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THESE

POUR L'OBTENTION DU GRADE DE
DOCTEUR DE L'UNIVERSITE EN
SCIENCES DE LA VIE ET DE LA SANTE

PAR

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CARACTERISATION CHEZ LES HIRUDINEES DE NEUROPEPTIDES SUSCEPTIBLES D'INTERVENIR SUR L'OSMOREGULATION.

LOCALISATION IMMUNOCYTOCHIMIQUE
DOSAGE - PURIFICATION - STRUCTURE

Présentée le 3 février 1993 devant la Commission d'examen :

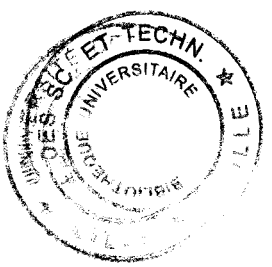
Président
Rapporteurs

Examinateurs

A Béatrice

A mes parents

A mes amis



La prévision est toujours très difficile, surtout lorsqu'elle concerne le futur

Niels Bohr

Annexe 1

Sigles servant à la dénomination des peptides de Vertébrés

AVP = Arginine-vasopressine.
A II = Angiotensine II.
LVP = Lysine-vasopressine.
MSH = Melanophore stimulating hormone.
NP = Neurophysine.
OT = Ocytocine.
PLGa = Prolyl-leucyl-Glycinamide.
TA = Acide tocinoïque.
VT = Vasotocine.

Sigles servant à la dénomination des peptides d'Invertébrés

CDCH = Caudo-dorsal cell hormone (hormone de ponte de *Lymnaea stagnalis*).
LC = Lysine conopressine.
RFa = Arg-Phe-amide.
SISP = Sodium influx stimulating peptide.

Annexe 2

Codes de 3 et de 1 lettres utilisés pour la dénomination des acides aminés.

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Acide aspartique	Asp	D
Cystéine	Cys	C
Glutamine	Gln	E
Acide glutamique	Glu	Q
Acide pyroglutamique	pGlu	pQ
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Méthionine	Met	M
Méthionine sulfoxyde	Met(O)	M(O)
Phénylalanine	Phe	F
Proline	Pro	P
Sérine	Ser	S
Thréonine	Thr	T
Tryptophane	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

SOMMAIRE

SOMMAIRE

INTRODUCTION

Ces dernières années ont connu une véritable explosion des connaissances relatives aux neuropeptides. Ces molécules sont largement répandues dans tout le règne animal y compris chez les Protozoaires (Roth et Le Roith, 1984). Des peptides identiques ou très proches se retrouvent à la fois chez les Invertébrés et chez les Vertébrés ce qui suppose qu'ils sont apparus très tôt au cours de l'évolution et que certains d'entre eux sont restés particulièrement stables. L'apparition de ces molécules aurait précédé celle du système nerveux qui les aurait alors utilisées dans les communications intercellulaires. La transmission synaptique ne serait apparue que secondairement (Scharrer, 1990). Chez les premiers Métazoaires, la coordination des différentes fonctions physiologiques aurait été sous la dépendance exclusive du système nerveux jusqu'à l'apparition, au cours de l'évolution, des glandes endocrines. Une telle situation se retrouve encore chez les Annélides où aucune glande endocrine n'a été mise en évidence jusqu'à présent. Les Annélides, premiers êtres cœlomates triblastiques bien organisés, occupent ainsi une position clef dans l'arbre évolutif ayant donné naissance aux cœlomates supérieurs. L'étude de leurs neuropeptides présente donc une grande importance phylogénique pour la compréhension des mécanismes par lesquels ces molécules se sont diversifiées et spécialisées au cours de l'évolution. Dans ce groupe des Annélides, le système nerveux central des Hirudinées est très étudié en raison de son organisation particulièrement favorable. Comparé à celui des Vertébrés, il comporte un nombre réduit de neurones (10^4), de plus la grande taille de certains d'entre-eux permet l'implantation d'électrodes et la réalisation d'injections intracellulaires.

Au niveau de la chaîne nerveuse, la métamérisation entraîne la répétition (x32) stéréotypée d'une même unité de base : le ganglion segmentaire formé d'environ 400 cellules. Les mêmes neurones se retrouvent d'un ganglion à un autre chez une même sangsue. Il faut néanmoins noter que les deux ganglions génitaux contiennent des cellules surnuméraires (Macagno, 1980). Du fait de la simplicité de ce système nerveux, et des facilités expérimentales qu'il offre, les caractéristiques morphologiques et physiologiques de plus de la moitié des neurones de la chaîne nerveuse ventrale sont dès à présent connues. Un des buts poursuivis par les chercheurs travaillant sur ce matériel biologique est l'établissement de tout le réseau neuronal de ces animaux. Des fonctions physiologiques contrôlées par les ganglions segmentaires comme la nage, le battement cardiaque et la nutrition sont déjà bien explorées (Sawyer, 1986).

Alors que les connaissances sont très avancées sur la chaîne nerveuse, le cerveau reste mal connu. Riche en cellules neurosécrétrices, il contrôle par

l'intermédiaire de neurohormones d'importantes fonctions physiologiques : la maturation génitale (Hagadorn *et al.*, 1963 ; Damas, 1968 ; Malecha, 1979 ; Kulkarni, 1980 ; Nagabhushanam et Kulkarni, 1980 ; Webb, 1980 ; Webb et Omar, 1981), le changement de coloration (Gersch et Richter, 1961), la consommation d'oxygène (Kulkarni *et al*, 1978a, b) et l'osmorégulation (Rosça *et al.*, 1958 ; Czechowicz, 1968 ; Rosça, 1972 ; Kulkarni et Nagabhushanam, 1978 ; Kulkarni *et al.*, 1979 ; Malecha, 1979, 1983). Cette dernière fonction fait l'objet de nos recherches chez la sangsue Rhynchobdelle *Theromyzon tessulatum*, espèce parasite des oiseaux aquatiques. Le maintien de l'équilibre hydrominéral chez cet animal met constamment en jeu des hormones de type diurétique et antidiurétique. Cependant, ces substances sont produites en plus grande abondance dans deux circonstances : après un repas de sang et au cours de la maturation génitale qui suit le troisième et dernier repas de sang. Ainsi, après un repas, l'eau et les ions en excès dans le sang sont excrétés en quelques heures, la quantité d'urine éliminée dans les 24 heures suivant une ingestion de sang est 8 fois supérieure à la normale (Zerbst-Boroffka, 1973). D'autre part, la gamétogenèse s'accompagne d'une importante rétention d'eau (l'animal peut doubler de volume) sous le contrôle d'une hormone anti-diurétique (Malecha, 1979, 1983) apparentée immunologiquement à l'ocytocine mammalienne (Malecha *et al.*, 1989a).

L'utilisation d'anticorps polyclonaux dirigés contre des peptides de Mammifères a permis de mettre en évidence dans le cerveau de *T. tessulatum* (Verger-Bocquet *et al.*, 1988) des cellules possédant des épitopes portés par des peptides intervenant dans l'osmorégulation chez les Vertébrés [l'angiotensine II (AII), la lysine-vasopressine (LVP) et l'ocytocine (OT)]. Ces peptides ont généralement une origine très ancienne et leur activité vis-à-vis d'une même fonction physiologique peut s'être quelquefois maintenue au cours de l'évolution (Mühlethaler *et al.*, 1984 ; Vincent et Simonnet, 1986). Il est cependant bien connu qu'il est impossible, sur des bases uniquement immunocytochimiques, d'identifier un peptide et de spéculer sur sa fonction physiologique (Veenstra, 1988).

Le but de notre travail a été l'isolement et la caractérisation biochimique des molécules susceptibles d'intervenir sur l'osmorégulation des Hirudinées. Cela a tout d'abord nécessité la mise au point de techniques de dosage ELISA fiables et sensibles afin d'évaluer les quantités de substances apparentées à l'OT, à l'AII, à la LVP et au FMRF-amide présentes aux différentes étapes de la vie des animaux, ceci afin de prélever le système nerveux central au moment le plus propice aux extractions biochimiques. Cette étude a montré que les quantités de substances immunoréactives présentes chez *T. tessulatum* sont généralement faibles. Le

nombre de sangsues nécessaires pour l'isolement d'une quantité suffisante des différentes molécules est incompatible avec la production de notre élevage de *T. tessulatum* (5 000 animaux par an) ou avec des récoltes dans la nature du fait de la rareté de l'espèce. A ce stade de notre travail, nous avons donc envisagé d'effectuer les extractions biochimiques sur une autre Hirudinée. En partant de la constatation maintenant bien établie que les neuropeptides fonctionnels sont souvent stables au cours de l'évolution (Scharrer, 1987, 1990), nous avons émis l'hypothèse que dans un groupe zoologique aussi homogène que celui des Hirudinées les molécules jouant un rôle physiologique important sont vraisemblablement les mêmes ou sont tout au moins très proches d'une espèce à une autre. Notre choix s'est porté sur *Erpobdella octoculata,* espèce particulièrement abondante en Europe et chez laquelle les dosages ELISA ont montré en moyenne 10 fois plus de substances immunoréactives que chez *T. tessulatum*. Cependant, avant de poursuivre notre travail, la validité de notre hypothèse a été vérifiée en montrant que des extraits bruts ou purifiés des molécules OT-like et AII-like d'*E. octoculata* injectés chez *T. tessulatum* entraînent une réponse physiologique (Salzet *et al.*, 1992b, 1992d, sous presse ; Salzet *et al.*, en préparation 1), et que le comportement chromatographique des substances reconnues par l'anti-OT et l'anti-AII est identique pour les deux espèces de sangsues considérées (Salzet *et al.*, en préparation 4). Les présomptions en faveur de molécules identiques à la fois chez *T. tessulatum* et *E. octoculata* étant très fortes, cette dernière espèce est devenue notre "matériel biochimique".

Selon l'hypothèse émise par Scharrer (1987), les neuropeptides actuels dérivent de précurseurs protéiques ancestraux qui, étape par étape, ont développé au cours de l'évolution la capacité de libérer des principes actifs (*i.e.* des peptides). Ce processus pourrait avoir une certaine analogie avec les différentes étapes de maturation des précurseurs de neuropeptides actuels. Ces molécules ancestrales n'existent plus, cependant d'un point de vue phylogénique la connaissance des précurseurs des peptides d'Annélides peut apporter des informations sur les mécanismes par lesquels ces molécules se sont diversifiées et spécialisées au cours de l'évolution. Leur étude a donc été abordée dans notre travail pour les différentes substances que nous avons isolées.

Les résultats sont présentés dans cette thèse sous la forme de publications soit, déjà éditées soit, en préparation. Ils sont précédés d'une présentation du matériel biologique, d'un bref rappel anatomique du système nerveux central des Hirudinées et plus particulièrement de celui des deux espèces étudiées (*T. tessulatum* et *E. octoculata*) et d'une mise au point sur les relations structures/

fonctions des peptides intervenant sur l'osmorégulation dans le règne animal (familles des angiotensines, des ocytocines/vasopressines et des RFamides) dont des épitopes ont été reconnus par voie immunocytochimique au niveau du ganglion supra-œsophagien de *T. tessulatum*.

GENERALITES

I - Le matériel biologique

Deux modèles ont été utilisés.

1. *Theromyzon tessulatum*

Parasite des oiseaux aquatiques, cette sangsue Rhynchobdelle Glossiphoniidée prélève du sang en s'introduisant dans les fosses nasales. Adulte, elle atteint une masse variant de 150 mg à plus d'1 g. Elle se reproduit une fois au cours de sa vie puis meurt (Wilkialis, 1970 ; Wilkialis et Davies, 1980a, b).

Les grandes étapes de la vie de *T. tessulatum* ont été définies en prenant comme repères les repas de sang (Malecha, 1983), lesquels sont généralement au nombre de trois [Fig. 1]. Les prises de nourriture définissent quatre stades (stade 0 : de l'éclosion au premier repas, stades 1, 2 et 3 : respectivement après les premier, deuxième et troisième repas). A l'issue du troisième repas, la croissance de cette sangsue se caractérise par une augmentation de masse considérable due à une importante rétention d'eau [Fig. 1]. Cependant, si le poids atteint après le troisième repas est inférieur à 150 mg, un repas supplémentaire peut être nécessaire, le cycle s'étendant alors sur 2 ans (Wilkialis et Davies, 1980a). Le stade 3 est très long et peut durer 1 an. Il a été subdivisé en prenant en compte les

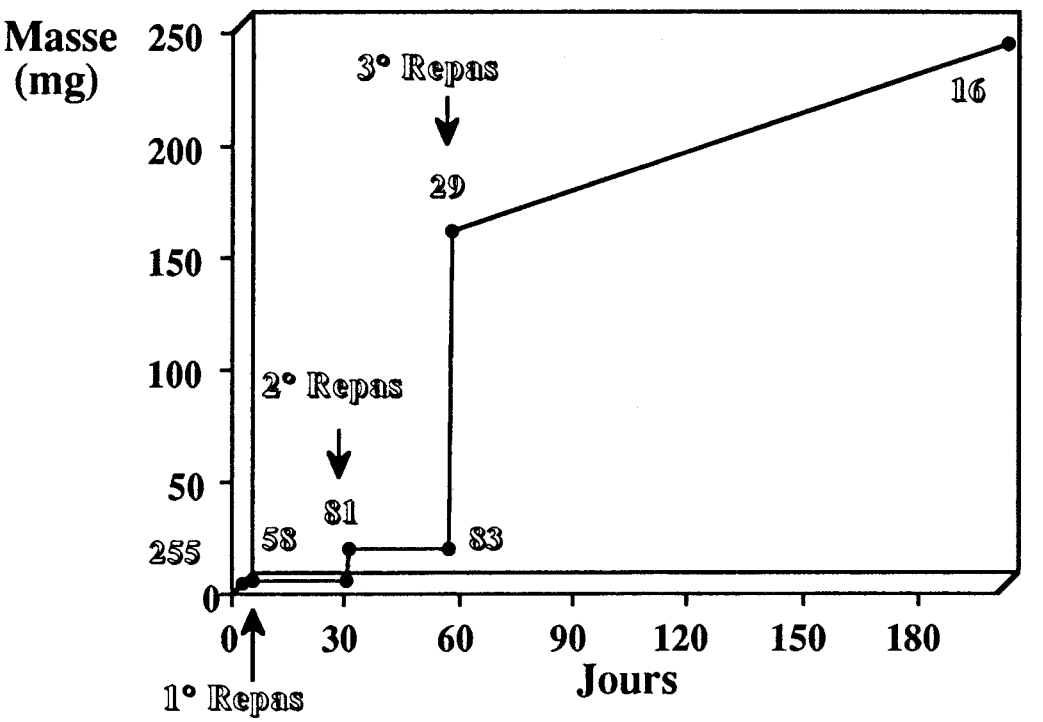


Figure 1- Croissance pondérale de *T. tessulatum* maintenues à la température du laboratoire et au rythme nycthéméral normal. Les chiffres en relief représentent les effectifs ayant servi à l'établissement de la masse moyenne (d'après Malecha, 1979).

Figure 2- Principales étapes du stade 3 de *T. tessulatum* . Dans tous les cas, l'animal est vu par sa face ventrale.

a Stade 3A. Le tube digestif est rempli de sang. Les organes génitaux ne sont pas discernables par transparence (X 10).

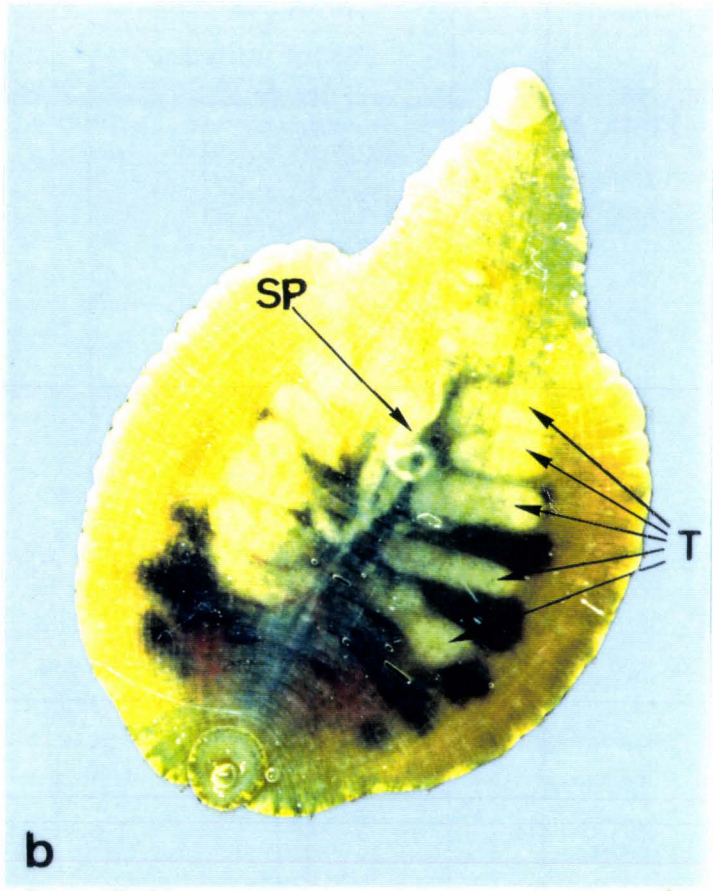
b Stade 3B. Les testicules (T) sont nettement visibles ainsi que les spermiductes (SP). Le tube digestif contient encore du sang (X 10).

c Stade 3D. Les testicules ont regréssé, le tube digestif est vide et les ovaires bourrés d'ovocytes mûrs sont visibles par transparence. L'orifice génital ♀ (OG ♀) apparaît très nettement à la suite du développement de cellules glandulaires (X 10).

d Ponte du deuxième cocon. Le premier cocon (1) est juste au-dessus de la ventouse caudale, le second (2) est en cours de remplissage (X 10).

e Stade 3E. La sangsue couve ici cinq cocons (X 2,5). [Photographie M. Guillon, Service du Cinéma Scientifique].


a


SP
T
b


OG♀
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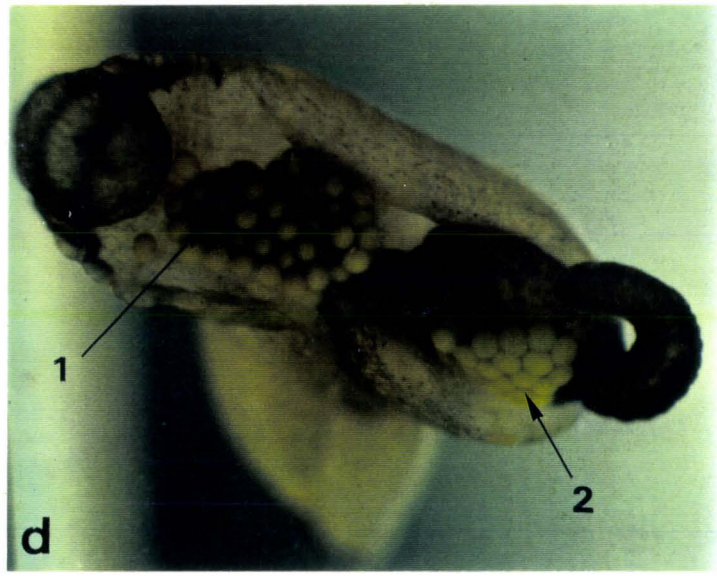
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2
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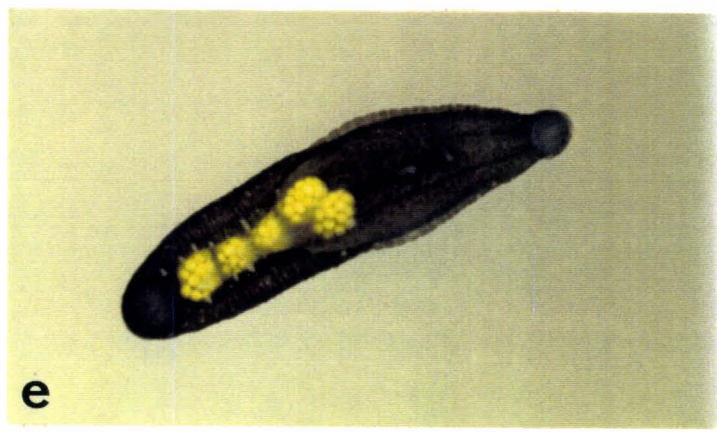
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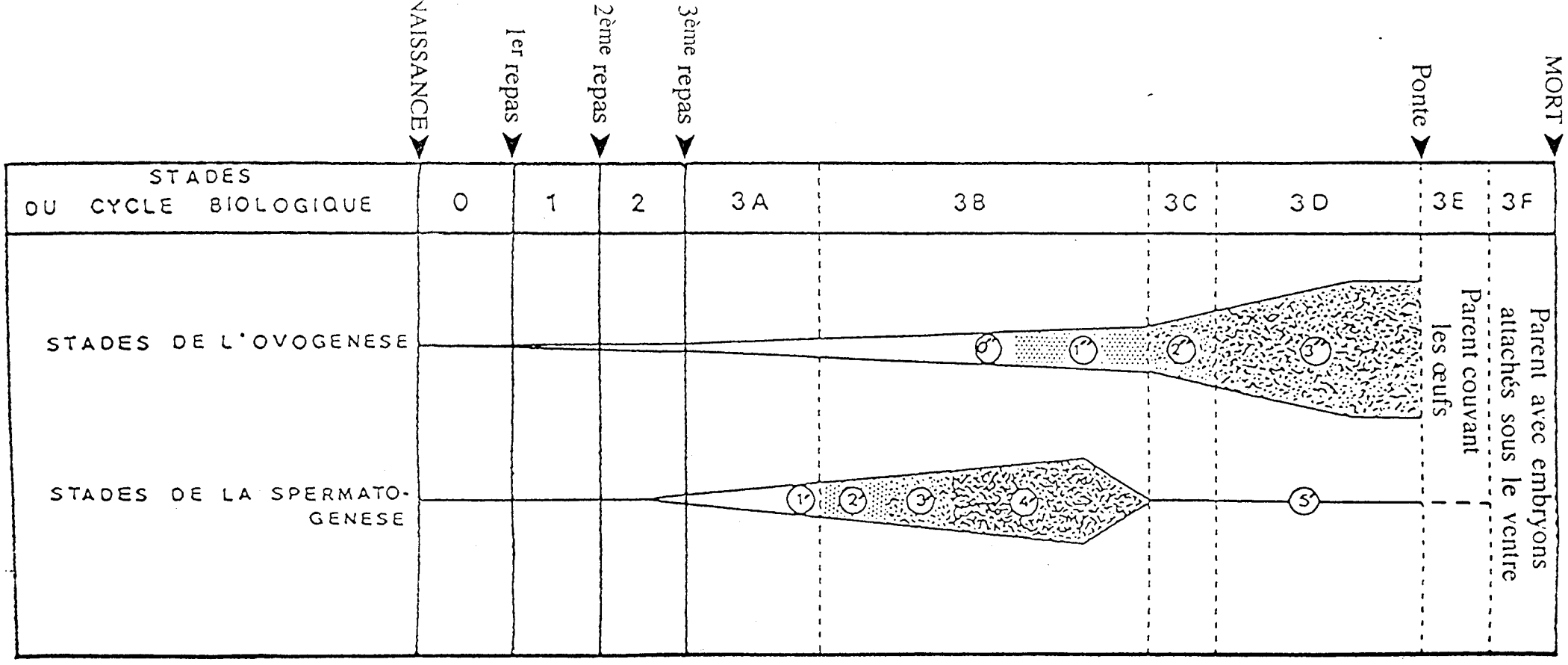


Figure 3- Représentation schématique du cycle biologique de *T. tessulatum* (d'après Malecha *et al.*, 1989b).
Les stades de l'ovogenèse (0"--->3") et les stades de la spermatogenèse (1'--->5'), ont été définis précisément par Malecha *et al.*, (1989b).
Il n'est pas tenu compte, dans ce schéma, de la durée des différents stades.

différentes étapes de la reproduction [Figs 2 et 3]. L'évolution de la spermatogenèse définit les stades 3A et 3B, celle de l'ovogenèse les stades 3C et 3D (Malecha *et al.*, 1989b). La couvaison des cocons caractérise le stade 3E qui est séparé du stade 3D par la ponte d'œufs riches en vitellus.

