for electron microscopy, as follows. They were postfixed with a 2% solution of osmium tetroxide in 0.12 M phosphate buffer, dehydrated through graded series of ethanol solutions, stained in 1% uranyl acetate and, after passage through propylene oxide, embedded in Epon as previously described (Sá and Madeira, 2005a,b; Sá et al., 2009). From each Epon-embedded block, two sets of 8 serial 2μm-thick sections were cut. Each semithin section was placed on a gelatin-coated microscope slide and stained with toluidine blue. Then, the tissue from each block was trimmed into a pyramidal shape and several ribbons of 8-10 serial ultrathin sections were cut, collected on Formvar-coated grids, and double-stained with uranyl acetate and lead citrate.

4.5. Tissue processing for immunocytochemistry

The brains were removed from the skulls, immersed in the fixative solution for 2 hr (4 ºC) and, then, transferred to a solution of 10% sucrose in phosphate buffer (4 ºC), where they were maintained overnight. After, they were trimmed and embedded in agar for the reasons given above. The agar-embedded blocks were mounted on a Vibratome with the rostral surface up and sectioned at 40 μm throughout its entire length. One set of sections, formed by sampling sections at regular intervals of 120 μm (one out of 3) throughout the rostrocaudal extent of VMN, was collected in phosphate-buffered saline (PBS) and used for the immunocytochemical detection of the PR-containing neurons in the VMNvl. Due to sampling scheme used, 10-12 sections were stained, on average, per animal. Another set, composed by sections adjacent to those in the first set, was stained with Giemsa and used for assessing the location of the knife cuts and the complete isolation of the VMN under microscopic observation. Only the brains in which the VMN was completely isolated and not damaged by the surgical cuts were used. Sections used for PR immunostaining were washed twice in PBS, treated with 3% H_2O_2 for 10 min to inactivate endogenous peroxidase, washed again and blocked with 10% normal horse serum for 45 min. In order to increase tissue penetration, all immunoreactions were made in a solution of 0.5% Triton X-100 in PBS. The antiserum against PRs (Anti-Progesterone Receptor, a.a. 922-933, clone 6A - MAB462, Millipore Corporate, Billerica, USA) was used at the dilution of 1:2,000. Sections were incubated for 72 hr, at 4 °C, with the primary antiserum. Biotinylated horse IgG anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA) was used as the secondary antibody, for 1 hr, at the dilution of 1:400. Sections were then treated with avidin-biotin peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories) diluted 1:800 and incubated for at least 1 hr at room temperature. After that, sections were incubated for 80 s in 0.05% diaminobenzidine (DAB; Sigma) to which 0.01% H_2O_2 was added. Sections were rinsed with PBS for at least 15 min between each step. Stained sections were mounted on gelatin-coated slides and air-dried. They were then dehydrated in a series of ethanol solutions (50%, 70%, 90% and 100%), cleared in xylol, and coverslipped using Histomount (National Diagnostics, Atlanta, GA, USA). The immunocytochemical staining of the sections from all groups analyzed was performed in parallel at the same time. The same procedure was followed for control sections, which were incubated without primary antiserum; no immunostaining was observed in these sections.

4.6. Estimation of the number of synapses per neuron

In the present study we used the number of synapses per neuron as the main estimator of the effect of ovariectomy and estrogen treatment on the synapses received by VMNvl neurons. The use of this estimator is convenient because it enables us to overcome the bias introduced by possible changes in the volume of the VMNvl or in the number of its neurons due to the surgical deafferentation.

The estimates were performed independently in the VMNvl of the deafferented hypothalamus (right VMNvl) and of the contralateral hypothalamus (left VMNvl), and in the left VMNvl of ovariectomized control rats. The total number of synapses per neuron was computed as the sum of the number of spine and dendritic synapses per neuron. The number of spine and dendritic synapses per neuron was calculated by dividing the numerical density of each type of synapse by the numerical density of VMNvl neurons.