Le transport des sangsues néonates jusqu'à leur premier repas définit le stade 3F qui peut durer plus de 5 mois, jusqu'à ce que le parent trouve un hôte favorable pour les jeunes sangsues qu'il porte. L'adulte meurt quelques jours après leur départ (Wilkialis et Davies, 1980b).

T. tessulatum est élevée au laboratoire, ce qui nous permet de disposer tout au long de l'année d'animaux à différents stades physiologiques. Ainsi, notre test biologique (variation de la masse des animaux après différents traitements) est réalisé sur des *T. tessulatum* au stade 3B, stade correspondant à une phase de rétention d'eau importante chez ces sangsues.

2. *Erpobdella octoculata*

E. octoculata est une sangsue Arhynchobdelle Pharyngobdelle. Présente en grande quantité en Europe, et notamment à Harchies (Belgique) où nous les récoltons dans les étangs du Centre de Recherches Biologiques, elle constitue un matériel de choix pour effectuer les extractions biochimiques.

Les Erpobdellidées sont des sangsues carnivores, se nourrissant de Vers, de Mollusques et de larves d'Insectes. Le cycle biologique d'*E. octoculata* comprend, deux générations, sur une année. Ces sangsues déposent des cocons (voir photo ci-dessous) contenant chacun environ 5 à 10 œufs alécithes. L'embryon se développe grâce à la présence dans le cocon de réserves issues des glandes clitelliennes du parent.

E. octoculata déposant ses cocons.

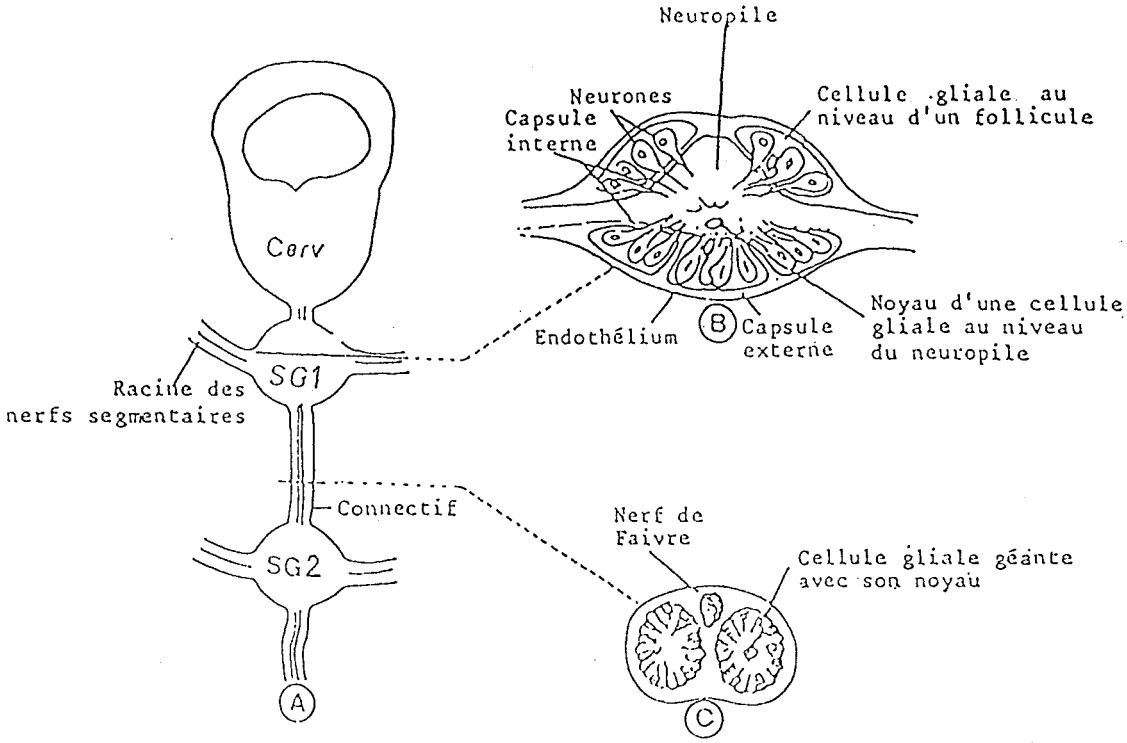


Figure 4- Diagramme du système nerveux central des Hirudinées (d'après Kuffer et Potter, 1964).

A- Cerveau (Cerv) et deux premiers ganglions segmentaires de la chaîne nerveuse (SG1 : 1er ganglion, SG2 : 2ème ganglion).

B, C- Sections au niveau de la chaîne nerveuse (côté dorsal vers le haut).
B- au niveau d'un ganglion segmentaire.
C- au niveau des connectifs interganglionnaires.

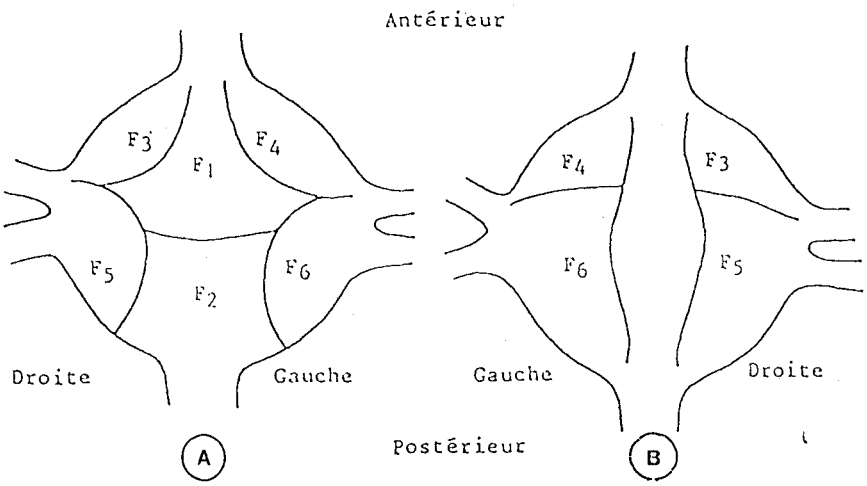


Figure 5- Représentation schématique des six follicules constituant un ganglion segmentaire d'Hirudinée. A : vue ventrale ; B : vue dorsale (d'après Macagno, 1980).
F1, 2, 3, 4, 5, 6 : numérotation des follicules.
F1 : follicule médian antérieur.
F2 : follicule médian postérieur.
F3, F4 : follicule latéral antérieur droit (F3) ou gauche (F4).
F5, F6 : follicule latéral postérieur droit (F5) ou gauche (F6).

II - Le système nerveux des Hirudinées

Le système nerveux central des Hirudinées se subdivise en trois grandes parties :

- une partie antérieure contenant le cerveau,
- une partie moyenne où les ganglions se répartissent à raison d'un par métamère,
- une partie caudale ou ganglion caudal.

1. Le cerveau

Le cerveau [Fig. 4A, 6A] est formé de deux masses nerveuses, l'une supra-œsophagienne (ganglion supra-œsophagien) et l'autre sous-œsophagienne (ganglion sous-œsophagien) formée par la fusion de 4 neuromères. Deux commissures péri-œsophagiennes relient les ganglions supra- et sous-œsophagiens.

Comparées aux connaissances acquises sur les ganglions segmentaires, celles relatives aux neurones cérébraux restent fragmentaires, peut-être en raison du fait que ces derniers contrôlent des fonctions plus complexes. Le cerveau comprend des groupes de neurones ou follicules séparés les uns des autres par des cloisons conjonctives.

2. La partie moyenne

A ce niveau, la chaîne nerveuse, contenue dans le sinus sanguin ventral, comprend 21 ganglions segmentaires (segmental ganglia : SG). Les SG, numérotés de SG1 à SG21, sont unis par deux connectifs ou cordons nerveux longitudinaux [Fig.4A]. Tout au long de la chaîne, dans le neurilème qui entoure les connectifs, court un cordon nerveux, médio-dorsal par rapport aux connectifs et parallèle à ces derniers, c'est le nerf de Faivre (Figs. 4C et 7D). Chaque ganglion renferme environ 400 neurones, avec quelques légères variations de ce nombre d'un ganglion à un autre (Macagno, 1980). Les 5ème et 6ème ganglions (SG5 et SG6) qui innervent les organes sexuels, d'où leur nom de ganglions génitaux (sex SG), sont particuliers. Ils contiennent des cellules surnuméraires, environ 300 dans le cas d'*Hirudo*, selon Macagno *et al.* (1986) [Tableau I].

Les neurones ganglionnaires sont unipolaires et leur corps cellulaire a un diamètre compris entre 10 et 60 µm. Ils sont répartis en 6 follicules : 4 latéraux (2 antérieurs et 2 postérieurs) et 2 médio-ventraux (1 antérieur et 1 postérieur) [Figs 5, 7C et 9C]. Leurs prolongements axonaux et arborisations terminales sont présents à l'intérieur d'un neuropile central [Fig. 4B]. Une cellule gliale unique enveloppe les neurones dans chaque follicule ; deux cellules gliales entourent les

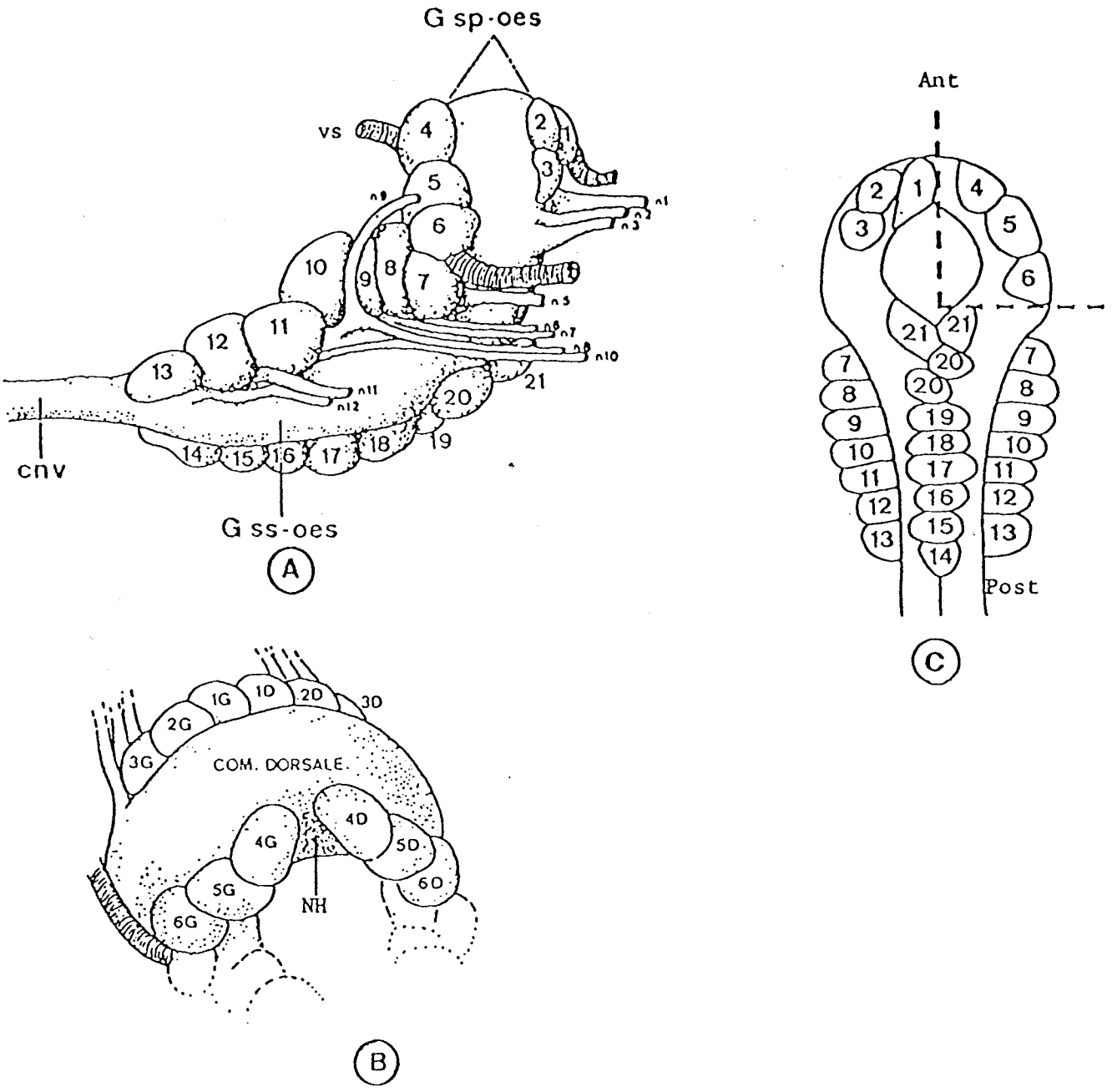


Figure 6- Le cerveau de *Theromyzon*

A Ganglions supra-œsophagien (Gsp-oes) et sous-œsophagien (Gss-oes) de *T. rude* (vue latérale droite, partie antérieure vers la droite).
cnv : chaîne nerveuse ventrale ; 1 à 20 : follicules du cerveau ; n1 à n12 : nerfs céphaliques ; vs : vaisseau sanguin (d'après Hagadorn, 1958).

B Vue dorsale du ganglion supra-œsophagien montrant les follicules antérieurs (N°S 1, 2 , 3) et postérieurs (N°S 4, 5, 6) droits (D) et gauches (G) de part et d'autre de la commissure dorsale (COM. DORSALE) sur laquelle se situe la zone neurohémale (NH) (d'après Hagadorn *et al.*, 1963).

C Représentation schématique du cerveau, figurant la numérotation des follicules (N°S 1 à 3 et N°S 7 à 21 : vue ventrale ; N°S4 à 6 : vue dorsale) (d'après Malecha *et al.*, 1989b).

axones au niveau du neuropile (Fig. 4B). De plus, deux cellules gliales sont présentes au niveau des connectifs interganglionnaires (une dans chaque connectif) (Figs 4C, 7D).

Tableau I- Nombre de neurones dans les ganglions segmentaires SG4 à SG7 d'*Hirudo medicinalis* (d'après Macagno *et al.*, 1986).

Ganglion	Nombre de cellules par ganglion
SG4	387 ± 9 (4)
SG5	735 ± 31 (4)
SG6	745 ± 36 (3)
SG7	399 ± 5 (3)

SG4, SG5, SG6 et SG7 : respectivement 4^{e}, 5^{e}, 6^{e} et 7^{e} ganglion de la chaîne nerveuse. Chaque valeur est une moyenne ± déviation standard obtenue à partir d'un nombre d'échantillons figurant entre parenthèses.

3. Le ganglion caudal

Il est formé par la fusion des neuromères des 7 derniers métamères. Sept paires de nerfs provenant de ce ganglion caudal innervent la ventouse caudale (Rubin, 1978).

4. Particularités du cerveau de *T. tessulatum* [Figs 6 et 7]

Comme chez *T. rude* (Hagadorn, 1958, 1962), les neurones du cerveau sont groupés en 36 follicules [Figs. 6A et C].

La partie supra-œsophagienne du cerveau (ganglion supra-œsophagien) [Figs. 6B et 7A, B] est composée d'une commissure dorsale, au niveau postéro-médian de laquelle se situe une zone neurohémale (Webb, 1980) et de 6 paires de follicules : 3 (N°^{s} 1, 2 et 3) sont situées à la bordure antérieure de la commissure, les 3 autres (N°^{s} 4, 5 et 6) lui sont postérieures.

La partie sous-œsophagienne du cerveau (ganglion sous-œsophagien) comprend 24 follicules disposés en 3 groupes : 2 dorso-latéraux disposés symétriquement et composés chacun de 7 follicules (N°^{s} 7 à 13) et un groupe médio-ventral composé de 6 follicules (N°^{s}14 à 19) auxquels s'ajoutent vers l'extrémité antérieure 2 autres paires (N°^{s} 20 et 21).

Deux vaisseaux sanguins sont associés au cerveau : l'un passe sous la commissure dorsale (Fig. 7A), en relation étroite avec le périneurium, l'autre est situé ventralement par rapport au tube digestif et, après s'être dichotomisé, traverse la masse sous-œsophagienne.

Figure 7- Le système nerveux central de *T. tessulatum*. (A, B : Coloration selon la technique de Clark, C, D : Triple coloration de Prenant).

A- Coupe transversale de la région antérieure du cerveau mettant en évidence les follicules postérieurs 4, 5 et 6 du ganglion supra-œsophagien et les follicules 7 du ganglion sous-œsophagien. (x 450).

On notera que les follicules 4 se situent de part et d'autre du vaisseau sanguin dorsal (vsd).

com.v : commissure ventrale, t : trompe.

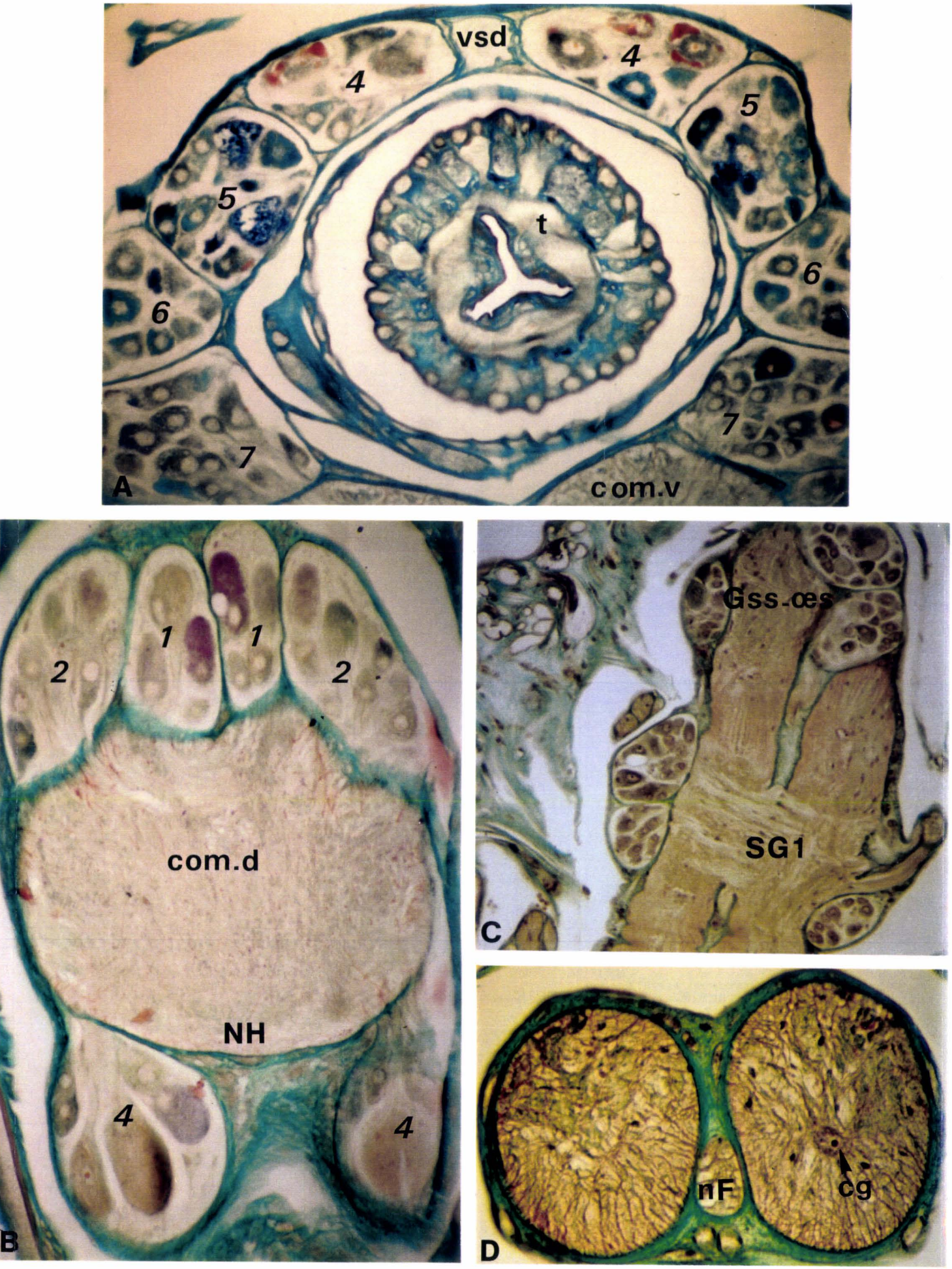
B- Coupe frontale au niveau du ganglion supra-œsophagien mettant en évidence les follicules antérieurs 1 et 2 (les follicules 3 ne sont pas visibles sur cette coupe) et une paire de follicules postérieurs (les follicules 4) placée au voisinage de l'aire neurohémale (NH).(x 450).

com. d : commissure dorsale.

C- Coupe frontale mettant en évidence une partie du ganglion sous-œsophagien (Gss-œs) et le premier ganglion segmentaire de la chaîne nerveuse ventrale (SG1).(x 180).

D-Coupe transversale de la chaîne nerveuse ventrale au niveau des connectifs interganglionnaires. (x 450).

c g : cellule gliale géante avec son noyau, nF : nerf de Faivre.


vsd
4
4
5
5
t
6
6
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7
A
com.v
1
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NH
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4
B
Gss-œs
SG1
C
nF
cg
D

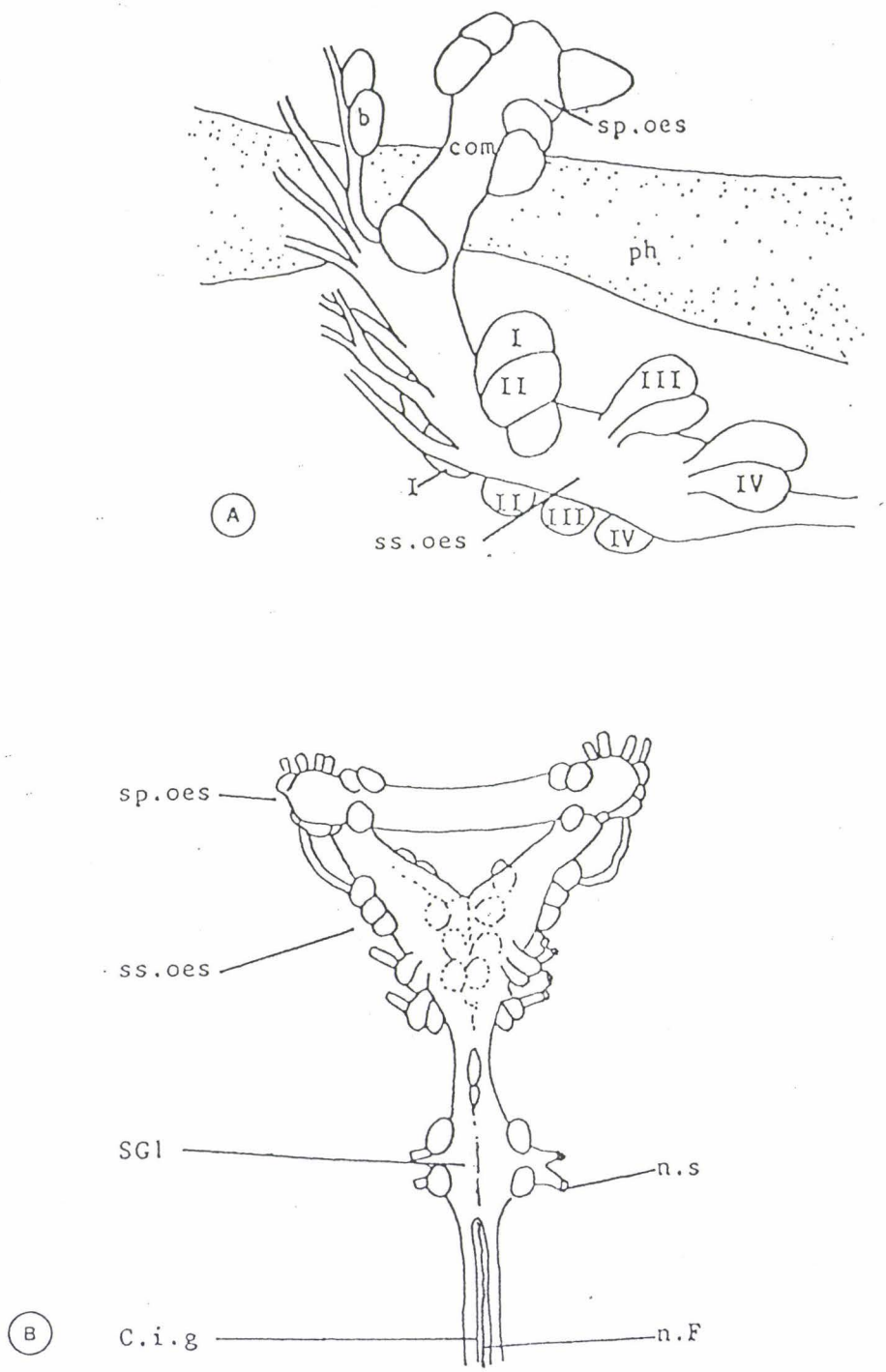


Figure 8- Le système nerveux central d'*Erpobdella octoculata*.

A Cerveau en vue latérale gauche (d'après Van Damme, 1977).
Les neuromères constituant la région sous-œsophagienne sont indiqués en chiffres romains (d'après la numérotation de Sawyer, 1986). com : commissure, b : ganglion buccal, ph : pharynx.

B Cerveau et premier ganglion segmentaire (SG1) de la chaîne nerveuse en vue dorsale (adapté d'après Bristol, 1898). C.i.g. : connectif interganglionnaire, n.F : nerf de Faivre, n.s : nerf segmentaire.

sp.oes : région supra-œsophagienne, ss.oes : région sous-œsophagienne

Une étude cytologique effectuée sur le cerveau de *T. rude* par Hagadorn (1962) a montré qu'à côté des neurones ordinaires (cellules δ), qui constituent 95 % des neurones, 2 types de cellules neurosécrétrices sont présents : des cellules α réagissant positivement à la fuchsine paraldéhyde et des cellules ß contenant un matériel de sécrétion coloré par le vert lumière ou l'orangé G. La figure 7A illustre ces types cellulaires chez *T. tessulatum.*

Une étude ultrastructurale des cellules α et β a démontré l'existence de plusieurs types de granules de sécrétion. Des terminaisons nerveuses contenant ces différentes sécrétions sont présentes dans la zone neurohémale (Hagadorn *et al.*, 1963).

5. Particularités du cerveau d'*E. octoculata* [Figs. 8 et 9]

Le complexe céphalique situé en arrière des yeux postérieurs comporte des massifs ganglionnaires ainsi que vers l'avant une paire de ganglions buccaux (système nerveux stomatogastrique) reliés au collier péri-œsophagien. Les massifs ganglionnaires dispersés autour du neuropile central sont très espacés et occupent 2 régions (supra- et sous-œsophagiennes) . La région supra-œsophagienne du cerveau comprend 6 paires de follicules situées symétriquement de part et d'autre de la commissure cérébroïde. Trois paires se trouvent en avant et trois paires en arrière de cette commissure. La région sous-œsophagienne du cerveau est constituée par les quatre premiers neuromères de la chaîne nerveuse.

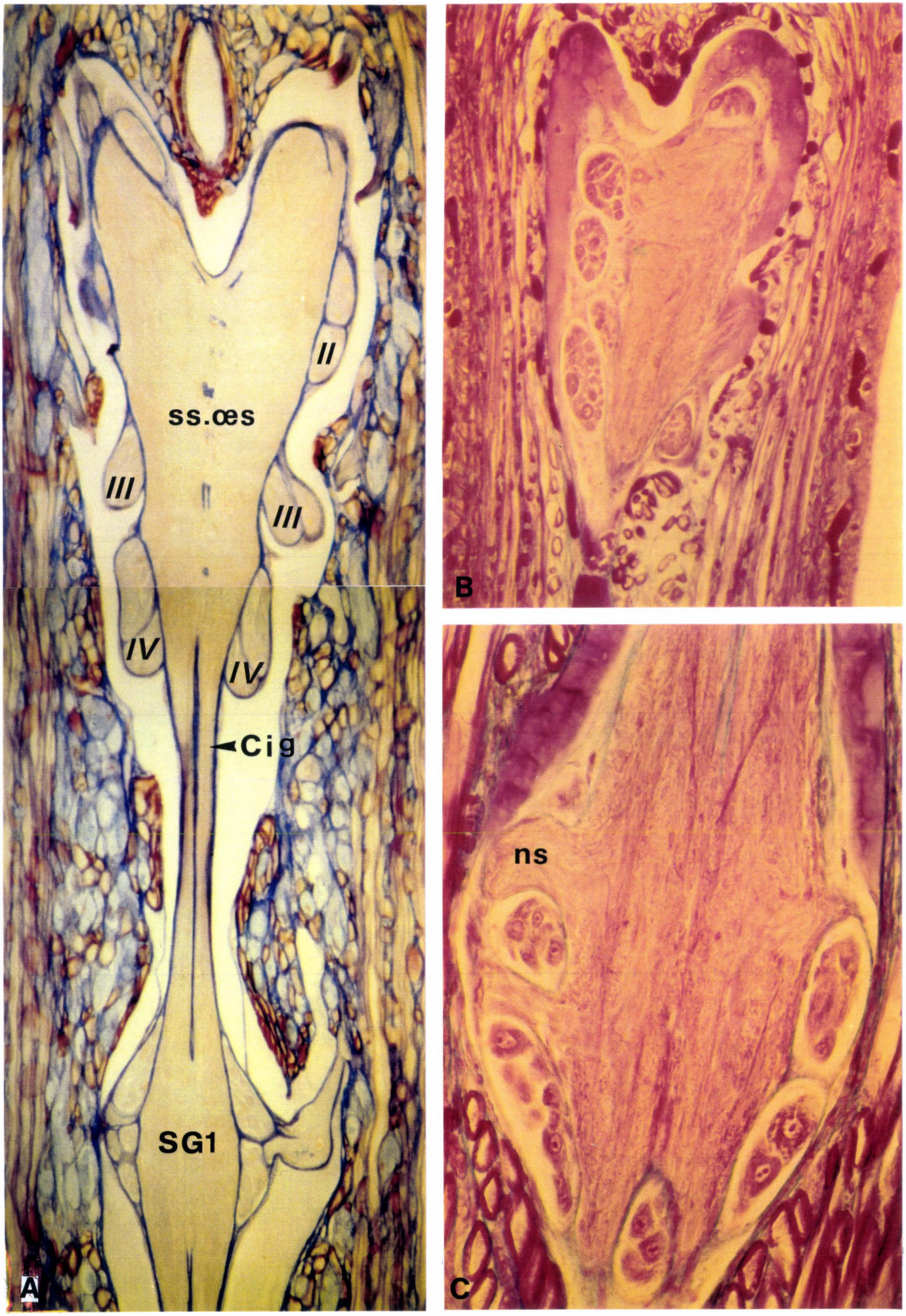
Figure 9 : Le système nerveux central d'*E. octoculata* (Coloration à l'Azan).

A- Coupe frontale passant par la région sous-œsophagienne du cerveau (SS. œs) et par le premier ganglion segmentaire (SG1) de la chaîne nerveuse ventrale.(x 180).

II, III, IV : neuromères numérotés selon Sawyer (1986), c.i.g : connectif interganglionnaire.

B- Coupe frontale légérement oblique au niveau de la région sous-œophagienne du cerveau mettant en évidence le neuropile et les massifs ganglionnaires.(x 180).

C- Coupe frontale d'un ganglion segmentaire de la chaîne nerveuse ventrale figurant le neuropile, les follicules latéraux et le follicule médian postérieur.(x 450).
ns : nerf segmentaire.


ss.œs
II
III
III
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C

III - La famille des angiotensines

L'angiotensine II a été découverte en 1940 chez les Vertébrés par Page et Helmer. Elle est composée de 8 acides aminés et est produite par une cascade de réactions [Fig. 10].

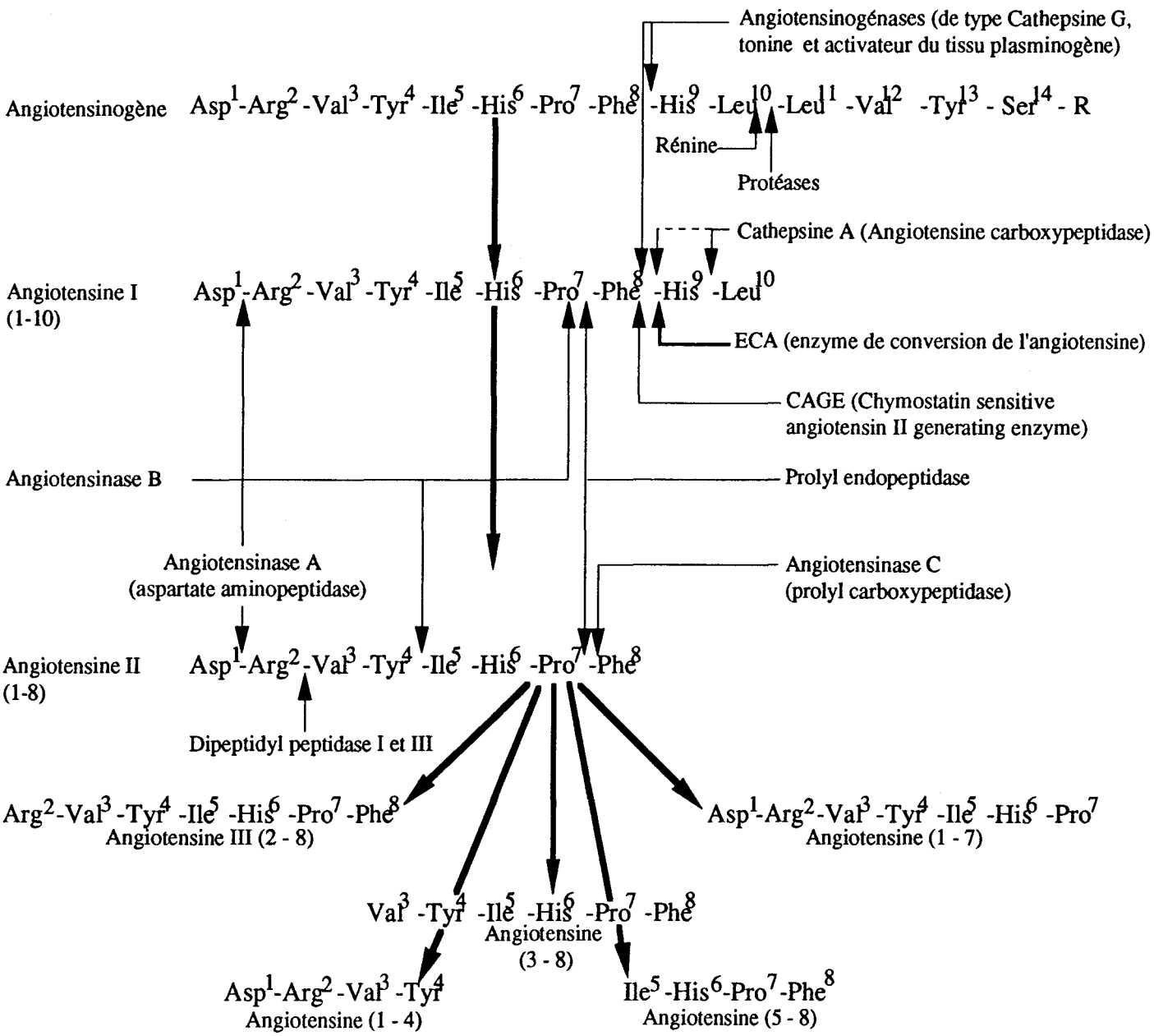


Figure 10- Métabolisme de l'angiotensine II (d'après Saavedra, 1992).

Le précurseur de cette hormone est l'angiotensinogène (α2 globuline) synthétisé au niveau du foie. C'est une protéine d'environ 60 kDa (Campbell *et al.*, 1991). Son gène a été cloné (Higenfeldt et Hachenthal, 1979). L'angiotensinogène libéré dans la circulation sanguine est clivé au niveau rénal par la rénine en un décapeptide inactif, l'angiotensine I (AI). Ce dernier est ensuite hydrolysé en angiotensine II (AII) par une carboxy-peptidase (enzyme de conversion), principalement au niveau des capillaires pulmonaires. L'AII ainsi libérée peut agir sur ses récepteurs spécifiques avant d'être dégradée. Une succession de coupures enzymatiques [Fig. 10] survient alors, ayant comme conséquence une perte de l'activité biologique (Abhold et Harding, 1988).

1. Mise en évidence des angiotensines dans le règne animal

Jusqu'à présent le système rénine/angiotensine n'a pu être mis en évidence que chez les Vertébrés mammaliens (Fitzsimons, 1979) et non mammaliens (Oiseaux : Takei, 1977 ; Reptiles : Fitzsimons et Kaufman, 1977 ; Amphibiens : Wilson, 1984 et Poissons : Takei *et al.*, 1979).

2. Rôle physiologique, récepteurs et mode d'action

L'ensemble des connaissances dans ce domaine est relatif aux Vertébrés.

a. Rôle physiologique de l'AII

L'élévation de la concentration d'AII circulante provoque de nombreuses réponses physiologiques (voir Tableau II). Hormis ces actions périphériques, l'AII qui est aussi synthétisée dans le cerveau (Philipps *et al.*, 1991) exerce à ce niveau différentes actions : régulation de la pression sanguine, de la soif et de la prise de sodium, stimulation de la synthèse de vasopressine et de corticotropine, stimulation de la secrétion de catécholamine, de vasopressine et de vasotocine (Saavedra, 1992).

Tableau II- Tissus cibles et actions physiologiques de l'angiotensine II chez les Mammifères (d'après Guillon, 1989).

Tissus cibles	Sous-type de récepteur	Fonction physiologique	Systèmes de transduction impliqués
Hippocampe		Neurotransmission	
Rein (glomérule du cortex)		Stimulation de la natriurèse et de la filtration glomérulaire	
Adipocyte		Action insuline-like	
Adénohypophyse		Potentialisation de l'effet CRF	
Tractus génital mâle (épididyme)		Contraction	
Foie		Stimulation de la glycogénolyse	Stimulation de la PC et d'une perméabilité calcique et inhibition de l'AC
Hypophyse		Sécrétion de prolactine	Stimulation de la PC
Surrénale		Sécrétion de minéralocorticoïdes	Stimulation de la PC et d'une perméabilité calcique
Cellule musculaire lisse		Contraction	Stimulation de la PC et d'une perméabilité calcique
Placenta (trophoblaste)		Sécrétion d'hPL	Stimulation de la PC
Cerveau		Stimulation de la prise d'eau et de sel. Régulation de la pression sanguine. Stimulation de la synthèse d'hormone pituitaire (AVP, ACTH, Prolactine, LH). Neuromodulation	
Cœur		Effet inotropique	Activation probable d'un canal Ca^{2+} voltage dépendant
Rein - cellule mésangiale - cellule du tubule distal	AT1	Contraction Réabsorption de l'eau et du NaCl	Activation PC Inhibition AC
Plaquette			

AC, Adénylate cyclase ; ACTH, Hormone corticotrope ; AVP, Arginine-vasopressine ; CRF, corticolibérine; LH, Hormone lutéinisante ; PC, Phospholipase C ; hPL, Hormone Lactogénique Placentaire.

b. Les récepteurs de l'AII

L'utilisation d'AII marquée a permis de caractériser les récepteurs spécifiques à l'AII au niveau de différents tissus cibles [Tableau II]. De même, les analogues de structure de l'AII ont été utilisés pour classer ces récepteurs en fonction de leur affinité (Capponi *et al.*, 1981). Des sous-types de récepteurs

différant par leurs profils pharmacologiques et par leurs mécanismes de transductions ont été mis en évidence [Fig. 11].

Sur la base de leur affinité et de leur interaction ou non avec la protéine G, deux types de récepteurs nommés respectivement AT1 et AT2 ont été identifiés. L'ADNc du récepteur AT1 a été isolé et séquencé (Murphy *et al.*, 1991). C'est une protéine de 41 kDa (Takayanagi *et al.*, 1992) qui est formée de 7 domaines transmembranaires liés à la protéine G. Les résidus cystéiques présents dans les 4 domaines extracellulaires sont importants pour la formation des ponts disulfures nécessaires à la conformation et à l'affinité du couplage ligand-récepteur. Son mécanisme de transduction est bien connu [Fig. 11]. A l'opposé, mis à part le fait qu'il n'est pas couplé à la protéine G (Bottari *et al.*, 1991), on ne dispose à l'heure actuelle que de données fragmentaires sur l'AT2.

Des études récentes ont permis de montrer que l'oncogène *mass* codait pour une protéine capable de produire certains effets de l'AII (Jackson *et al.*, 1988).

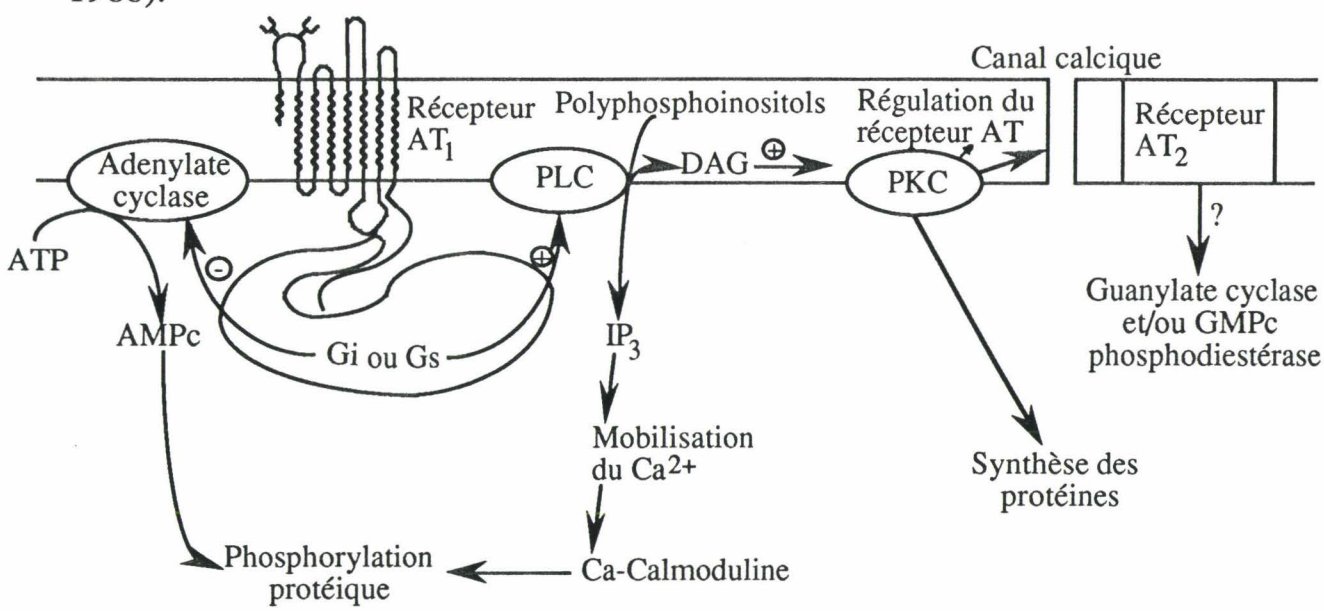


Figure 11 Schéma, proposé par Saavedra (1992), des mécanismes de transduction des récepteurs à angiotensine II (AT_1 et AT_2).
Le récepteur AT_1 peut, soit inhiber l'adénylate cyclase via des protéines de type G inhibitrices (Gi), soit stimuler via une protéine de type G stimulatrice (Gs) une phospholipase C (PLC) via des phosphoinositols conduisant à la formation de 2 seconds messagers : le diacyl glycérol (DAG) et l'inositol triphosphate (IP_3). L'IP_3 permet la libération du calcium intracellulaire et de DAG et augmente l'affinité de la phosphokinase C (PKC) au calcium.
Le récepteur AT_2 serait lié à un système guanylate cyclase et/ou GPMc phosphodiestérase.

c. Mode d'action de l'AII

A partir de l'étude des récepteurs, il a pu être déduit qu'au niveau des tissus cibles, l'AII peut provoquer 3 types d'actions distinctes [Tableau II] :

l'activation d'une phospholipase C, l'inhibition de l'adénylate cyclase et la stimulation d'un influx calcique (voir revue de Saavedra, 1992).

d. Modulation des récepteurs à l'AII

Les études, réalisées sur de nombreux tissus cibles de l'AII, ont montré que la densité et l'affinité de ces récepteurs peuvent évoluer en fonction de divers paramètres physiologiques. Tout agent capable de moduler la concentration d'AII circulante (inhibiteur ou activateur de la rénine ou de l'enzyme de conversion) aura une influence sur la densité des sites à l'AII (Summers et Fregly, 1987).