The numerical density (Nv) of VMNvl neurons was estimated from the series of semithin sections obtained as described above, by applying the physical disector method (Madeira and Paula-Barbosa, 1993; Sterio, 1984). Because the disector was made

from pairs of alternate sections and each section was used in turn as the reference section or the look-up section, 45 disectors were performed, on average, per nucleus. The sections were analyzed using a modified Olympus BH-2 microscope interfaced with a color video camera and equipped with a Heidenhain ND 281 microcator (Traunreut, Germany), a computerized stage, and an object rotator (Olympus, Albertslund, Denmark). A computer fitted with a framegrabber (Screen Machine II, FAST Multimedia, Germany) was connected to the monitor. By using the C.A.S.T. – Grid system software (Olympus), two counting frames equivalent in shape and area (15,947 μm^2) were superimposed onto the tissue images on the screen. The image of one section was frozen on the left half side of the screen and compared with the image of the adjacent section displayed on the right side of the screen. Neurons were counted, at final magnification of 800×, when their nuclei (the counting unit) were visible in the reference section (the right sided image), but not in the look-up section (the left sided image), within the counting frame without being intersected by the exclusion edges or their extensions. An average of 200 neurons was counted per each VMNvl.

The number of synapses per unit volume of neuropil (numerical density, Nv) was estimated by using the physical disector method applied to electron micrograph prints (Madeira and Paula-Barbosa, 1993; Sá and Madeira, 2005a; Sterio, 1984). For this purpose, two series of 7 electron micrographs of corresponding fields of the neuropil were taken at primary magnification of 5,400× from the ultrathin sections obtained as described above. The micrographs were then enlarged photographically to a final magnification of 16,200×. Disectors were made from micrographs obtained from pairs of alternate sections. Because each section was used in turn as the reference section, 40 disectors were made per animal. A transparency with an unbiased counting frame was superimposed onto the reference section micrograph. A synapse was counted whenever its postsynaptic density (the counting unit) was seen entirely or partly within the counting frame without intersecting the forbidden lines and their extensions in the reference section, but not in the look-up section. Synapses were identified by the presence of synaptic densities, at least three synaptic vesicles at the presynaptic site and a synaptic cleft (Colonnier, 1968; Gray and Guillery, 1966). Because the number of symmetrical synapses received by the dendritic trees of VMNvl neurons is very low (Field, 1972; Nishizuka and Pfaff, 1989), for the purpose of the estimations herein performed no distinction was made between symmetrical and asymmetrical synapses. The mean thickness of the ultrathin sections, estimated using the minimal fold technique (Small, 1968), was 70 nm. On average, 200 synapses were counted per animal.

4.7. Estimation of the total number of PR-immunoreactive neurons

The estimates were obtained using the computerized stereology system described above and by applying the optical fractionator method (Madeira et al., 1997; West et al., 1991) to an average of 11 sections per VMNvl analyzed. In each section, the fields of view were systematically sampled using an interframe distance of 70 μm along the x and y axes. The disector used had a counting frame area of 1482 μm^2 at the tissue level and a fixed depth of 10 μm. The estimations were performed, at final magnification of 2000×. Positive PR immunoreactivity was identified as dark brown nuclear staining. On average, 350 PR-immunoreactive cells were counted per nucleus; the mean coefficient of error (CE; Gundersen et al., 1999) of the estimates was 0.07.

4.8. Statistical analyses

The Student´s t-test for independent variables was used to examine the influence of EB treatment in hormone levels and uterine weights. A two-way analysis of variance (ANOVA) with deafferentation and EB treatment as the independent variables was applied to the remaining data. Whenever significant results were found from the overall ANOVA, pair-wise comparisons were subsequently made using the post hoc Tukey's HSD test. Differences were considered significant if $p<0.05$.