IV - La famille des ocytocines/vasopressines

La structure des peptides de cette famille a été élucidée par Du Vigneaud (1954). Ce sont des nonapeptides constitués d'une partie cyclique et d'une partie linéaire. Chez les Vertébrés, seuls 2 acides aminés varient, par contre 4 varient chez les Invertébrés [Fig. 12]

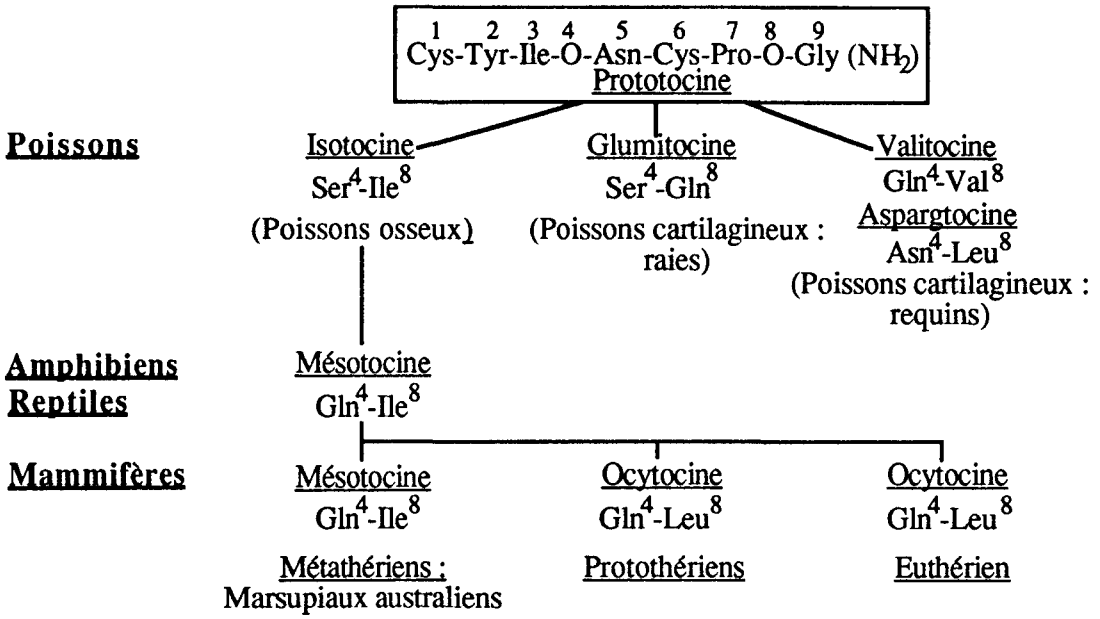
Peptides apparentés à l'ocytocine

Cys	Tyr	**Phe**	**Arg**	Asn	Cys	Pro	**Ile**	Gly	NH_2	Céphalotocine	Mollusque Céphalopode (*Octopus vulgaris*)
Cys	Tyr	Ile	**Asn**	Asn	Cys	Pro	Leu	Gly	NH_2	Aspargtocine	Poissons cartilagineux
Cys	Tyr	Ile	**Ser**	Asn	Cys	Pro	**Gln**	Gly	NH_2	Glumitocine	Poissons cartilagineux
Cys	Tyr	Ile	Gln	Asn	Cys	Pro	**Val**	Gly	NH_2	Valitocine	Poissons cartilagineux
Cys	Tyr	Ile	**Ser**	Asn	Cys	Pro	**Ile**	Gly	NH_2	Isotocine	Poissons osseux
Cys	Tyr	Ile	Gln	Asn	Cys	Pro	**Ile**	Gly	NH_2	Mésotocine	Mammifères, Oiseaux, Reptiles, Amphibiens
Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Leu	Gly	NH_2	Ocytocine	Mammifères

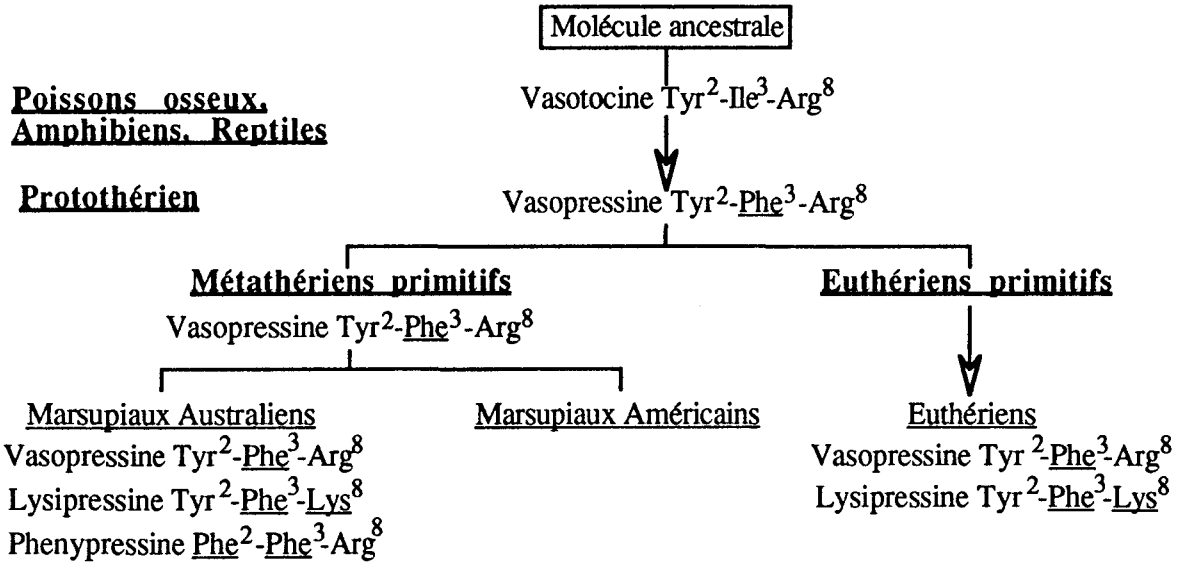
Peptides apparentés à la vasopressine

Cys	**Phe**	**Ile**	**Arg**	Asn	Cys	Pro	**Lys**	Gly	NH_2	Lys-Conopressine	Mollusques Gastéropodes (*Aplysia kurodai*) (*Conus geographus*) (*Lymnaea stagnalis*)
Cys	**Ile**	**Ile**	**Arg**	Asn	Cys	Pro	Arg	Gly	NH_2	Arg-Conopressine	Mollusque Gastéropode (*Conus striatus*)
Cys	**Leu**	**Ile**	**Thr**	Asn	Cys	Pro	Arg	Gly	NH_2	Arg-Vasopressine	Insecte (*Locusta migratoria*)
Cys	Tyr	**Ile**	Gln	Asn	Cys	Pro	Arg	Gly	NH_2	Vasotocine	Vertébrés non mammalien
Cys	**Phe**	Phe	Gln	Asn	Cys	Pro	Arg	Gly	NH_2	Phénylpressine	Mammifères
Cys	Tyr	Phe	Gln	Asn	Cys	Pro	**Lys**	Gly	NH_2	Lys-Vasopressine	Mammifères
Cys	Tyr	Phe	Gln	Asn	Cys	Pro	Arg	Gly	NH_2	Arg-Vasopressine	Mammifères

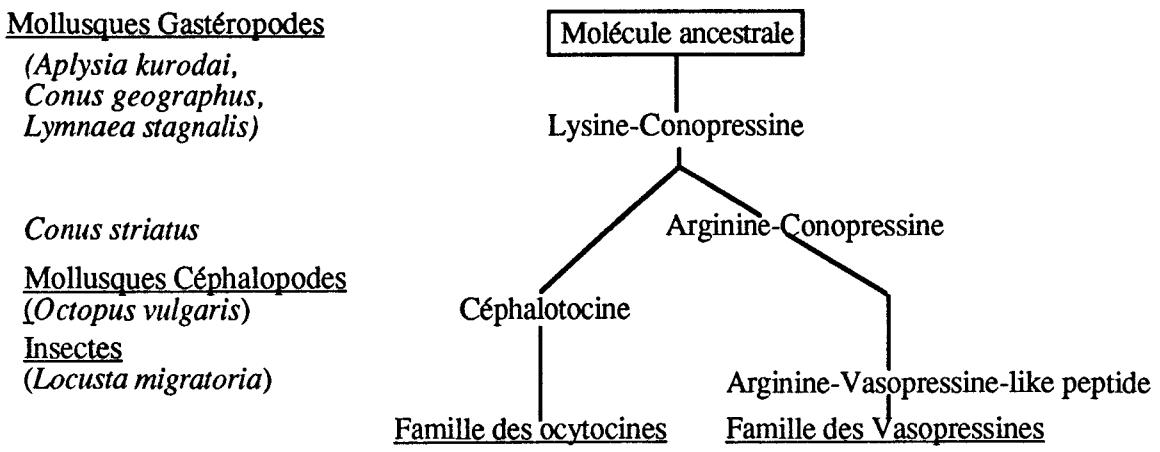
Figure 12- Séquences primaires des molécules appartenant à la famille des ocytocines/vasopressines



A - Evolution possible des molécules apparentées à l'ocytocine chez les Vertébrés (d'après Acher *et al.*, 1985).



B - Evolution possible des molécules apparentées à la vasopressine chez les Vertébrés (d'après Acher *et al.*, 1985)



C - Evolution possible des molécules de la famille des ocytocines/vasopressines chez les Invertébrés (basée sur l'hypothèse de Van Kesteren *et al.*, 1992).

Figure 13- Evolution possible des molécules apparentées à la famille des ocytocines/vasopressines dans le règne animal.

1. Etude structurale

a. Chez les Vertébrés

Six molécules apparentées à l'ocytocine et quatre apparentées à la vasopressine ont été identifiées (Acher *et al.*, 1985) [Fig. 12].

α *La famille des ocytocines*

Dans cette famille, des substitutions ont lieu uniquement au niveau des acides aminés en position 4 et/ou 8 [Fig. 13A]. En position 4, un résidu polaire (glutamine, sérine ou asparagine) est toujours présent. De même, un résidu non polaire (leucine, isoleucine ou valine) est toujours présent en position 8, exception faite d'une glutamine dans la glumitocine.

Une hypothèse a été émise selon laquelle une molécule ancestrale nommée prototocine aurait donné l'isotocine chez les Poissons osseux, la glumitocine chez les raies, la valitocine et l'aspargtocine chez les requins (Acher *et al.*, 1985) (Fig. 13A). La mésotocine dériverait de l'isotocine, elle est présente chez les Amphibiens, les Reptiles, les Oiseaux, et les Marsupiaux australiens mais elle est remplacée par l'ocytocine chez les Protothériens et chez les Euthériens (Acher *et al.*, 1973, 1985) [Fig. 13A].

β La famille des vasopressines

L'arginine-vasotocine se retrouve chez tous les Vertébrés, à l'exception des Mammifères [Fig. 13B]. Du fait de la présence d'arginine-vasopressine chez les Protothériens, il est possible que la substitution en position 3 de l'isoleucine en phénylalanine ait eu lieu précocement au cours de l'évolution des Mammifères. L'arginine-vasopressine est conservée chez les Euthériens avec quelques changements occasionnels en lysine-vasopressine (substitution en position 8). Chez les Métathériens, l'arginine-vasopressine est retrouvée dans quelques familles telles que les *Phalangeridae*. Dans d'autres familles, telles que les *Macropodidae*, une duplication du gène suivie d'une mutation a conduit à la Lys^8-vasopressine (lysipressine) et à la Phe^2-vasopressine (phénypressine) (Acher *et al.*, 1973, 1985) [Fig. 13B].

b. Chez les Invertébrés

Des immunoréactivités à l'ocytocine [Tableau III] et à la vasopressine [Tableau IV] ont été détectées dans la majorité des phyla. Cependant, seules 6 molécules apparentées à la famille des ocytocines/ vasopressines ont jusqu'à

Tableau III- Immunoréactivité à l'anti-ocytocine dans le tissu nerveux d'Invertébrés.

Espèces	Références
PROTOSTOMIENS	
Cœlentérés	
Hydra magnipapillata	Grimmelikhuijzen *et al.* (1982)
Hydra attenuata	Grimmelikhuijzen *et al.* (1982)
Annélides	
Neanthes japonica	Mizuno et Takeda (1988a)
Pheretima hilgendorfi	Kinoshita et Kawashima (1986)
Pheretima communissima	Mizuno et Takeda (1988a)
Theromyzon tessulatum	Malecha *et al.* (1986)
Mollusques	
Limax marginatus	Mizuno et Takeda (1988a)
Lymnaea stagnalis	Schot *et al.* (1981)
Meretrix lamarckii	Mizuno et Takeda (1988a)
Octopus vulgaris	Martin *et al.* (1980)
Oncidium verrucosum	Mizuno et Takeda (1988a)
Arthropodes	
Baratha brassicae	Mizuno et Takeda (1988a)
Periplaneta americana	Verhaert *et al.* (1984)
DEUTEROSTOMIENS	
Procordés	
Cheylosoma productum	Sawyer (1959)
Ciona intestinalis	Dodd et Dodd (1966)
Pyura haustor	Sawyer (1959)

Tableau IV - Immunoréactivité à l'anti-arginine-vasotocine/argine-vasopressine dans le tissu nerveux d'Invertébrés.

Espèces	Références
PROTOSTOMIENS	
Cœlentérés	
Hydra magnipapillata	Mizuno et Takeda (1988b)
Hydra attenuata	Grimmelikhuijzen *et al.* (1982)
Annélides	
Neanthes japonica	Mizuno et Takeda (1988b)
Pheretima communissima	Mizuno et Takeda (1988b)
Pheretima hilgendorfi	Kinoshita et Kawashima (1986)
Theromyzon tessulatum	Verger-Bocquet *et al.* (1988)
Tubifex tubifex	Yahya *et al.* (1983)
Mollusques	
Achatina fulica	Mizuno et Takeda (1988b)
Aplysia californica	Moore *et al.* (1981)
Aplysia kurodai	Mizuno et Takeda (1988b)
Ariolimax columbianus	Sawyer *et al.* (1984)
Bradybaena similaris	Mizuno et Takeda (1988b)
Limax marginatus	Mizuno et Takeda (1988b)
Limax maximus	Sawyer *et al.* (1984)
Meretrix lamarckii	Mizuno et Takeda (1988b)
Octopus vulgaris	Martin *et al.* (1980)
Oncidium verrucosum	Mizuno et Takeda (1988b)
Pomacea canaliculata	Mizuno et Takeda (1988b)
Arthropodes	
Achata domesticus	Strambi *et al.* (1979)
Armadillidium vulgare	Takeda *et al.* (1986)
Baratha brassicae	Mizuno et Takeda (1988b)
Clitumnus extradentatus	Rémy *et al.* (1977)
Gryllus bimaculatus	Mizuno et Takeda (1988b)
Gnorimosphaeroma rayi	Mizuno et Takeda, 1988b)
Hemigrapsus sanguineus	Mizuno et Takeda (1988b)
Leptinotarsa decemlineata	Veenstra (1984)
Ligia exotica	Takeda *et al.* (1986)
Locusta migratoria	Rémy *et al.* (1977)
Palaemon serratus	Van Herp et Bellon-Humbert (1982)
Periplaneta americana	Verhaert *et al.*, 1984)
Porcellio scaber	Takeda *et al.* (1986)
DEUTEROSTOMIENS	
Echinodermes	
Asterina pectinifera	Mizuno et Takeda (1988b)
Procordés	
Ciona intestinalis	Dodd et Dodd (1966)
Halocynthia roretzi	Mizuno et Takeda (1988b)

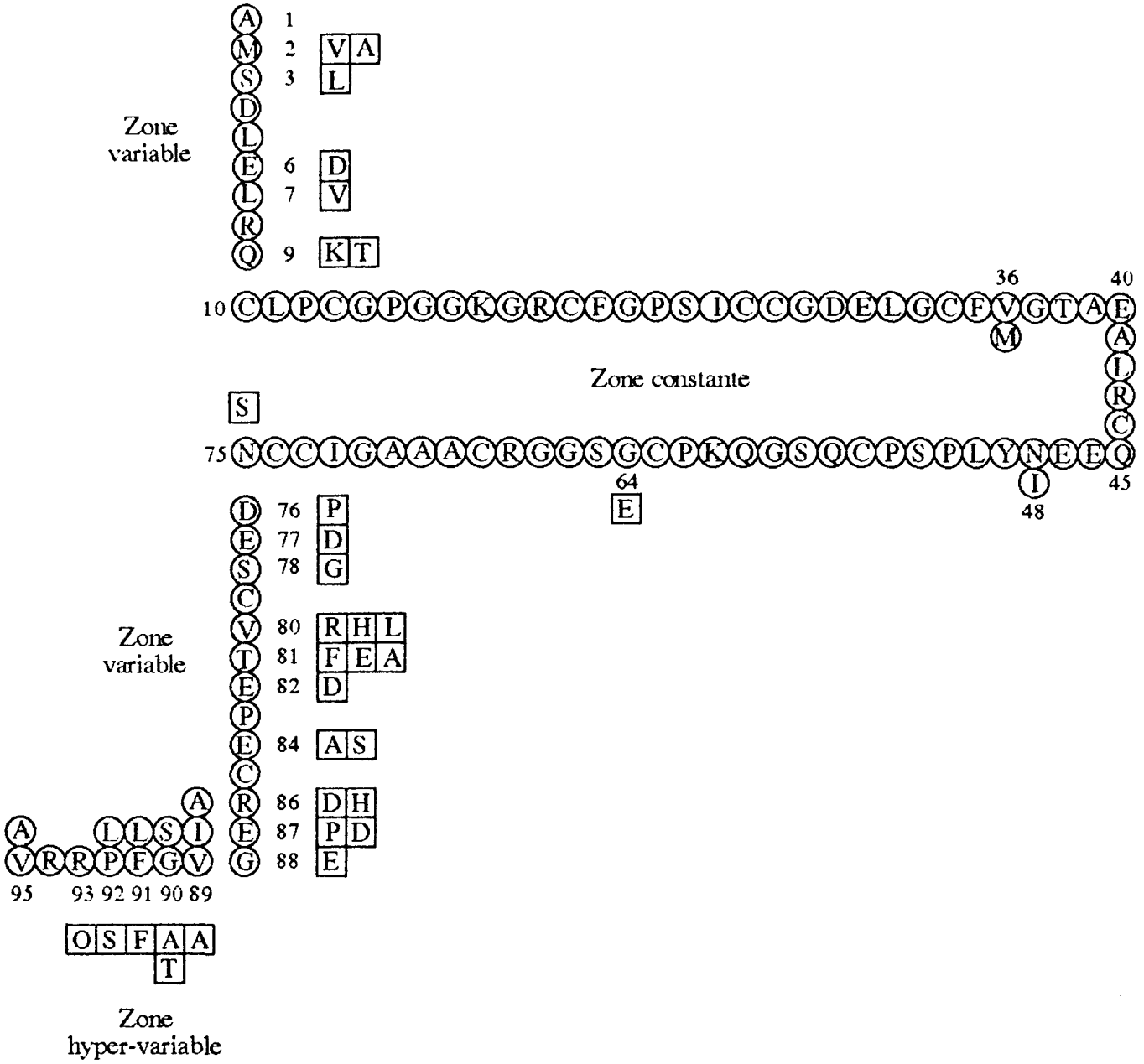


Figure 14- Comparaison entre les deux familles de neurophysines (MSEL-neurophysines○ et VLDV-neurophysines□). La zone variable change d'une famille à l'autre, la zone hypervariable change à l'intérieur d'une même famille et d'une famille à l'autre (d'après Acher *et al.*, 1985).

présent pu être isolées et séquencées. Parmi celles-ci, cinq sont apparentées à la vasotocine et une est apparentée à la mésotocine [Figs 12 et 13C].

Parmi, les 5 molécules apparentées à la vasotocine qui ont été isolées, celles des Mollusques Gastéropodes *Aplysia kurodai*, *Conus geographus* et *Lymnaea stagnalis* sont identiques, elles correspondent à la lysine-conopressine. Cette molécule présente, par rapport à la vasotocine, une phénylalanine à la place d'une tyrosine en position 2, une arginine à la place d'une glutamine en position 4 et une lysine à la place d'une arginine en position 8 (Cruz *et al.*, 1987 ; Van Kesteren *et al.*, 1992 ; McMaster *et al.*, 1992). Les 2 autres molécules isolées respectivement chez le Mollusque Gastéropode *Conus striatus* et chez l'Insecte *Locusta migratoria* présentent chacune uniquement des substitutions en positions 2 et 4 (Cruz *et al.*, 1987 ; Proux *et al.*, 1987).