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DISCUSSÃO GERAL

Em trabalhos anteriores realizados no Instituto de Anatomia tinha-se verificado que o volume do VMNvl aumentava em cerca de 20% entre as fases de diestro 1 e de proestro, e que esta variação se devia ao aumento do volume dos corpos celulares dos seus neurónios, não sofrendo o neurópilo alterações significativas do seu volume (Madeira *et al.*, 2001). Esta plasticidade morfológica induzida pelos esteróides sexuais nas fêmeas justificava que as diferenças sexuais no volume tanto do VMNvl como dos corpos celulares dos seus neurónios também variassem ao longo do ciclo ovárico, sendo máximas quando os machos eram comparados com fêmeas em fase de diestro 1 e mínimas, no caso do volume do VMNvl, ou até mesmo nulas no caso de volume somático dos seus neurónios, quando a comparação era feita com fêmeas em fase de proestro.

O estudo quantitativo, cujos resultados constam do TRABALHO 1, permitiu demonstrar que o aumento do volume somático dos neurónios do VMNvl entre a fase de diestro 1 e a de proestro, período durante o qual as concentrações plasmáticas de estrogénios e de progesterona aumentam para atingir a fase de pico no proestro (Butcher *et al.*, 1974), se devia à sua activação metabólica. Esta conclusão baseou-se na verificação de que o volume nuclear e o volume e área dos organelos citoplasmáticos envolvidos na síntese proteica, e.g., o retículo endoplasmático rugoso e o aparelho de Golgi, aumentavam de modo significativo entre estas duas fases do ciclo ovárico. O número de poros nucleares também se encontrava aumentado, o que reforça esta interpretação dado saber-se que o número de poros nucleares está associado à actividade metabólica nuclear, designadamente à importação para o núcleo de pequenas proteínas ribonucleares e à exportação para o citoplasma de diferentes tipos de RNA (revisto Corbett *et al.*, 1997; Görlich e Kutay, 1999). Embora a comparação dos resultados obtidos nas fases de diestro 1 e proestro não tenha permitido discriminar os efeitos dos estrogénios dos da progesterona, a verificação de que as variações que ocorrem ao longo do ciclo ovárico são em tudo idênticas às provocadas pela administração de estrogénios a ratos ovariectomizados (descritos e referenciados em detalhe na Introdução desta dissertação) sugere que a progesterona não influencia, pelo menos de modo marcado, estes aspectos da morfologia dos neurónios do VMNvl.

Apesar de nas fêmeas os neurónios do VMNvl terem um corpo celular menor ou idêntico em volume, dependendo da fase do ciclo ovárico, ao dos machos, o comprimento das suas arborizações dendríticas é semelhante ou maior que nos machos quando as fêmeas estão em fase de diestro 1 ou de proestro, respectivamente, devido ao maior comprimento, nesta fase, dos dendritos terminais (Madeira *et al.*, 2001). Sabia-se também que a densidade das espinhas dendríticas era maior nas fêmeas que nos machos, independentemente da fase do ciclo ovárico, e que o seu valor aumentava em cerca de 30% entre as fases de diestro 1 e de proestro (Madeira *et al.*, 2001). Esta observação sugeria que a complexidade da organização sináptica do VMNvl era maior nas fêmeas que nos machos e, naquelas, maior quando os níveis de esteróides sexuais se encontravam em fase de pico (proestro) do que quando os seus níveis plasmáticos eram baixos (diestro 1).

É do conhecimento geral que as espinhas dendríticas são estruturas muito dinâmicas, com grande capacidade de modificar as suas dimensões, forma e número em resposta a variações da actividade neuronal e que, no cérebro adulto, são a estrutura neuronal sobre a qual se estabelece a maioria das sinapses excitatórias (para revisão, ver Harris e Kater, 1994; Calabrese *et al.*, 2006; Bourne e Harris, 2008). Porém, no VMNvl, onde os neurónios ou não possuem ou são muito pobres em espinhas dendríticas (Millhouse, 1973), cerca de 3/5 dos contactos sinápticos fazem-se sobre os troncos dendríticos (Field, 1972; Nishizuka e Pfaff, 1989) e, destes, cerca de 85% são de tipo assimétrico (Nishizuka e Pfaff, 1989) e, portanto, presumivelmente excitatórios. Por este motivo, no TRABALHO 2 procedeu-se à quantificação do número de sinapses estabelecidas sobre as espinhas dendríticas, os troncos dendríticos e os corpos celulares dos neurónios do VMNvl de ratos em fase de diestro 1 e de proestro, e também de machos. Estimou-se o número de sinapses por neurónio, parâmetro estereológico que permitiu ultrapassar as limitações de interpretação inerentes à determinação de parâmetros quantitativos intermediários, como é o caso das densidades. Estes dados permitiram corroborar a ideia de que as sinapses axodendríticas eram mais numerosas que as axospinhosas (Matsumoto e Arai, 1986b; Nishizuka e Pfaff, 1989), mas mostraram que essa predominância não era tão acentuada como estes autores tinham sugerido com base na determinação de densidades de superfície. Para estes autores, o número de sinapses axodendríticas era 3 a 4 (Matsumoto e Arai, 1986b) ou 2 (Nishizuka e Pfaff, 1989) vezes superior ao das sinapses axospinhosas, mas a estimativa do número de sinapses por neurónio mostrou que os contactos sinápticos sobre os troncos dendríticos são apenas 1,5 vezes mais

numerosos que os estabelecidos sobre as espinhas dendríticas. Apenas nos machos o número de sinapses axodendríticas era cerca de 2 vezes superior ao das sinapses axospinhosas. Os dados obtidos permitiram ainda concluir que as sinapses axossomáticas, que são predominantemente inibitórias, são em número muito reduzido, representando apenas 2-3% do número total de sinapses estabelecidas sobre cada neurónio do VMNvl.

Os resultados deste estudo permitiram também demonstrar que o aumento dos níveis de esteróides sexuais entre a fase de diestro 1 e a de proestro provoca um aumento de aproximadamente 40% no número total de sinapses por neurónio, sendo semelhante a contribuição das sinapses axospinhosas e axodendríticas para este aumento. Embora sendo expectáveis, devido aos relatos existentes na literatura sobre as variações no número de espinhas dendríticas em resposta aos estrogénios (Frankfurt *et al.*, 1990; McEwen e Wooley, 1994; Calizo e Flanagan-Cato, 2000; Madeira *et al.*, 2001), estes resultados permitiram demonstrar pela primeira vez que o aumento dos níveis circulantes de esteróides sexuais se associa à formação de novos contactos sinápticos sobre as espinhas dendríticas dos neurónios do VMNvl. De facto, os resultados obtidos por Nishizuka e Pfaff (1989) mostravam que a administração de 17β-estradiol a ratos ovariectomizados não alterava a densidade de sinapses axospinhosas e levava apenas a um aumento discreto da densidade das sinapses axodendríticas.

Verificou-se também que o número de sinapses axossomáticas era maior nas fêmeas em proestro que em diestro 1, mas esta variação era de 32% e, portanto, proporcionalmente menor que a observada nos restantes tipos de sinapses. Dado que no VMNvl praticamente todos os contactos sinápticos sobre as espinhas dendríticas e a grande maioria dos que se estabelecem sobre os troncos dendríticos são excitatórios, os resultados que constam do TRABALHO 2 permitiram concluir que o aumento dos níveis circulantes de esteróides sexuais entre as fases de diestro 1 e de proestro do ciclo ovárico está associado a um aumento importante das influências excitatórias exercidas sobre os neurónios do VMNvl.

Curiosamente, nos machos, as sinapses axodendríticas e axospinhosas eram em número idêntico ao das fêmeas em diestro 1, ao contrário das axossomáticas cujo número não diferia significativamente do das fêmeas em proestro. Por outro lado, a dimensão das sinapses e a percentagem de plasmalema ocupada por estas densidades eram significativamente maiores nos machos, sobretudo no caso dos contactos sinápticos estabelecidos sobre os somas. Estas observações demonstram que o padrão de organização sináptica do VMNvl é sexualmente dimórfico e sugerem que sobre os neurónios dos machos se fazem sentir menos

influências excitatórias que sobre os das fêmeas, independentemente da fase do ciclo ovárico em que estas se encontrem.