La molécule du Mollusque Céphalopode *Octopus vulgaris* (Céphalotocine) possède 78 % d'homologie avec la mésotocine et l'isotocine, ce qui la classe dans la lignée des ocytocine-like (Reich, 1992).

Ainsi, une molécule apparentée à la vasotocine a été retrouvée chez les Mollusques Gastéropodes. Par contre, chez le Mollusque Céphalopode *O. vulgaris,* une molécule apparentée à la famille des ocytocines a été isolée. Chez les Insectes, une vasopressine-like a été purifiée. Ces résultats tendent à impliquer une duplication d'un gène ancestral pour les molécules apparentées aux OT et VP qui aurait peut-être eu lieu il y a 400 millions d'années (Van Kesteren *et al.*, 1992).

2. Les Précurseurs

L'organisation générale des précurseurs de la famille des ocytocines/vasopressines est bâtie selon un schéma identique pour les 2 molécules. Les peptides (OT ou VP) sont associés à une petite protéine acide de 93 à 95 résidus : la neurophysine (NP). Deux types distincts de neurophysines ont été mis en évidence. [Fig. 14] : les MSEL- et les VLDV-neurophysines, ainsi dénommées en se basant sur la nature des acides aminés en position 2, 3, 6 et 7 (Acher *et al.*, 1985). En fait, ces neurophysines diffèrent entre elles par leurs séquences N-terminale (1 à 9) et C-terminale (76-95), leur partie centrale (10-75) étant identique. Chez les 2 précurseurs, la position des cystéines est constante ainsi que celles des 7 ponts disulfures. Les MSEL-neurophysines ont été séquencées chez 6 espèces de Mammifères (mouton, porc, cheval, rat, bœuf et baleine). Elles contiennent toutes 95 résidus. Des variations par substitution ont été trouvées uniquement au niveau de 7 acides aminés (Chauvet *et al.*, 1981). Les VLDV-neurophysines ont été séquencées chez 4 espèces de Mammifères (mouton, porc,

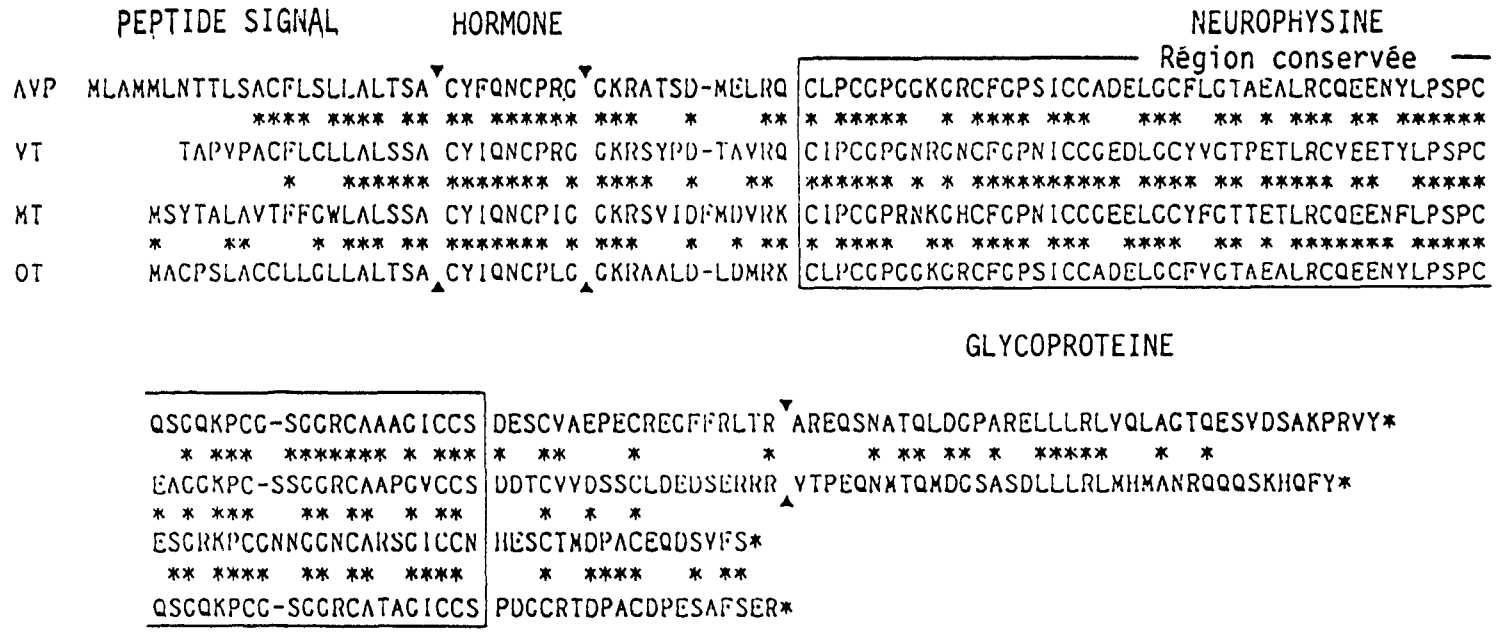


Figure 15- Comparaison des séquences d'acides aminés des précurseurs de la vasotocine (VT) et de la mésotocine (MT) du crapaud et de l'arginine-vasopressine (AVP) et de l'ocytocine (OT) du rat (d'après Nojiri *et al.*, 1987).

Les acides aminés identiques sont indiqués par des astérisques.
Les régions conservées des neurophysines sont encadrées.
Les flèches indiquent la limite entre les domaines constituant les précurseurs.

cheval et rat). Elles contiennent toutes 93 résidus et, en prenant comme référence le précurseur isolé chez le porc, le nombre de substitutions sur l'ensemble de la séquence varie de 6 à 9.

a. Le précurseur de l'ocytocine

C'est une protéine d'environ 11 kDa, constituée de l'ocytocine (≈ 1 kDa) associée à la VLDV-neurophysine (≈ 10 kDa) [Fig. 15]. Le gène de celui-ci [Fig. 16] comprend 3 exons (A, B, C) et 2 introns (I, II). La taille respective de chacun d'entre eux est de 157 pb pour l'exon A, 202 pb pour l'exon B, 116 pb pour l'exon C, 220 pb pour l'intron I et 84 pb pour l'intron II. Le premier exon code pour le peptide signal, l'ocytocine et les 9 premiers résidus de la VLDV-neurophysine, le second pour la partie centrale du précurseur (résidus 10-76) et le dernier pour la partie C-terminale (résidus 77-93).

Le clivage de la pro-ocytocine s'effectue au sein de la vésicule de sécrétion de la façon suivante [Fig. 17]. La pro-ocytocine est d'abord clivée par une endopeptidase (PCE) coupant spécifiquement les résidus dibasiques en 2 molécules intermédiaires : l'ocytocine (OT)-Gly-Lys-(Arg) et la neurophysine (NP)-Arg-COOH. L'OT-Gly-Lys-(Arg) subit ensuite l'attaque d'une carboxy-peptidase (CPB) et d'une endoamineoxydase pour donner le produit fini, l'OT. En ce qui concerne la NP-Arg-COOH, deux enzymes interviennent : une carboxy-peptidase (CPB) et une aminopeptidase (AP). Le clivage de la NP-Arg-COOH s'effectue par la CPB, puis par l'AP ou vice-versa, ceci afin de donner le produit fini, la VLDV-NP.

b. Le précurseur de la vasopressine

C'est une protéine de 15 à 17 kDa constituée de la vasopressine attachée à la MSEL-neurophysine, l'ensemble étant associé à une glycoprotéine (GP) de 39 acides aminés (Michel *et al.*, 1990) [Fig. 15]. Son gène est organisé comme celui de l'ocytocine : il comprend 3 exons et 2 introns. L'exon A possède 163 pb, l'exon B 202 pb, l'exon C 210 pb, l'intron I 1000 pb et l'intron II 227 pb [Fig. 16]. L'exon A code pour le peptide signal, la vasopressine et les 9 premiers acides aminés de la MSEL-neurophysine, l'exon B pour la partie centrale de la MSEL-neurophysine (10-77) et l'exon C pour la glycoprotéine de la partie C-terminale (78-93) (North, 1987).

Le clivage de la pro-vasopressine est comparable à celui de l'OT. Une PCE la clive en 2 intermédiaires : la VP-Gly-Lys-Arg et la NP-Arg-GP. La VP-Gly-Lys-Arg est coupée en VP-Gly-Lys puis en VP-Gly par une carboxypeptidase. Une α-amide E (endoamineoxydase) agit ensuite pour libérer la vasopressine. La

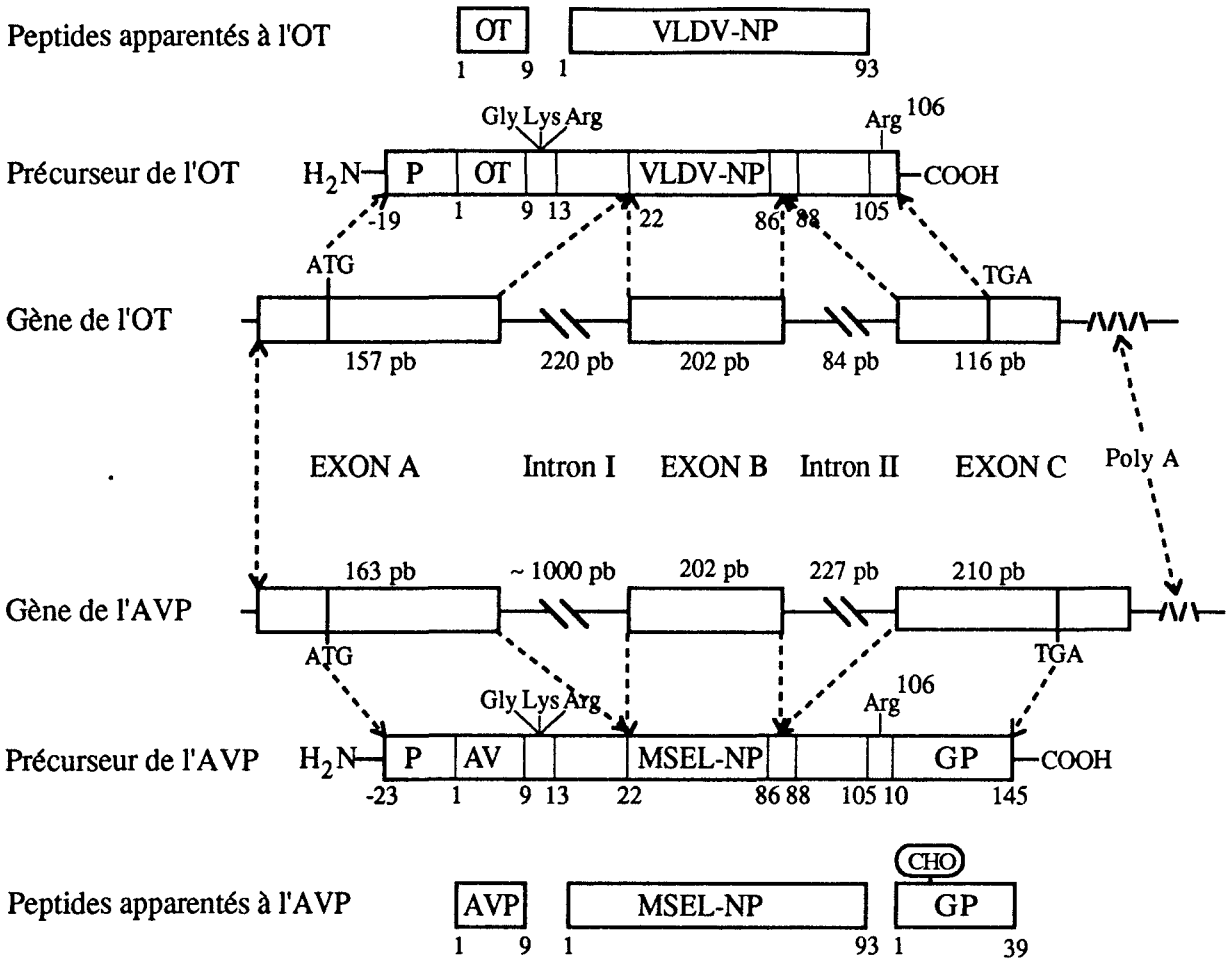


Figure 16- Organisation des gènes de l'ocytocine (OT) et de l'arginine- vasopressine (AVP) chez le rat, relations entre ces gènes et leurs précurseurs respectifs (préprohormones), et produits finis (peptides) (d'après Gainer *et al.*, 1988).
ATG : site d'initiation de la traduction ; CHO : groupement associé à la glycoprotéine ; GP : glycoprotéine ; VLDV-NP : VLDV-neurophysine ; MSEL-NP : MSEL-neurophysine ; pb : paire de bases ; PS : peptide signal, TGA : site d'arrêt de la traduction.

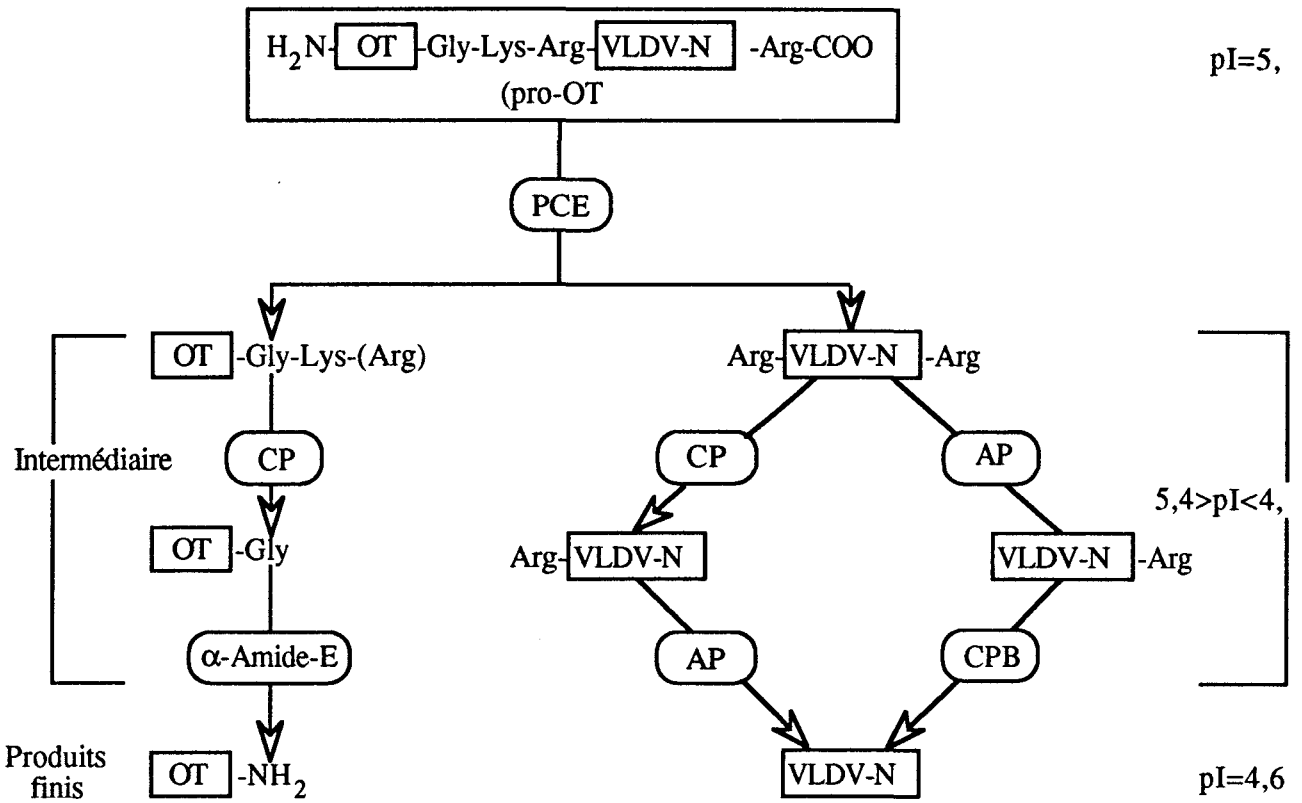


Figure 17- Représentation schématique du clivage de la pro-ocytocine (pro-OT) (d'après Gainer *et al*, 1988).
Les produits issus du clivage [ocytocine (OT), VLDV-neurophysine (VLDV-NP)] sont représentés par des rectangles. Seuls les acides aminés excisés durant le"processing" sont indiqués dans le précurseur et dans les formes intermédiaires (*e.g.* Gly-Lys-Arg). PCE : enzyme de conversion de la prohormone (endopeptidase) ; CPB : carboxypeptidase ; AP : aminopeptidase B ; α-Amide-E : endoamineoxydase. Les pIs (points isoélectriques de la prohormone, des intermédiaires de la neurophysine et de la neurophysine) sont indiqués sur la droite de la figure.

NP-Arg-GP subit, quant à elle, l'attaque enzymatique de 2 proneurophysinases pour libérer la NP et la GP [Fig. 18].

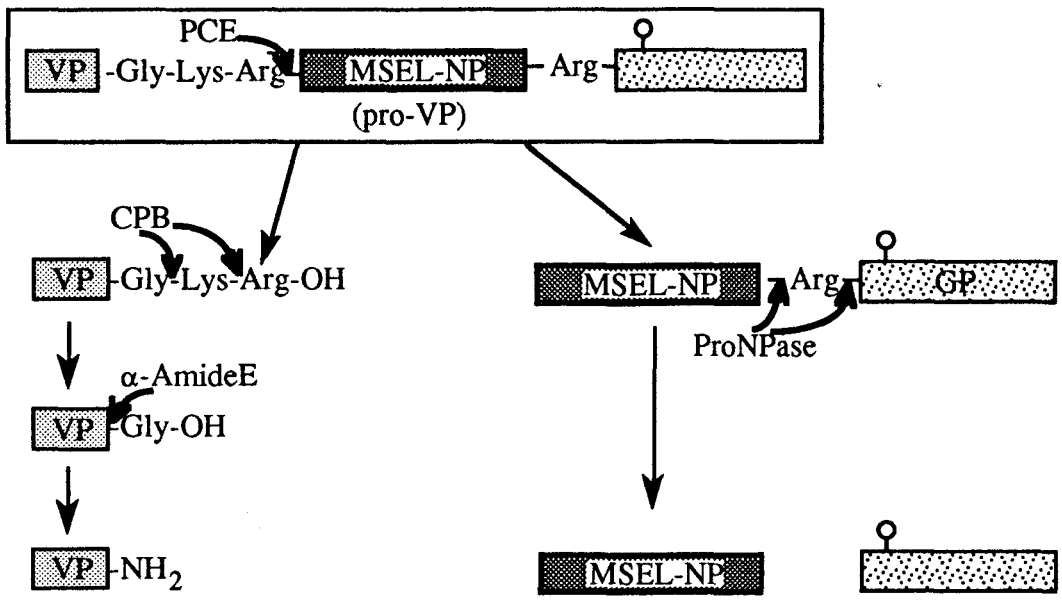


Figure 18- Représentation schématique des voies de clivage principales de la pro-vasopressine (pro-VP) (d'après North, 1987).
Les flèches courbes indiquent les sites clivés par les enzymes (PCE : enzyme de conversion de la prohormone (endopeptidase), CPB : carboxypeptidase, α-AmideE : endoamineoxydase, ProNPase : proneurophysinase). GP : glycoprotéine, MSEL-NP : MSEL-neurophysine, VP : vasopressine.

c. Les précurseurs au cours de l'évolution

La figure 19 présente une évolution possible des précurseurs de la famille des OT/VP proposée par Acher *et al.*, (1985).

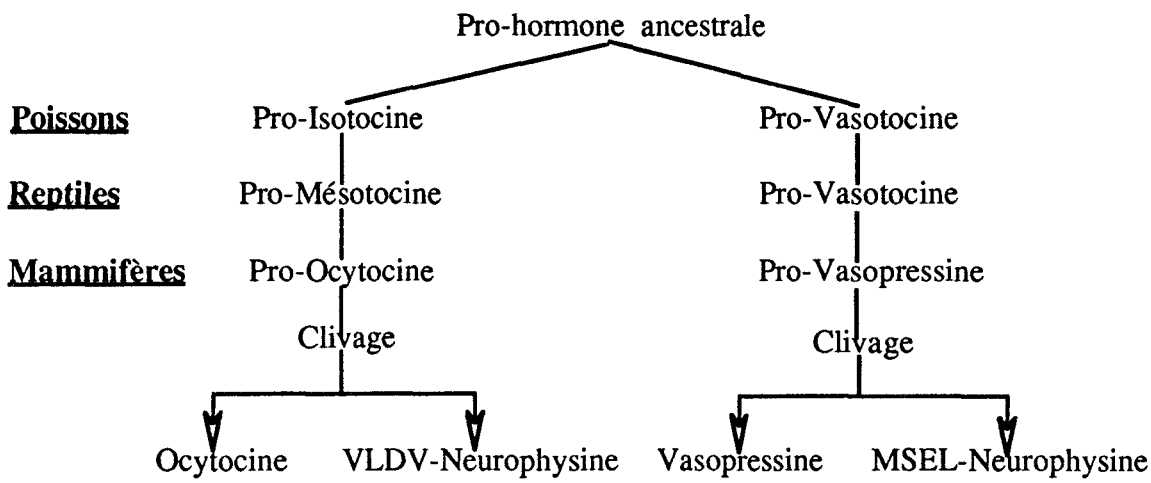


Figure 19 - Evolution possible des précurseurs de la famille des ocytocines/vasopressines chez les Vertébrés (d'après Acher *et al.*, 1985).

Chez les Invertébrés, des études immunohistochimiques au niveau du cerveau de l'Insecte *L. migratoria* ont montré une colocalisation de 2 épitopes au sein des mêmes cellules, l'un reconnu par l'anticorps anti-neurophysine, l'autre par l'anticorps anti-vasopressine (Romeuf *et al.*, 1986). De plus, les résultats récents de Van Kesteren *et al.* (1992) ont établi un pourcentage d'homologie de respectivement 49 % et 45 % entre le précurseur de la lysine-conopressine trouvé chez la limnée et les précurseurs de la vasopressine et de l'ocytocine humaines. Le précurseur de la lysine-conopressine des Gastéropodes est donc bâti sur un plan comparable à celui des Vertébrés. De plus, le fait que le précurseur de la lysine-conopressine de limnée présente un pourcentage d'homologie sensiblement équivalent avec ceux de la vasopressine et de l'ocytocine laisse présager l'existence d'un précurseur commun aux 2 lignées [Fig. 13C]. Les résultats des séquençages des peptides chez le Céphalopode *O. vulgaris* (Reich, 1992) et chez l'Insecte *L. migratoria* (Proux *et al.*, 1987) sont en faveur d'une duplication d'un gène ancestral qui aurait généré d'un côté la lignée de l'isotocine et de l'autre celle de la vasotocine. Chaque lignée aurait ensuite subi des substitutions indépendantes ayant conduit chez les Mammifères aux couples ocytocine/VLDV-neurophysine et vasopressine/MSEL-neurophysine (Van Kesteren *et al*, 1992).