Sabe-se que os esteróides sexuais levam à formação e à perda cíclica de sinapses não só no VMNvl, mas também em áreas que não têm conotação funcional com a reprodução, como é o caso da formação do hipocampo. Os estudos realizados nesta região revelaram que os dois esteróides sexuais mais abundantes nas fêmeas, os estrogénios e a progesterona, estão ambos envolvidos na variação do número de sinapses que ocorre em cada ciclo ovárico. Com efeito, foi demonstrado que a administração de estrogénios a ratos adultos ovariectomizados estimula a formação de novos contactos sinápticos sobre as espinhas dendríticas dos neurónios da região CA1 do hipocampo (Woolley e McEwen, 1992) e que a subsequente administração de progesterona provoca, numa fase inicial, aumento do número de espinhas dendríticas e ulteriormente, 12 a 24 horas mais tarde, redução do número de sinapses cuja formação tinha sido induzida pela administração de estrogénios (Gould *et al.*, 1990; Woolley e McEwen, 1993). Pelo contrário, no núcleo arqueado onde o número de sinapses axossomáticas também flutua ao longo do ciclo ovárico, com valores mínimos na fase de estro e máximos na de metestro, verificou-se que os níveis circulantes de estrogénios eram o único factor regulador desta variação (Naftolin *et al.,* 1996). Estas observações são particularmente interessantes porque, ao contrário do hipocampo, onde apenas um número moderado de células expressa o mRNA dos receptores para a progesterona, no núcleo arqueado o número de neurónios positivos para o mRNA destes receptores é elevado (Hagihara *et al.,* 1992).

Apesar de no VMNvl a expressão de receptores para a progesterona ser, tal como no núcleo arqueado, elevada (Warembourg *et al.*, 1986; Olster e Blaustein, 1990; Hagihara *et al.*, 1992), sobretudo após exposição aos estrogénios (Parsons *et al.*, 1982), a progesterona não está envolvida nas variações que ocorrem ao longo do ciclo ovárico no número de sinapses estabelecidas sobre os neurónios do VMNvl. Com efeito, os resultados apresentados no TRABALHO 3 mostram que a administração de progesterona a ratos ovariectomizados não altera o número de sinapses relativamente ao observado em ratos injectados apenas com veículo, e que a administração de progesterona após tratamento com benzoato de estradiol não modifica o número de sinapses relativamente ao detectado em ratos injectados apenas com benzoato de estradiol. A observação de que a administração de benzoato de estradiol precedida de RU486, um bloqueador dos receptores da progesterona, provocava um aumento

do número de sinapses idêntico ao resultante da administração isolada de benzoato de estradiol veio reforçar a conclusão de que a progesterona não é um factor regulador da organização sináptica do VMNvl, apesar de ser essencial, em condições fisiológicas, para a expressão do comportamento sexual feminino.

Tendo-se concluído que a formação e a perda cíclica de sinapses no VMNvl depende das variações que ocorrem ao longo do ciclo ovárico nos níveis circulantes de estrogénios, importava analisar se estas hormonas actuariam através da activação dos clássicos receptores nucleares de estrogénios e identificar qual das isoformas, o ERα ou o ERβ, estaria envolvida nessa acção. A verificação de que a administração dos agonistas específicos de cada um dos receptores, o PPT e o DPN respectivamente, aumentava o número de sinapses axodendríticas e axospinhosas para valores idênticos aos resultantes da administração de doses fisiológicas de benzoato de estradiol mostrou que o mecanismo de acção destas hormonas é genómico e mediado pela activação de ambas as isoformas dos receptores nucleares de estrogénios.