3. Rôle physiologique, récepteurs et mode d'action

a. Rôle physiologique

α *Chez les Vertébrés*

L'ocytocine est synthétisée au niveau du cerveau (hypothalamus) et libérée dans la circulation sanguine générale au niveau hypophysaire. Chez les Mammifères, elle a 3 effets principaux : elle stimule la contraction utérine, provoque la montée de lait et augmente la synthèse de prolactine hypophysaire. La présence de récepteurs ocytociques chez les mâles tend à indiquer un rôle physiologique plus large : contrôle possible de certaines fonctions rénales, modulation de la synthèse de progestérone au niveau testiculaire, action neuromodulatrice au niveau du système nerveux central (Laevitt *et al.*, 1987).

La vasopressine est synthétisée dans les noyaux supra-optiques et paraventriculaires de l'hypothalamus et, tout comme l'ocytocine, elle est libérée dans la circulation sanguine générale au niveau du lobe neural de l'hypophyse. Elle a 2 rôles physiologiques majeurs : la réabsorption de l'eau au niveau rénal et une action pressique au niveau des cellules musculaires de la paroi des vaisseaux sanguins.

β *Chez les Invertébrés*

Les études les plus approfondies ont été réalisées chez l'Insecte *L. migratoria*. Chez cet animal, la diurèse est la principale fonction régulant la déperdition hydrique. Celle-ci résulte des actions successives de deux organes, les tubes de Malpighi et le rectum. Les tubes de Malpighi jouent un rôle diurétique en excrétant l'urine primaire dans laquelle se concentrent des anions et cations inorganiques (K^+, Na^+,Cl^-...) ou organiques (acide hippurique, sulfones et alcaloïdes). Cette urine se déverse ensuite dans le tube digestif entre les intestins moyen et postérieur et se mêle aux produits de la digestion. Au niveau du rectum intervient une fonction antidiurétique. Une partie de l'eau et de certains ions contenus dans les excréments est réabsorbée avant que ceux-ci ne soient évacués à l'extérieur (Picquot et Proux, 1987). Un test biologique basé sur la propriété physiologique des tubes de Malpighi a été mis au point par Mordue et Goldsworthy (1969). Il a permis la purification d'une substance vasopressine-like (Schooley *et al.*, 1987). Cette molécule est présente sous 2 formes : monomérique et dimérique. Si le monomère ne présente pas d'activité biologique, le dimère (forme anti-parallèle), par contre, a un effet diurétique, contrairement à ce que l'on constate chez les Vertébrés où il a un rôle antidiurétique. Proux et Hérault (1988) ont montré que l'AVP-like augmente le taux d'AMPc et l'excrétion urinaire au niveau des tubes de Malpighi isolés, ce qui laisse présager un système de transduction utilisant l'AMPc. Récemment, Picquot et Proux (1990) ont démontré que la substance AVP-like est secrétée sous forme de monomère au niveau du ganglion sous-œsophagien et se transforme à ce niveau en dimère. Le monomère et le dimère sont relargués en même temps dans l'hémolymphe où la transformation du monomère en dimère continue de s'effectuer. Le dimère est ensuite transporté au niveau des tubes de Malpighi (tissu cible) où s'exerce son action biologique, avant d'être dégradé par une cascade enzymatique.

Chez le Mollusque *A. californica*, un effet indirect de la substance vasotocine-like sur l'osmorégulation a pu être démontré. Il s'exerce *via* les neurones dénommés R15 qui réagissent en libérant une neurohormone se fixant sur des osmorécepteurs situés au niveau des néphridies (Moore *et al.*, 1981).

b. Les récepteurs

Le point des connaissances relatives aux récepteurs est détaillé dans la revue de Guillon (1989). Nous rappellerons ici les données essentielles, elles concernent les Mammiféres.

Tableau V - Tissus cibles et actions physiologiques de la vasopressine et de l'ocytocine chez les Mammifères. (d'après Guillon, 1989).

Hormone	Tissus cible	Sous-type de récepteur	Fonction physiologique	Systèmes de transduction impliqués
Vasopressine	Rein	V2	Réabsorption de l'eau	Stimulation de l'AC
	Foie	V1a	Stimulation de la glycogénolyse	Stimulation de la PC et d'une perméabilité calcique membranaire
	Surrénale	V1a	Sécrétion de minéralocorticoïdes	
	Cellule musculaire lisse	V1a	Contraction	
	Testicule (cellule de Leydig)	V1	Synthèse de progestérone	Stimulation de la PC et inhibition de l'AC
	Plaquette	V1a	Agrégation	
	Tractus génital mâle -vésicule séminale -épididyme	V2? V1	Contraction	Stimulation de la PC
	Vessie	V1		
	Tractus génital femelle (myomètre)	V1a		Stimulation de la PC
	Adénohypophyse	V1b	Sécrétion d'ACTH	Stimulation de la PC
	Hippocampe	V1a	Neurotransmission ?	Stimulation de la PC
	Ganglion supérieur	V1a	Neurotransmission ?	Stimulation de la PC
	Lymphocyte	V1?	Modulation de la synthèse de lymphokine	
Ocytocine	Glande mammaire		Ejection du lait	Stimulation de la PC
	Tractus génital femelle		Contraction	Stimulation de la PC et probablement d'une perméabilité calcique et inhibition d'une $(Ca^{2+}Mg^{2+})$ATPase

AC = Adénylate Cyclase
ACTH = Hormone corticotrope
PC = Phospholipase C

α *Les récepteurs à l'ocytocine*

* Localisation des récepteurs

Les récepteurs ocytociques spécifiques ont été découverts au niveau des organes cibles traditionnels : l'utérus, la glande mammaire (Tableau V). De plus, la présence de sites à OT a été relatée au niveau du cortex rénal, des cellules de Leydig, des adipocytes et de zones bien précises du cerveau (Laevitt *et al.*, 1987).

* Caractérisation des récepteurs [Fig. 20]

L'utilisation d'analogues de structure de l'OT a permis de distinguer 2 types de récepteurs avec des affinités différentes (Guillon, 1989) : l'un à forte affinité retrouvé au niveau de l'utérus et de la glande mammaire, l'autre avec une affinité plus faible interagissant sur les récepteurs à VP. A l'heure

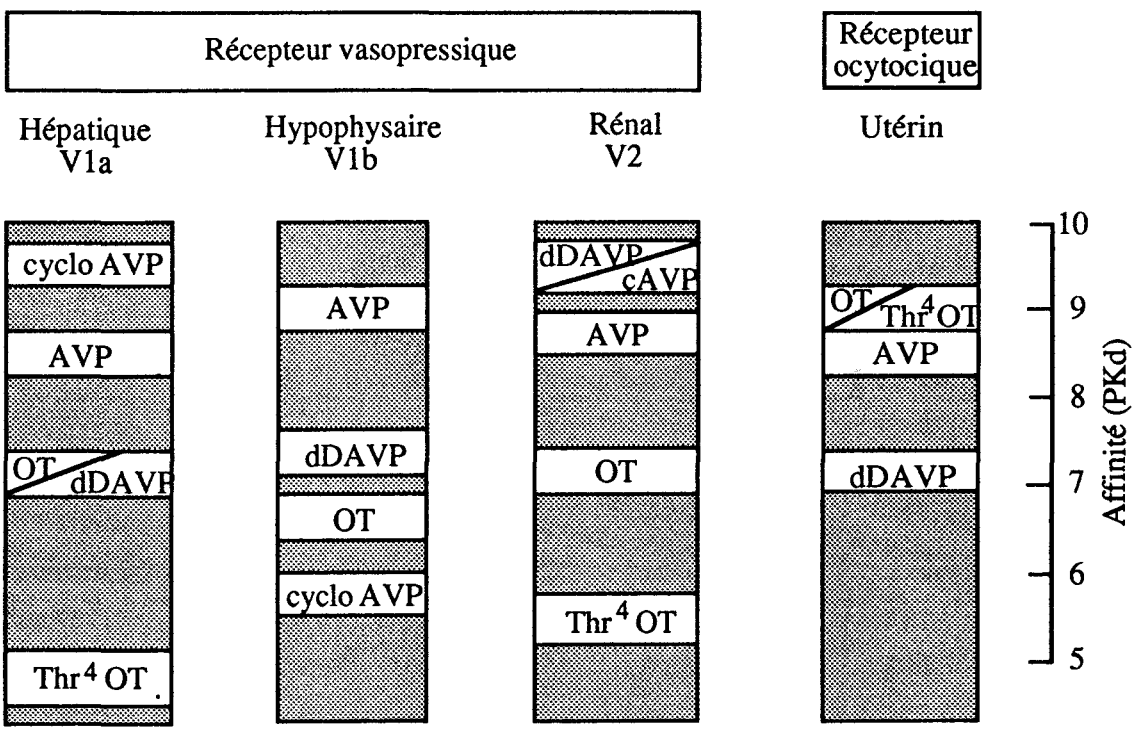


Figure 20- Pharmacologie comparative des récepteurs vasopressiques et ocytociques chez le rat (Tiré de Guillon, 1989).
Les affinités de 5 analogues structuraux de la vasopressine et de l'ocytocine, choisis pour leur sélectivité vis-à-vis des divers types de récepteurs étudiés, ont été mesurées sur des membranes de foie de rat (récepteur vasopressique V1a), sur des membranes d'hypophyse de rat (récepteur vasopressique V1b), sur des membranes de rein de rat (récepteur V2) ou sur des membranes d'utérus de rate (récepteur ocytocique). Les résultats sont exprimés en pKd (-log(Kd))pour les 4 tissus étudiés. AVP : Arginine^{8} Vasopressine, OT : Ocytocine, Thr OT : Thréonine^{4} Glycine^{7} Ocytocine, dDAVP : diamino (8-Darginine)-Vasopressine, CycloAVP : (acide propionique 1ß-mercapto-ßß cyclopentaméthylène 2-O-éthyl Tyrosine 4-Valine) Arginine Vasopressine.

actuelle, les travaux relatifs au récepteur à forte affinité ont permis, en utilisant plusieurs analogues de structures, de l'isoler au niveau du myomètre et de la glande mammaire de rate. Il a déjà été solubilisé et l'estimation de sa masse moléculaire est de 60 kDa dans les 2 cas (Soloff *et al.*, 1988).

* Mode d'action de l'ocytocine

Cette hormone agit principalement en augmentant la concentration de calcium intracellulaire. Plusieurs mécanismes sont responsables de cette action [voir Tableau V] : la stimulation d'une phospholipase C, l'inhibition d'une $(Ca^{2+}Mg^{2+})$ATPase présente dans les membranes plasmiques de myomètre de rate et une possible stimulation hormonale d'un influx calcique. L'augmentation de la concentration du calcium cytosolique résultant de ces 3 types de processus stimule des kinases, calmodulines dépendantes, et conduit aux effets physiologiques associés à l'ocytocine.

* Modulation des récepteurs

Une des propriétés importantes des récepteurs ocytociques de la glande mammaire et de l'utérus des mammifères est leur grande plasticité. Ainsi par exemple, lors de la grossesse, la densité des sites OT de l'utérus augmente de 10 à 50 fois selon les espèces. Elle atteint son maximum juste avant la parturition et revient à son état basal quelques jours après (Laevitt *et al.*, 1987).

β *Les récepteurs à la vasopressine*

* Localisation des récepteurs

A côté des organes cibles déjà connus (le rein et les cellules musculaires lisses), un grand nombre de tissus d'origines diverses possèdent des récepteurs spécifiques à la vasopressine [Tableau V].

* Caractérisation des récepteurs [Fig. 20]

La classification des récepteurs à la vasopressine est basée sur le mécanisme de transduction : stimulation d'une adénylate cyclase (AC) (cas des récepteurs rénaux) ou stimulation d'une phospholipase C (PC) (cas des autres récepteurs). Compte tenu de ces données, Michel *et al.* proposèrent en 1979 le terme de V1 pour les récepteurs vasopressinergiques couplés à la PC et celui de V2 pour ceux couplés à l'AC. Un second critère, basé sur la structure du site de liaison du récepteur, a été pris en compte [Tableau V]. La mesure de l'affinité d'une série d'analogues structuraux de la vasopressine a permis de comparer leurs propriétés

Tableau VI- Membres de la famille des peptides apparentés au FMRF-amide (RFamides) dans le règne animal (d'après Walker, 1992).

Groupe zoologique	Peptide
Cœlentérés	pQGRFa
	QGRFa
Plathelminthes	PDKDFIVNPSDLVLDNKAALRDYLRQINEYFAIIGRPRFa
Némathelminthes	KNEFIRFa
	KHEYLRFa
	SDPNFLRFa
Annélides	FMRFa
	FLRFa
	FTRFa
	YMRFa
	YLRFa
	GGKYMRFa
Mollusques	FLRFa
	FMRFa
	pQDPFLRFa
	SDPFLRFa
	NDPFLRFa
	SGQSWRPQGRFa
	GDPFLRFa
	SDPYLRFa
	SDPFFRFa
	ALTNDHFLRFa
	GSLFRFa
	SSLFRFa
	TFLRFa
	AFLRFa
	YGGFMRFa
	ALAGDHFFRFa
Arthropodes	FLRFa
	PDVDHVFLRFa
	pQDVDHVFLRFa
	pQRPhPSLKTRFa
	EQFEDYsGHMRFa
	pEDVVHSFLRFa
	TNRNFLRFa
	SDRNFLRFa
Vertébrés	LPLRFa
	YGGFMRFa
	FLFQPQRFa
	YVMGHRFRWDRFa
	AGEGLSSPFWSLAAPQRFa

intrinsèques, ce qui a permis de subdiviser les récepteurs V1 en 2 sous-types : V1a (récepteurs dont le profil s'apparente au récepteur du foie) et V1b (récepteurs au profil proche du récepteur hypophysaire) [Fig. 20]. A l'heure actuelle, le récepteur V1a du foie de rat est presque totalement purifié. Cette molécule possède une masse moléculaire de 60 kDa (Dickey *et al.*, 1987).

* Mode d'action de la vasopressine

Lors de la fixation de la vasopressine sur son récepteur spécifique, essentiellement 3 types de réactions biochimiques peuvent survenir selon le récepteur considéré [Tableau V] : couplage positif (cas des récepteurs rénaux V2) ou négatif (cas de certains récepteurs V1) à l'adénylate cyclase, couplage positif à la phospholipase C (cas de la majorité des récepteurs V1).

* Modulation des récepteurs

La densité de récepteurs à la vasopressine est dépendante de la présence de nombreuses hormones. Une élévation de la concentration d'arginine-vasopressine circulante conduit à une diminution significative de ses récepteurs. Cet effet s'observe ausssi bien pour les V2 que pour les V1.

V- La famille des peptides apparentés au FMRF-amide (RFamides)

Le FMRF-amide (FMRFa) a été isolé pour la première fois à partir du système nerveux central du Lamellibranche *Macrocallista nimbosa* par Price et Greenberg (1977). A la suite d'études immunocytochimiques et biochimiques, ce tétrapeptide s'est révélé être ubiquitaire dans tout le règne animal. L'isolement de ce produit à l'aide d'anticorps dirigés contre la partie C-terminale (RFamide: RFa) a conduit à l'identification de toute une famille de peptides, celle des RFa, qui comprend le FMRFa et des peptides qui lui sont apparentés par cette séquence C-terminale [Tableau VI]. Ainsi, deux grandes classes de RFa ont pu être mises en évidence : la première est constituée par des tétrapeptides (FMRFa, FLRFa, YMRFa, YLRFa); la seconde correspond aux tétrapeptides possédant une extension N-terminale.

1. Précurseurs des RFa peptides

Chez les Mollusques *Aplysia californica* et *L. stagnalis*, les gènes des précurseurs du FMRFa ont été clonés et séquencés (Taussing et Scheller, 1986 ; Linacre *et al.*, 1990). Chez les 2 espèces, ils codent plusieurs peptides. En effet,

chez *A. californica* [Fig. 21], 28 copies du FMRFa, une copie du FLRFa, des séquences homologues au CRF (corticolibérine), à la α-MSH (melanophore stimulating hormone) et au CLIP (corticotropin-like intermediate lobe peptide) ont été trouvées. Chez *L. stagnalis*, le précurseur du FMRFa est 2 fois plus petit que celui d'*A. californica* (Linacre *et al.*, 1990). Il est associé, de façon contiguë du côté 3', au gène codant pour les heptapeptides [GDPFLRFa/SDPFLRFa] isolés en 1987 par Eberrink *et al.* (Saunders *et al.*, 1991).

De même, chez l'Insecte *Drosophila melanogaster*, Schneider et Taghert, (1988) ont isolé le gène du FMRFa. Il renferme des multicopies d'un même peptide (5 copies du DPKQDFMRFa) et 13 séquences peptidiques apparentées au FMRFa.

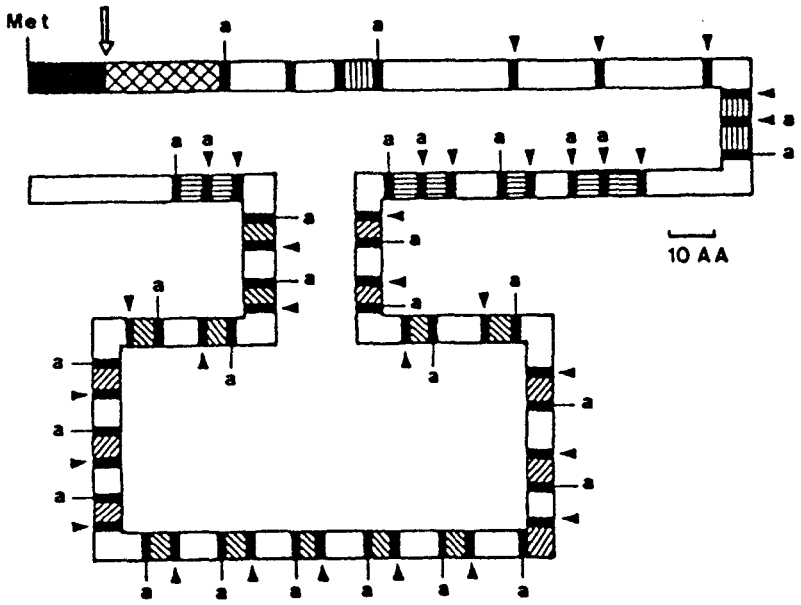


Figure 21- Représentation hypothétique du précurseur du FMRF-amide chez le Mollusque *Aplysia* californica (d'après Taussing et Scheller, 1986).
La méthionine d'initiation à l'extrémité N-terminale (Met) est suivie de la séquence du signal hydrophobe (zone noircie). La large flèche indique le site au niveau duquel cette séquence se sépare du précurseur et le début du peptide GYLRF-amide (zone quadrillée). Les barres verticales noires représentent les résidus d'acides aminés basiques, sites potentiels de coupure. Les sites de clivage simple sont indiqués par les pointes de flèche noires, ceux possédant un signal d'amidation (résidu glycine) sont signalés par la lettre "a". La seule copie de FLRF-amide est hachurée verticalement. Les copies de FMRF-amide localisées dans la région "ancestrale" du précurseur sont hachurées horizontalement, celles de la région itérative sont hachurées obliquement.

2. Activité biologique des RFa peptides

Si les fonctions des peptides de cette famille sont multiples [Tableau VII], essentiellement trois actions se retrouvent tout au long du règne animal : neurotransmission/neuromodulation, action myogénique et effet cardio-excitateur.

Tableau VII - Principales fonctions biologiques des RFamides dans le règne animal.

Groupe zoologique	Fonctions biologiques
Cœlentérés, Plathelminthes et Némathelminthes	Neuromodulation/Neurotransmission Action sur le système musculaire
Annélides	Neurotransmission/Neuromodulation Action sur le système musculaire Effet cardioexcitateur
Mollusques	Neurotransmission/Neuromodulation Action sur le système musculaire Effet cardioexcitateur Action hormonale : - au niveau de la reproduction - au niveau de l'alimentation - au niveau de l'osmorégulation
Crustacés	Neurotransmission/Neuromodulation Action sur le système musculaire Effet cardioexcitateur Action sur le processus de libération des hormones chromotropes Intégrations des informations visuelles et olfactives
Insectes	Neurotransmission/Neuromodulation Effet cardioexcitateur
Vertébrés	Neurotransmission/Neuromodulation Action sur le système musculaire Effet cardioexcitateur Rôle d'antagoniste des opioïdes endogènes Action hormonale (inhibition de la décharge d'insuline et de somatostatine) Action sur la fonction gastro-intestinale Action sur l'osmorégulation Action sur le comportement (dressage, amnésie)

Récemment, une action des RFa sur l'osmorégulation a été envisagé. Celle-ci peut être directe comme suggéré chez le Mollusque *Helisoma duryi* où les résultats de Saleuddin *et al.* (1992) plaident en faveur d'un effet antidiurétique du FMRFa, ou indirect, comme chez les Vertébrés où Majane et Yang (1991) ont mis en évidence un effet d'un RFa (YGGFMRFa) sur l'arginine-vasopressine en réponse à un stimulus osmotique.

3. Relations Structures/Fonctions

Des études réalisées sur différents tissus de Mollusques(musculaires, et nerveux) ont montré que l'activité biologique des RFa peptides dépend de leur structure. Ainsi, la substitution d'un ou de plusieurs des quatre résidus Phe^1, Met^2, Arg^3, Phe^4, modifie l'activité de ces molécules.

Pour garder leur activité biologique, il est indispensable que les RFa peptides possèdent à la fois l'amidation C-terminale, un acide aminé amidé en position 3, un résidu non polaire en position 2 et un acide aminé possédant un groupement aromatique et non polaire en position 1.

La structure des RFa conditionne la liaison et la discrimination des récepteurs à ces peptides.