Embora se saiba desde os estudos realizados com métodos autorradiográficos, no início dos anos 70, que os neurónios do VMNvl possuem receptores para os estrogénios (Pfaff e Keiner, 1973), só recentemente, e após identificação das duas isoformas destes receptores, se verificou que os neurónios do VMNvl expressam abundantemente quer o mRNA quer a proteína do ERα, sendo relativamente escassa a expressão do mRNA do ERβ, quer isoladamente quer em colocalização com o ERα (Shugrue *et al.*, 1997, 1998; Shughrue e Merchanthaler, 2001). Por este motivo, a observação de que a administração de DPN, o agonista específico dos receptores de tipo β, provocava um aumento do número de sinapses que não diferia significativamente do resultante da administração quer do agonista do ERα, PPT, quer de benzoato de estradiol, sugeria que a influência dos estrogénios na plasticidade sináptica exibida pelos neurónios do VMNvl não era mediada pela activação dos receptores expressos pelos neurónios do VMNvl e apontava para um provável envolvimento das aferências ao VMN provenientes de núcleos/regiões que, no seu conjunto, expressassem as duas isoformas dos receptores de estrogénios.

As experiências levadas a cabo em ratos que, após ovariectomia, foram submetidos a deaferenciação cirúrgica do VMN, e que estão descritas em detalhe no TRABALHO 4, demonstraram que a integridade das aferências nervosas ao VMN é fundamental para que os

estrogénios promovam a formação de novas sinapses no VMNvl. Com efeito, os resultados aí descritos mostram que a administração de doses fisiológicas de benzoato de estradiol a ratos cujo VMN tinha sido previamente deaferenciado não produz qualquer variação no número de sinapses axodendríticas e axospinhosas, sendo o número de sinapses nestes ratos idêntico ao dos injectados com veículo. Os dados obtidos neste TRABALHO mostraram também que a perda de sinapses provocada pela deaferenciação do VMN era cerca de 2 vezes superior para as sinapses axospinhosas do que para as axodendríticas, o que permite concluir que embora os terminais axonais envolvidos na formação de sinapses tenham origem intrínseca e extrínseca ao VMN, i.e., pertençam a interneurónios e a neurónios de projecção, o principal alvo das aferências extrínsecas ao VMNvl são as espinhas dendríticas dos seus neurónios. Estes dados sugerem que estímulos conduzidos pelas aferências que mediam a influência dos estrogénios na plasticidade estrutural sináptica patenteada pelos seus neurónios em resposta aos estrogénios são sobretudo de natureza excitatória.

Outro sinal paradigmático da plasticidade induzida pelos estrogénios no VMNvl é a indução de receptores para a progesterona. Durante o ciclo ovárico, a expressão do comportamento sexual feminino requer a exposição sequencial do VMN aos estrogénios e à progesterona (Boling e Blandau, 1939; Rubin e Barfield, 1983; Ogawa *et al.*, 1994). Embora se tenha demonstrado experimentalmente que a administração de doses suprafisiológicas de estradiol é suficiente para a expressão dos comportamentos típicos da fase receptiva (copulatória) do comportamento sexual feminino, designadamente o reflexo de lordose (Brandling-Bennett *et al.*, 1999), a expressão dos comportamentos proceptivos (paracopulatórios), também eles parte integrante do comportamento sexual das fêmeas e necessários à reprodução, requerem a acção da progesterona (Fadem *et al.*, 1979; Tennent *et al.*, 1980).

Apesar da formação de novas sinapses e da expressão de receptores para a progesterona serem ambos sinais indiciadores de estimulação estrogénica do VMNvl e serem, portanto, ambos presumivelmente necessários à sua normal funcionalidade, os mecanismos subjacentes à sua expressão são, no entanto, marcadamente distintos. De facto, as observações realizadas em ratos ovariectomizados e depois submetidos a deaferenciação do VMN, e cuja descrição detalhada consta do TRABALHO 4, mostraram que a deaferenciação não interfere com o aumento, induzido pela administração de doses fisiológicas de benzoato de estradiol, do número total de neurónios do VMNvl que são imunorreactivos para o receptor da

progesterona. Daqui se pode concluir que, ao invés do que acontece com o padrão de organização sináptica dos neurónios do VMNvl, os estímulos veiculados pelas aferências nervosas ao VMN não estão envolvidos na mediação da indução pelos estrogénios da expressão de receptores para a progesterona.