Chez *L. stagnalis*, un seul type de récepteur au niveau des cellules caudo-dorsales (CDCs) des ganglions cérébroïdes serait impliqué dans l'induction de la réponse hyperpolarisante et dans la suppression de l'excitabilité des CDCs (Brussaard *et al.*, 1989). Par contre, chez *Helix aspersa,* des sites récepteurs d'affinité et de spécificité différentes seraient présents au sein de divers organes et notamment au niveau du cœur et du cerveau (Payza, 1987 ; Payza *et al.*, 1989). Chez *H. aspersa*, les heptapeptides et tétrapeptides n'interagiraient pas sur les mêmes sites récepteurs. Ainsi, à faible dose, les heptapeptides ont des effets cardioexcitateurs plus importants que le FMRFa. D'autre part, au niveau des muscles rétracteurs, le FMRF-amide provoque une contraction alors que l'heptapeptide pQDPFLRF-amide entraîne un relâchement du muscle contracté (Payza *et al.*, 1989). Les effets distincts des tétra- et des hepta-peptides sur les neurones du système nerveux central d'*H. aspersa* seraient dus à l'existence de sites récepteurs multiples (Cottrell et Davies, 1987).

L'ensemble de ces résultats montre que la partie active des peptides de la famille des RFa est la partie C-terminale Arg-Phe-amide et que les substitutions ou extensions N-terminales des RFa peptides servent à la fixation et à la reconnaissance des récepteurs.

RESULTATS

Cartographie immunologique du follicule 4 du ganglion supra-œsophagien de *T. tessulatum.*

Article présenté

(1) Verger-Bocquet, M., Wattez, C., Salzet, M. and Malecha, J., Immunocytochemical identification of peptidergic neurons in compartment 4 of the supraesophageal ganglion of the leech *Theromyzon tessulatum* (O. F. M.), *Can. J. Zool.*, (1992), 70, 856-865 .

La cartographie du follicule 4 de *T. tessulatum* réalisée à l'aide d'anticorps polyclonaux spécifiques d'hormones peptidiques de Mammifères et d'anticorps monoclonaux spécifiques de molécules issues de neurones du ganglion supra-œsophagien de cette sangsue a permis la caractérisation de plus de la moitié des 30 cellules présentes dans ce follicule. Sept types cellulaires ont été identifiés au stade 3B du cycle biologique de l'animal : (1) un groupe de 4 à 5 cellules immunopositives aux anticorps polyclonaux dirigés contre l'angiotensine II (a-AII) et la γ-mélanostimuline (a-γ MSH) et aux anticorps monoclonaux Tt7 et Tt159, (2) un groupe de 5 cellules immunoréactives à l'anti-somatostimuline (a-GRF), (3) trois cellules immunoréactives à l'anti-motiline, (4) une cellule immunopositive à l'anti-met-enképhaline, (5) une cellule immunoréactive à la fois à l'anti-ocytocine (a-OT) et à l'anticorps monoclonal Tt1, (6) une cellule immunoréactive à l'anti-lysine-vasopressine (a-LVP) et à l'anticorps monoclonal Tt9, (7) six cellules immunoréactives à l'anti-FMRF-amide (a-FMRFa) (Salzet *et al.*, inédit).

Le nombre de cellules immunoréactives détectées dans ce follicule n'est pas toujours constant, il peut varier pour deux raisons essentielles. La première est l'état physiologique de l'animal. Ainsi, une variation du nombre de cellules immunoréactives à l'anti-motiline a pu être observée chez *T. tessulatum* en fonction du degré de maturité sexuelle des animaux (Malecha *et al.*, 1989a). La seconde raison est la variabilité de la position de la cloison conjonctive séparant les follicules.

Cette étude montre l'hétérogénéité des neurones constituant le follicule 4. La colocalisation d'épitopes reconnus à la fois par des anticorps polyclonaux et par des anticorps monoclonaux reflète la diversité des substances existant au sein d'un même neurone.

La présence d'épitopes réactifs à l'a-AII, l'a-OT, l'a-LVP et l'a-FMRFa au sein du follicule 4, qui est connu pour être impliqué dans la régulation de la balance hydrique (Malecha, 1979, 1983), permet d'envisager une éventuelle intervention des molécules reconnues par ces anticorps dans le contrôle de l'homéostasie hydrominérale.

Immunocytochemical identification of peptidergic neurons in compartment 4 of the supraesophageal ganglion of the leech *Theromyzon tessulatum* (O.F.M.)

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The use of polyclonal antibodies directed against mammalian peptide hormones and of monoclonal antibodies raised against molecules of supraesophageal ganglion neurons of the leech *Theromyzon tessulatum* has led to the identification of more than half of the 30 neurons present in compartment 4 of the supraesophageal ganglion. Six cellular types were characterized at stage 3B of the life cycle: (1) a group of four or five large angiotensin II and γ-melanocyte stimulating hormone (γ-MSH) immunopositive cells also immunoreactive with monoclonal antibodies Tt-7 and Tt-159 (cells of class I), (2) a group of five small growth hormone releasing factor (GRF) positive cells, (3) three motilin-positive cells, (4) one met-enkephalin-positive cell, (5) one oxytocin-positive cell that also immunoreacts with monoclonal antibody Tt-1, and (6) one vasopressin-positive cell immunoreactive with monoclonal antibody Tt-9. This study shows the heterogeneity of the neurons constituting compartment 4 and demonstrates that most of them have secretions of a peptidergic nature. The co-localization of epitopes recognized by anti-γ-MSH and anti-angiotensin II is demonstrated in cells of class I. The number of immunoreactive cells found in compartment 4 is not always constant and can vary for the following reasons: (*i*) changes in the physiological status of the leech, as is the case with anti-GRF and anti-motilin, (*ii*) individual variations for some cellular types (cells of class I), (*iii*) variability in the situation of the connective-tissue septum separating compartments 4 and 5.

VERGER-BOCQUET, M., WATTEZ, C., SALZET, M., et MALECHA, J. 1992. Immunocytochemical identification of peptidergic neurons in compartment 4 of the supraesophageal ganglion of the leech *Theromyzon tessulatum* (O.F.M.). Can. J. Zool. **70** : 856–865.

L'utilisation d'anticorps polyclonaux spécifiques à des hormones peptidiques de mammifères et d'anticorps monoclonaux des molécules de neurones de ganglions supraesophagiens de la sangsue *Theromyzon tessulatum* a permis l'identification de plus de la moitié des 30 neurones présents dans le follicule 4 du ganglion supraoesophagien. Six types cellulaires ont été caractérisés au stade 3B du cycle biologique: (1) un groupe de quatre ou cinq grandes cellules immunopositives en présence des anticorps polyclonaux de l'angiotensine II et de la γ-mélanostimuline (anti-γ-MSH) et en présence des anticorps monoclonaux Tt-7 et Tt-159 (cellules de classe I), (2) un groupe de cinq petites cellules immunopositives en présence de l'anticorps du facteur de libération de l'hormone de croissance (anti-GRF), (3) trois cellules immunopositives en présence de l'anti-motiline, (4) une cellule immunopositive en présence de l'anti-met-enképhaline, (5) une cellule immunopositive en présence de l'anticorps polyclonal de l'ocytocine et de l'anticorps monoclonal Tt-1, (6) une cellule immunopositive en présence de l'anticorps polyclonal de la vassopressine et de l'anticorps monoclonal Tt-9. Cette étude démontre l'hétérogénéité des neurones qui constituent le follicule 4 et la nature peptidique des sécrétions de la plupart d'entre eux. La colocalisation d'épitopes reconnus par l'anti-γ-MSH et par l'anti-angiotensine II a été démontrée dans les cellules de la classe I. Le nombre de cellules immunoréactives détectées dans le follicule 4 n'est pas toujours constant et peut varier pour les raisons suivantes: (*i*) l'état physiologique de la sangsue (c'est le cas pour l'anti-GRF et l'anti-motiline), (*ii*) les variations individuelles chez certains types cellulaires (cellules de classe I), (*iii*) la variabilité de la position de la cloison conjonctive séparant le follicule 4 du follicule 5.

Introduction

The central nervous system of Hirudinea is built on a clearly metamerized framework, and numerous cells can be precisely identified from one preparation to another. Moreover, the number of neurons is relatively small and some of the cells are large, making them accessible to electrophysiological techniques and allowing circuits to be analyzed. For these reasons, neurons constituting the segmental ganglia have been extensively investigated in recent years, and the morphological and (or) physiological characteristics of more than 50% of them are now well known (Müller *et al.* 1981; Sawyer 1986). However, there have been very few studies of the supraesophageal ganglion, which, in the leech *Theromyzon*, is made up of about 500 cells distributed in six pairs of compartments, three anterior (numbered 1–3) and three posterior (numbered 4–6) Hagadorn 1958). Through neurohormones, this ganglion controls important functions: gametogenesis, chromatic adaptation, oxygen consumption, and osmoregulation (Sawyer 1986). To date, the primary structure of these neurohormones is still unknown. Nevertheless, studies on various animals in recent years give clear evidence that peptides play a fundamental role in nervous and neuroendocrine communications (Scharrer 1987*a*, 1987*b*). The diversity of the roles of these neuropeptides might be explained by their number, estimated to be on the order of a few hundred (De Loof and Schoofs 1990).

The aim of the present study is to map immunocytochemically the cells containing epitopes recognized by antibodies raised against vertebrate peptide hormones, in compartment 4 of the supraesophageal ganglion of the leech *Theromyzon tessulatum*. For this purpose, we used polyclonal antibodies raised against oxytocin, vasopressin, met-enkephalin, angiotensin II (AII), γ-melanocyte stimulating hormone (γ-MSH), motilin, and growth hormone releasing factor (GRF). The results reported here extend those previously obtained in the same species (Malecha *et al.* 1986; Verger-Bocquet *et al.* 1987; Verger-Bocquet *et al.* 1988; Malecha *et al.* 1989*a*). However, among the peptides that could play a physiological role in the leech, very few are likely to be identical with vertebrate pep-

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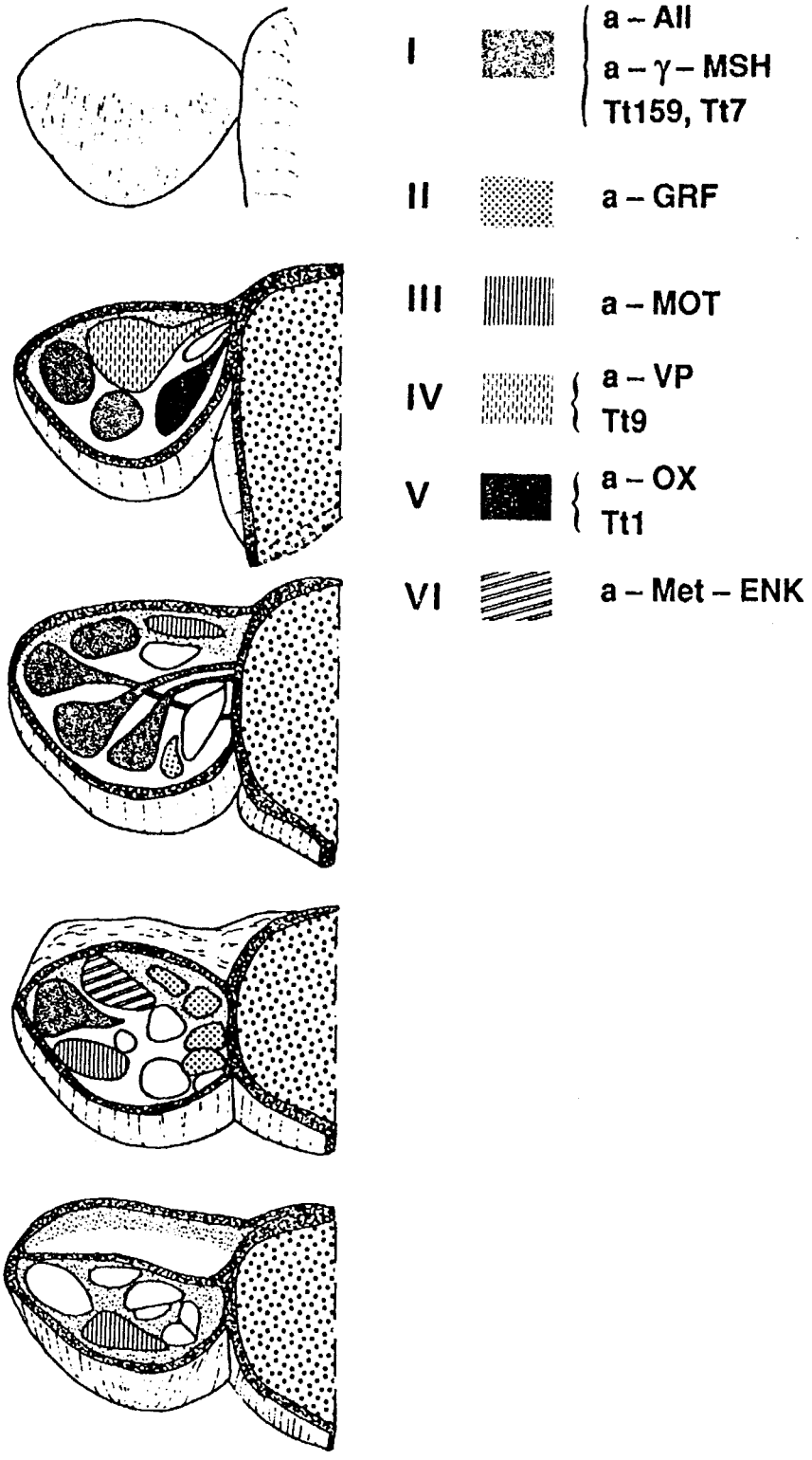

FIG. 1. Diagrammatic representation of compartment 4 of the brain of *T. tessulatum*. Frontal sections from the dorsal side (top) to the ventral side (bottom) show the location of the six cellular types (I, II, III, IV, V, and VI) characterized with the polyclonal antibodies anti-lysine-vasopressin (a-VP), anti-oxytocin (a-OX), anti-met-enkephalin (a-Met-ENK), anti-human growth hormone releasing factor (a-GRF), anti-motilin (a-MOT), anti-angiotensin II (a-AII), and anti-γ-melanocyte stimulating hormone (a-γ-MSH) and with monoclonal antibodies (Tt-1, Tt-7, Tt-9, and Tt-159) raised against molecules of supraesophageal ganglia neurons of *T. tessulatum*.

ide hormones; some must be very different and species-specific. For this reason, monoclonal antibodies produced by immunization of mice with extracts of supraesophageal ganglia, of *T. tessulatum* were used to complement this study. These monoclonal antibodies are able to recognize antigenic determinants that are specific to this leech and localized in the secretory granules of its neurons (Boilly-Marer *et al.* 1987).

Our study focuses on the pair of paramedial compartments

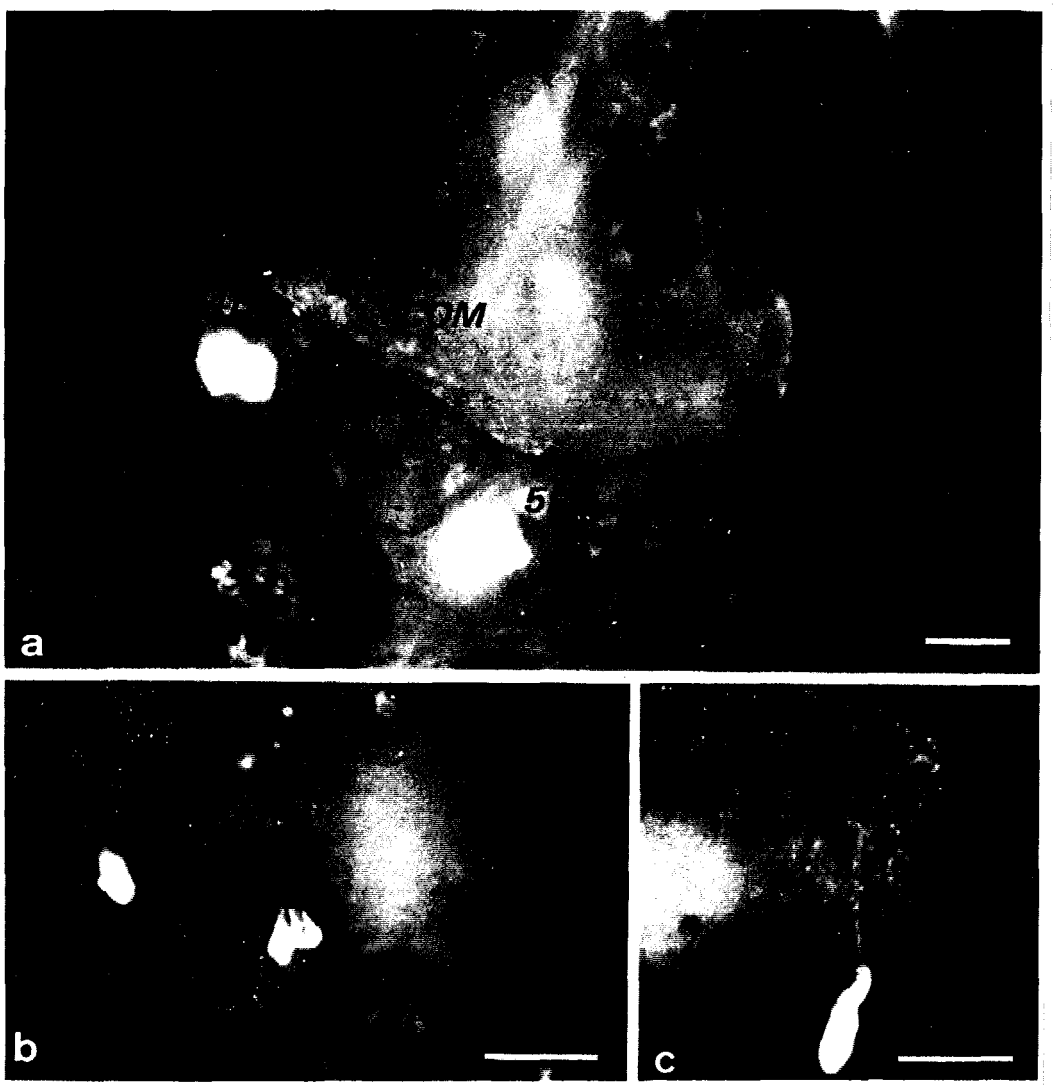

FIG. 2. Immunohistochemical fluorescence micrographs of whole-mount preparations of supraesophageal ganglia of *T. tessulatum* treated with different polyclonal antibodies (dorsal view). (*a*) AII-like cells located in both the left compartment 4 (4L) and the right compartment 5 (5R). (*b*) Cells immunoreactive with anti-GRF, located in compartment 4 close to the dorsal commissure. (*c*) Cell of compartment 4 immunoreactive with anti-oxytocin that sends its process directly into the dorsal commissure where immunoreactivity is strong. COM, dorsal commissure. Scale bars = 50 μm.

(compartment 4) where the cells responsible for the secretion of an antidiuretic hormone are most likely located (Malecha 1979, 1983).

Materials and methods

Animals

Laboratory-bred specimens of the leech *T. tessulatum* were used. Culture conditions were reported previously (Malecha 1983). The life cycle of these animals comprises the following stages: stage 0, from hatching till the first blood meal; stage 1, after the first blood meal; stage 2, after the second blood meal; stage 3, after the third blood meal. Stage 3 is subdivided into six substages (3A, 3B, 3C, 3D, 3E, 3F) defined elsewhere (Malecha *et al.* 1989*a*).

Production of antibodies

Polyclonal antibodies

Polyclonal antisera, kindly provided by Dr. G. Tramu (Université de Bordeaux I, Laboratoire de Neurocytochimie fonctionnelle, Talence, France), were obtained by immunization of rabbits with synthetic peptides coupled to human serum albumin or thyroglobulin and emulsified with Freund's complete adjuvant. Controls for the specificity of these sera were done with preabsorption by the homologous antigen. Use of these antibodies for immunohistochemical and RIA studies has been reported previously. These antibodies are directed against met-enkephalin (Tramu *et al.* 1981; Verger-Bocquet *et al.* 1987), oxytocin (Malecha *et al.* 1986), lysine-vasopressin (Tramu *et al.* 1983*a*), AII (Aguirre *et al.* 1989), GRF (Tramu *et al.* 1983*a*), motilin (Malecha *et al.* 1989*a*), and γ-MSH (Verger-Bocquet *et al.* 1988).