Em resumo, com o conjunto de trabalhos incluídos na presente dissertação demonstrou-se que as variações que ocorrem ao longo do ciclo ovárico na anatomia do VMNvl reflectem os efeitos dos estrogénios na complexidade da sua organização estrutural e no estado de activação dos seus neurónios. Demonstrou-se, também, que os efeitos dos estrogénios sobre a organização sináptica do VMNvl são indirectos e exercidos por via trans-sináptica, através da activação de neurónios que projectam para o VMN e que, por possuírem receptores para os estrogénios de tipo α e/ou β, são sensíveis a estas hormonas. Por fim, demonstrou-se que a indução pelos estrogénios da expressão de receptores para a progesterona pelos neurónios do VMNvl, necessária para a manifestação do reflexo de lordose, não é influenciada pelas aferências neuronais mas, pelo contrário, resulta da acção directa dos estrogénios sobre os neurónios do VMNvl.

CONCLUSÕES

Os resultados obtidos permitiram demonstrar que

- as variações ao longo do ciclo ovárico nas dimensões do VMNvl reflectem modificações do estado de activação dos seus neurónios. E,

- na presença de elevadas concentrações de esteróides sexuais os organelos citoplasmáticos envolvidos na síntese proteica estão hipertrofiados e o tráfico núcleo-citoplasmático aumentado, traduzindo um estado de estimulação neuronal. De igual modo,

- o aumento das concentrações plasmáticas de esteróides sexuais está associado a um aumento marcado do número de sinapses estabelecidas sobre os neurónios do VMNvl, que na sua maioria são de tipo assimétrico e, logo, maioritariamente excitatórias. Acresce que,

- a formação de novas sinapses em cada ciclo ovárico é da responsabilidade dos estrogénios e não da acção conjunta dos estrogénios e da progesterona. E,

- a acção estimuladora dos estrogénios sobre o padrão de organização sináptica dos neurónios do VMNvl é de natureza genómica e mediada pela activação dos receptores de estrogénios α e β. Porém,

- a acção estimuladora dos estrogénios sobre o padrão de organização sináptica dos neurónios do VMNvl é indirecta e mediada pelas aferências ao VMNvl que provêm de núcleos/regiões que expressam receptores para os estrogénios. Pelo contrário,

- a indução pelos estrogénios dos receptores para a progesterona, necessários à normal expressão do comportamento sexual feminino, resulta da acção local dos estrogénios e não é influenciada pelas aferências neuronais.

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RESUMO

Com os resultados constantes desta dissertação pôde caracterizar-se a influência dos esteróides sexuais na indução da plasticidade neuronal manifestada pela divisão ventrolateral do núcleo ventromedial do hipotálamo (VMNvl) ao longo do ciclo ovárico e identificar os mecanismos subjacentes à acção neurotrófica dos estrogénios. Os estudos foram realizados em ratos jovens da estirpe Wistar. As variações da morfologia e do padrão de conectividade dos neurónios do VMNvl durante o ciclo ovárico foram analisadas, a nível ultrastrutural, em ratos em fase de diestro 1 e de proestro. A caracterização da influência dos estrogénios e da progesterona na indução de plasticidade sináptica foi conseguida através da quantificação dos contactos sinápticos estabelecidos sobre os neurónios do VMNvl em ratos ovariectomizados tratados com estradiol e progesterona, isoladamente ou em associação, e com o antagonista dos receptores da progesterona RU486. A identificação da isoforma dos receptores de estrogénios envolvida na mediação dos efeitos sinaptogénicos desta hormona foi efectuada em ratos ovariectomizados tratados com o agonista específico dos receptores de tipo α ou de tipo β. A importância das aferências neuronais na mediação dos efeitos dos estrogénios quer na formação de novas sinapses quer na indução de receptores para a progesterona foi estudada em ratos ovariectomizados que, após deaferenciação cirúrgica do VMN, foram tratados com estradiol.

Os resultados obtidos mostram que as dimensões dos organelos citoplasmáticos e o número de poros nucleares e de sinapses estabelecidas sobre os neurónios do VMNvl se correlacionam positivamente com os níveis circulantes de esteróides sexuais, sendo este o factor determinante da flutuação, ao longo do ciclo ovárico, da expressão de diferenças sexuais em vários parâmetros morfológicos do VMNvl. Verificou-se também que a progesterona não tem funções sinaptogénicas no VMNvl, sendo a formação cíclica de sinapses promovida exclusivamente pelos estrogénios. A semelhança entre os efeitos sinaptogénicos do estradiol e os dos agonistas específicos dos receptores de tipo α e β permitiu concluir que esta acção dos estrogénios é mediada pela activação dos seus receptores nucleares. Porém, a observação de que a deaferenciação do VMN impedia a formação de novas sinapses pelo estradiol mostrou que os efeitos sinaptogénicos dos estrogénios no VMNvl são indirectos e mediados pelos estímulos veiculados por aferentes com origem em regiões do SNC sensíveis aos estrogénios. Por fim, a verificação de que a deaferenciação do VMN não interferia com a indução pelo estradiol dos receptores da progesterona revelou que esta acção dos estrogénios, necessária para a expressão do comportamento sexual feminino, é exercida directamente sobre os neurónios do VMNvl.

ABSTRACT

The studies incorporated in the present thesis contributed to clarify the role of sexual steroids in inducing the plastic changes displayed by neurons of the ventrolateral division of the hypothalamic ventromedial nucleus (VMNvl) across the ovarian cycle and to identify the mechanisms by which estrogens exert this neurotrophic action. The studies were performed in young Wistar rats. The variations in the morphology and connectivity pattern of VMNvl neurons across the ovarian cycle were analyzed, at the ultrastructural level, in rats at diestrus 1 and proestrus phases of the ovarian cycle. The quantification of the synaptic contacts established upon the VMNvl neurons of ovariectomized rats treated with estradiol or progesterone, alone or in association with RU486, allowed to characterize the effects of estrogen and progesterone in the induction of synaptic plasticity. In order to sort out the estrogen receptor isoform through which estrogens act to induce synaptogenesis, ovariectomized rats were treated with the specific agonists of the estrogen receptors α and β. To assess the role of neuronal afferents as mediators of the estrogen influence in the induction of new synapses and in the expression of progesterone receptors in the VMNvl, studies were done in ovariectomized rats that, after VMN deafferentation, were treated with estradiol.

The results showed that there is a positive correlation between the circulating levels of sex steroid hormones and the size of the cytoplasmic organelles involved in protein synthesis and the number of nuclear pores and synapses established upon VMNvl neurons. In addition, they evinced that these effects are the leading cause of the variations in the magnitude of the sexual dimorphisms displayed by several morphological features of the VMNvl along the ovarian cycle. Data obtained also demonstrated that progesterone does not promote the formation of new synapses in the VMNvl, and that the cyclic variations that occur in the number of VMNvl synapses over the estrous cycle is exclusively dependent on estrogen actions. The finding that the synaptogenic effects of estradiol and of the specific agonists of the α and β subtypes of estrogen receptor were very similar allowed to draw the conclusion that these estrogen effects are mediated by the activation of its nuclear receptors. Moreover, the observation that VMN deafferentation prevented the estradiol-induced formation of new synapses showed that the synaptogenic effects of estrogens in the VMNvl are indirect and mediated by the neural afferents originating from regions of the brain that are estrogen-sensitive. Lastly, the finding that VMN deafferentation did not interfere with the induction of progesterone receptors by estradiol showed that to promote this effect, known to be critical for the induction of the female sexual behavior, estrogens act directly upon VMNvl neurons.