Monoclonal antibodies

Preparation of the immunogen and immunization procedures are described elsewhere (Boilly-Marer *et al.* 1987). After testing the activity of the sera of the mice immunized with *T. tessulatum* supraesophageal ganglion, spleen cells of the immunized mice were isolated and fused with $Sp_2/0$ myeloma cells (Shulman *et al.* 1978) according to the Köhler and Milstein procedure (1975). For the initial screening, culture supernatants from the resultant hybrid clones were assayed for the presence of antibodies by means of an enzyme-linked immunosorbent assay (ELISA) with supraesophageal-ganglion extract as bound antigen (1 ganglion-equivalent per well). In a second step, the supernatants positive in ELISA were tested for the presence of specific antibodies by immunocytochemistry on tissue sections of supraesophageal ganglia of stage 3 leeches. Monoclonal antibodies

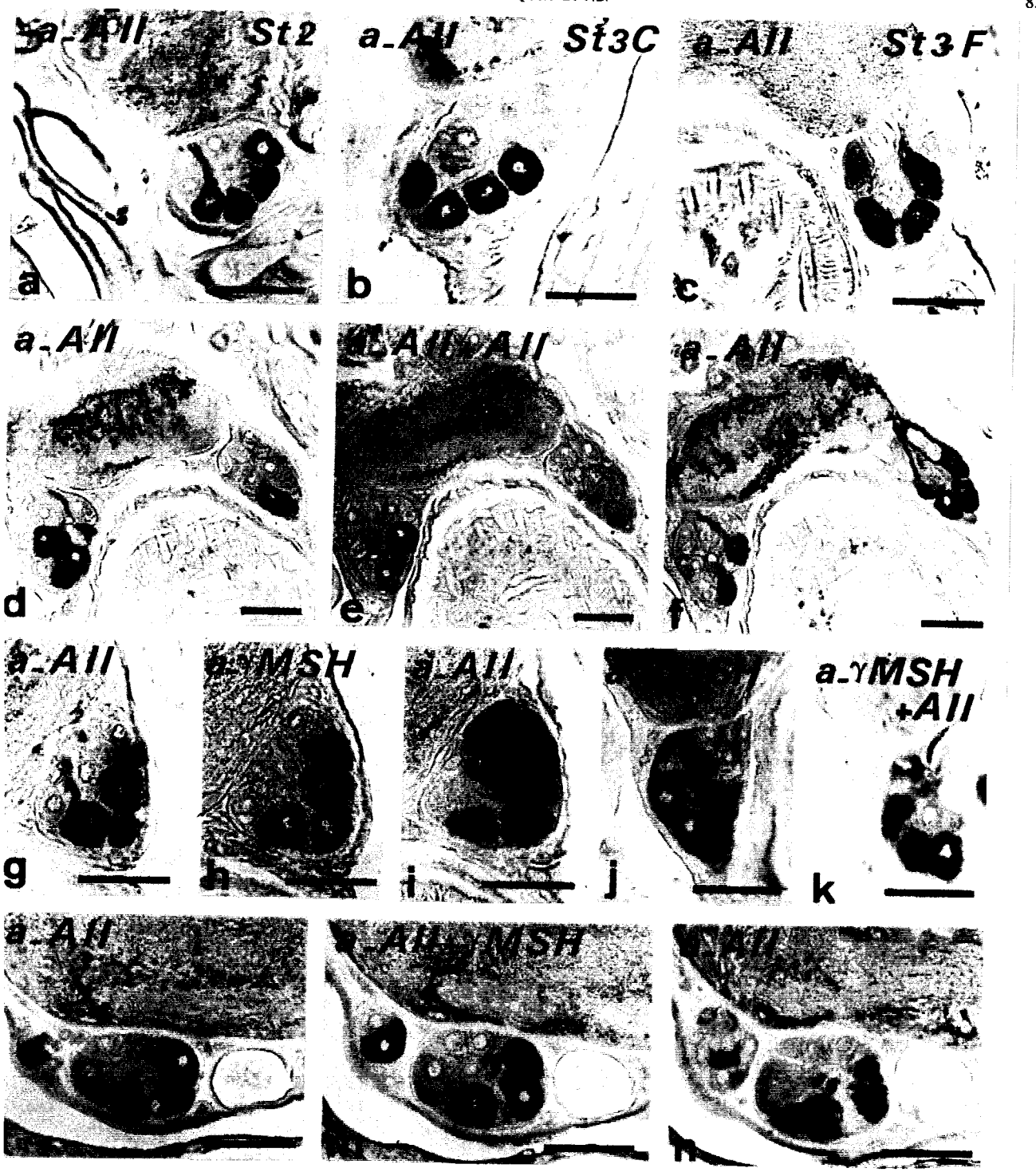

FIG. 3. (*a*–*c*) Immunostaining of class I cells with anti-AII at different life stages of the leech. St2, stage 2; St3C, stage 3C; St3F, stage 3F. (*d*–*f*) Three adjacent sections at the level of compartment 4 treated either with anti-AII (*d*, *f*) or with anti-AII absorbed with AII (*e*). It can be seen that class I cells and numerous fibers in the dorsal commissure stain positively with anti-AII (*d*, *f*) and that the anti-AII staining capacity is abolished after absorption with AII (*e*). (*g*–*i*) Adjacent sections through compartment 4 of supraesophageal ganglion, showing colocalization of AII-like (*g*, *i*) and γ-MSH-like (*h*) immunoreactivities in class I cells. (*j*, *k*) Adjacent sections through compartment 4 of supraesophageal ganglion incubated either with anti-γ-MSH (*j*) or with anti-γ-MSH preabsorbed with AII (*k*). Class I cells are stained in both cases. (*l*–*n*) Three adjacent sections through compartment 4 of supraesophageal ganglion incubated either with anti-AII (*l*, *n*) or with anti-AII preabsorbed with γ-MSH (*m*). Class I cells react positively in both cases. a-AII, anti-AII; a-γ-MSH, anti-γ-MSH. Scale bars = 50 μm.

used in this study (Tt-1, Tt-7, Tt-9, and Tt-159) were selected according to their ability to recognize in the supraesophageal ganglion the secretory granules of cells immunoreactive with polyclonal antibodies raised against peptides involved in osmoregulation (oxytocin, vasopressin, and AII). Selected hybridomas were cloned twice by limited dilution.

Immunocytochemical procedures

Both sectioned material and whole mounts were employed. Immunohistochemical controls for the specificity of staining were performed by incubating the diluted antiserum or the hybridoma supernatant with homologous or heterologous synthetic peptides prior to application.

TABLE 1. Immunohistochemical tests of antibodies used to stain class I cells of compartment 4

Antibody	Staining reaction	
	Blocked by:	Unaffected by:
Anti-AII (1:300)	AII (350 μg/mL)	γ-MSH (350 μg/mL)
Anti-γ-MSH (1:200)	γ-MSH (350 μg/mL)	AII (350 μg/mL)
Tt-7 (undiluted supernatant)		AII, γ-MSH (2 mg/mL)
Tt-159 (undiluted supernatant)		AII, γ-MSH (2 mg/mL)

NOTE: Numbers in parentheses represent the dilution of the antibody or the concentration of the peptide used (micrograms or milligrams per millilitre of undiluted serum or supernatant). Incubations with the different peptides are at 4°C for 6 h.

Whole mounts

Immunocytology of whole mounts of brains taken at stage 3B was carried out using a method (see Salzet *et al.* 1992) derived from that followed by Pearson and Lloyd (1989) and Shankland and Martindale (1989). The primary antibody was diluted 1:500. Whole mounts were examined and photographed under uv fluorescence with a Zeiss Axioskop microscope.

Sections

The anterior parts of the leeches (including the brain) were fixed overnight at 4°C in Bouin–Hollande fixative (and 10% $HgCl_2$ saturated solution), then they were embedded in paraffin and serially sectioned at 7 μm.

For studying immunoreactivity with the different antibodies, the animals were all fixed at stage 3B of their life cycle (Malecha *et al.* 1989*a*). In some cases, the anterior parts were also fixed at different stages of the life cycle (1, 2, 3A, 3C, 3D, 3E, 3F). Each antibody was tested on a minimum of 6 animals at a given stage, and the number of immunoreactive cells given is a mean.

After removal of the paraffin with toluene, the sections were stained by an indirect immunocytochemical peroxidase technique. Sections of supraesophageal ganglia were incubated overnight at room temperature with either polyclonal antiserum (dilution 1:200 or 1:300) or undiluted hybridoma supernatant. They were then treated for 1 h at 20°C with either goat anti-rabbit (for polyclonal antibodies) or rabbit anti-mouse (for monoclonal antibodies) Ig conjugated to horseradish peroxidase (Nordic, dilution 1:40). Peroxidase activity was visualized with a solution of 4-chloro-1-naphthol (40 mg/100 mL 0.1 M tris buffer, pH 7.6) containing 0.01% H_2O_2.

To establish whether co-localization of immunoreactivities occurs, consecutive sections mounted on different slides were stained.

Results

Among the roughly 30 cells constituting compartment 4, six cellular types were characterized according to their immunoreactivity with different antibodies. Their locations are shown in Fig. 1. In the following sections, the number of detected cells is given at stage 3B, unless stated otherwise.

The angiotensin II-like and γ-melanocyte stimulating hormone-like immunoreactive cells (class I cells)

This is a group of four, five, or six large cells (25–40 μm) (Figs. 3*g*–3*i*, 4*a*–4*d*) whose cellular bodies are located at the periphery of compartment 4. Their secretion accumulates into masses that are especially large at the periphery of the cells. A study of these cells at different life stages of the animal shows that immunoreactivity with anti-AII occurs throughout the leech life-span (Figs. 3*a*–3*c*).

Immunoreactivity with anti-γ-MSH polyclonal antibody is not always detected in stage 3 leeches, but this could be because some of the precise physiological stages of the leech might not yet be known. Preabsorptions (Table 1) by their homologous antigen completely abolished the staining capacity of anti-AII (Figs. 3*d*–3*f*) and anti-γ-MSH sera, whereas an anti-AII antibody absorbed by γ-MSH (Figs. 3*l*–3*n*) or an anti-γ-MSH antibody absorbed by AII (Figs. 3*j*, 3*k*) still produced a positive reaction. These results suggest that the two antibodies bind to different antigenic determinants: an AII-like one and a γ-MSH-like one.

These cells are also (Figs. 4*a*, 4*c*) immunoreactive with two monoclonal antibodies (Tt-7 and Tt-159), which also recognize neurons containing an AII-like substance in other compartments of the supra- and sub-esophageal ganglia. Absorptions of Tt-7 and Tt-159 with either AII or γ-MSH did not abolish immunostaining (Table 1, Figs. 4*e*–4*k*).

The location of these cells is variable. In most cases, they are located in compartment 4, but occasionally they can be distributed in both compartments 4 and 5 (Fig. 2*a*). More rarely, they are found only in compartment 5.

The growth hormone releasing factor-like immunoreactive cells (class II cells)

A group of five small cells (10–20 μm) (Figs. 5*a*, 5*c*) positioned at the periphery of compartment 4 close to the dorsal commissure (Fig. 2*b*) was identified. These cells are in a different location than the anti-AII immunoreactive cells (Figs. 5*a*–5*d*). In animals at stages 3C and 3D there can be up to 10 immunoreactive cells in this class.

The motilin-like immunoreactive cells (class III cells)

Three medium-sized cells (20–25 μm) were found that were not co-localized with the cells immunoreactive with anti-AII (Figs. 5*h*–5*k*). Their number varies throughout the life-span of the leech: one at stage 1, two at stages 2 and 3A, three at stages 3B, 3C, and 3D, and two at stages 3E and 3F (Malecha *et al.* 1989*a*).

The vasopressin-like immunoreactive cell (class IV cell)

Dorsally located and pyriform in shape (Figs. 5*f*, 6*b*), this cell is the largest cell in compartment 4 (35–40 μm). It is also immunoreactive with monoclonal antibody Tt-9 (Fig. 6*a*) but not with polyclonal antibody anti-AII (Figs. 5*e*–5*g*).

The oxytocin-like immunoreactive cell (class V cell)

This medium-sized cell (20–25 μm) (Fig. 6*d*), located close to the large vasopressin-like cell (Malecha *et al.* 1986), sends it process directly into the dorsal commissure of the supraesophageal ganglion (Fig. 2*c*). It is also immunoreactive with monoclonal antibody Tt-1 (Fig. 6*c*).

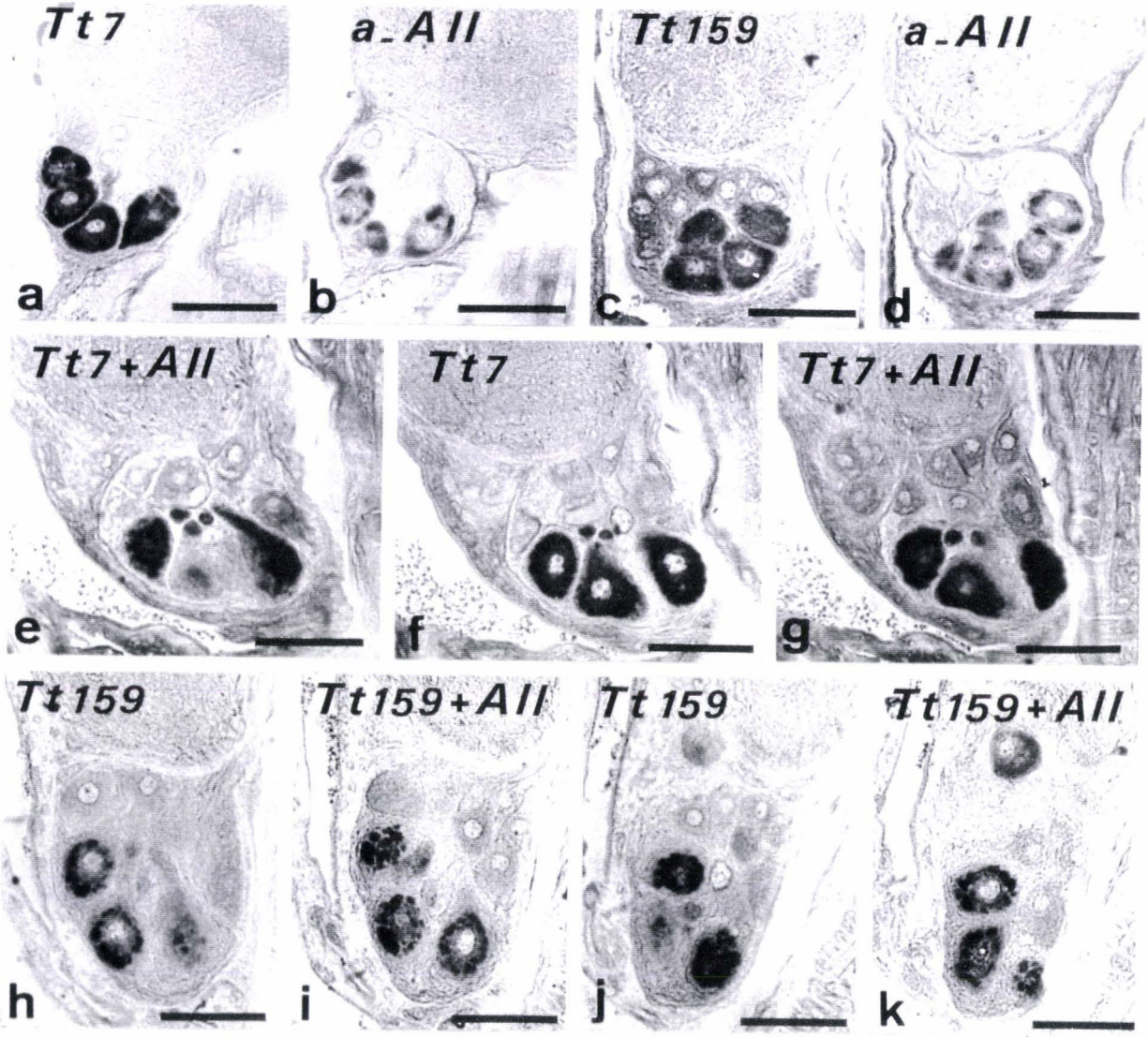

FIG. 4. (*a*, *b*) Consecutive sections showing class I cells that immunoreact with monoclonal antibody Tt-7 (*a*) and polyclonal antibody anti-AII (*b*). (*c*, *d*) Consecutive sections showing class I cells that immunoreact with monoclonal antibody Tt-159 (*c*) and polyclonal antibody anti-AII (*d*). (*e*–*g*) Three adjacent sections treated either with monoclonal antibody Tt-7 (*f*) or with Tt-7 after preabsorption with AII (*e*, *g*). Immunostaining of class I cells with Tt-7 is not affected by preabsorption with the peptide. (*h*–*k*) Adjacent sections treated either with monoclonal antibody Tt-159 (*h*, *j*) or with Tt-159 after preabsorption with AII (*i*, *k*). The positive staining of cells of class I with Tt-159 is not quenched by synthetic AII. a-AII, anti-AII. Scale bars = 50 μm.

The met-enkephalin-like immunoreactive cell (class VI cell)

A single immunoreactive cell of medium size (20–25 μm) is present in compartment 4. The met-enkephalin-like cells of the brain of *T. tessulatum* were described previously (Verger-Bocquet *et al.* 1987).

Discussion

Description of cellular types

With the use of specific markers (polyclonal and monoclonal antibodies), more than half of the cells constituting compartment 4 have been characterized. Six cellular types were identified and mapped in this compartment (Fig. 1). In this study, only one fixative was used. As the immunoreactivity of peptidergic cells is influenced by the type of fixation (Schot *et al.* 1984; Zipser and Schley 1984; Boer and van Minnen 1985), the use of different fixatives could have resulted in slight variations in the immunoreactivity observed for some antibodies.

Compartment 4 contains five large cells immunoreactive with anti-AII, anti-γ-MSH, Tt-7, and Tt-159, five small cells immunoreactive with anti-GRF, three cells immunoreactive with anti-motilin, one cell immunoreactive with anti-met-enkephalin, one cell immunoreactive with anti-oxytocin and Tt-1, one cell immunoreactive with anti-vasopressin and Tt-9, and about 15 cells that are not immunoreactive with these antibodies. Some of the cells of compartment 4 are also immunopositive with other polyclonal antibodies not considered in this study, such as anti-cholecystokinin-8, anti-gastrin, and anti-luteinizing hormone releasing hormone (Dhainaut-Courtois *et al.* 1985; Verger-Bocquet *et al.* 1986, 1988).

It is worth noting that the great majority of the cells constituting compartment 4 are peptidergic, which is in line with the hypothesis of Scharrer (1985) and Platt and Reynolds (1988) regarding the importance of this type of neuron in the nervous system of invertebrates. Also of interest is that for some of these neurons (those immunoreactive with anti-AII,

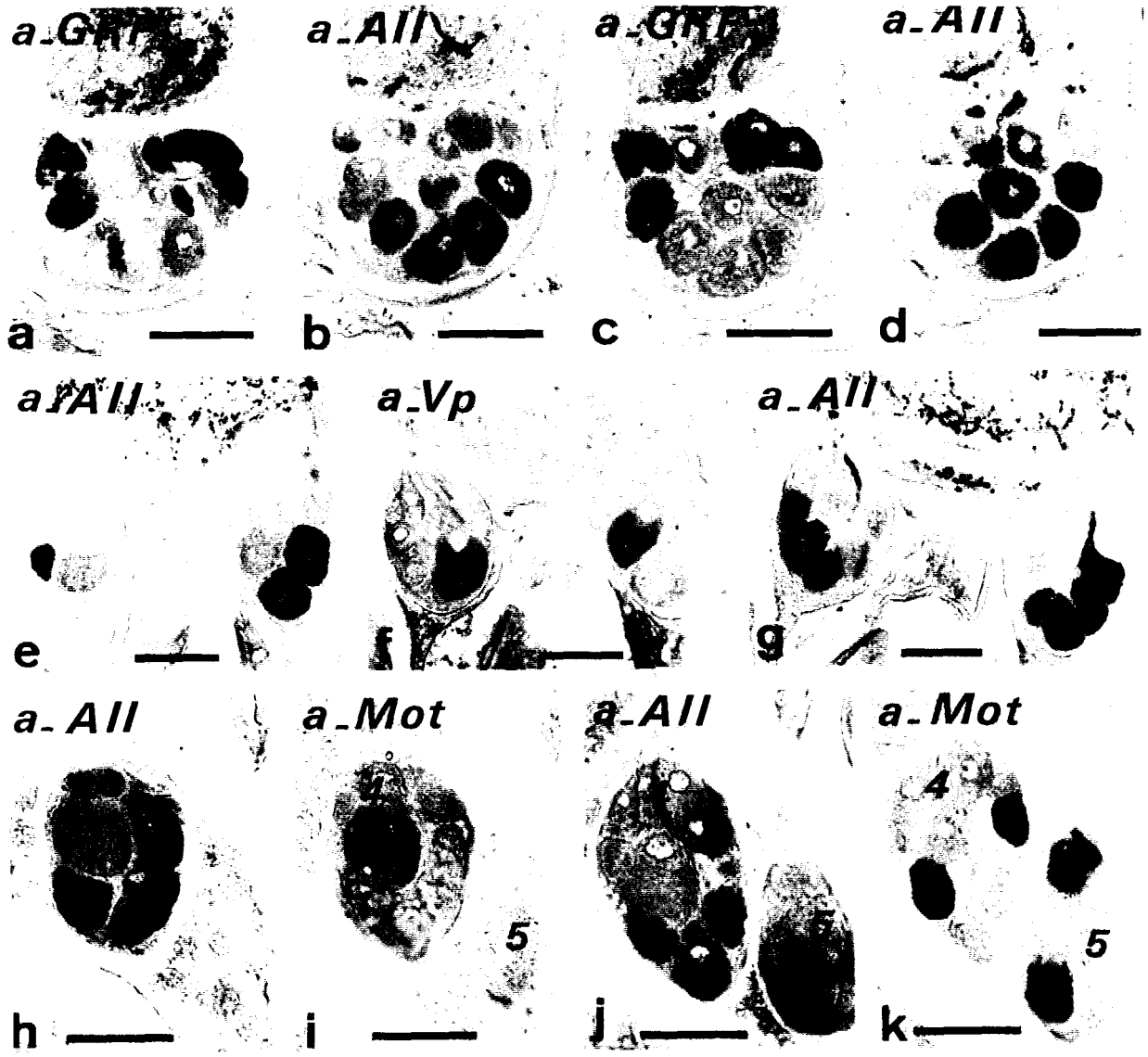

FIG. 5. (*a*–*d*) Consecutive sections through compartment 4 of the supraesophageal ganglion, showing the localization of AII-like (*b*, *d*) and GRF-like (*a*, *c*) immunoreactive cells. (*e*–*g*) Consecutive sections through compartment 4 of the supraesophageal ganglion, showing the localization of AII-like (*e*, *g*) and vasopressin-like (*f*) immunoreactive cells. (*h*–*k*) Consecutive sections through compartments 4 and 5 of the supraesophageal ganglion revealing AII-positive (*h*, *j*) and motilin-positive cells (*i*, *k*). 4, compartment 4; 5, compartment 5. Other abbreviations as in Fig. 1. Scale bars = 50 μm.

anti-motilin, and anti-oxytocin), we observed cellular processes in the dorsal commissure region where the neurohemal area is located. Some of these substances could therefore be released into the circulation and play a hormonal role, others could act as a neurotransmitter or neuromodulator at the level of neuron endings located in this zone.

Cells immunoreactive with the antibodies used (poly- and mono-clonal) are found in other compartments of the supraesophageal ganglion, subesophageal, and segmental ganglia (Malecha *et al.* 1986; Boilly-Marer *et al.* 1987; Verger-Bocquet *et al.* 1987, 1988); those of compartment 4 thus represent a population that is integrated into a larger whole. The one exception is the cellular type immunoreactive with anti-AII: its cytological characteristics were not found in any other area of the central nervous system, although other neurons immunoreactive with anti-AII exist in the other compartments.

Variability in the number of immunoreactive cells

The aim of this work is to characterize the cells constituting compartment 4, but the number of cells, estimated at 30 (27 in *Theromyzon rude*, Hagadorn 1958), fluctuates from animal to animal. The location of the AII-like cells, given particular consideration because their size and cytological characteristics make them easily recognizable, is variable. Most often they are located in compartment 4, but they can also be found in compartment 5 alone or in both compartments 4 and 5. Conversely, cells of compartment 5, such as the large anti-AII immunopositive cells, can be found in compartment 4. These variations do not result from a change in the position of the cells at the level of the supraesophageal ganglion, but more likely reflect variability in the location of the connective tissue septum separating compartments 4 and 5. The cells proximal to this septum are randomly situated either in compartment 4

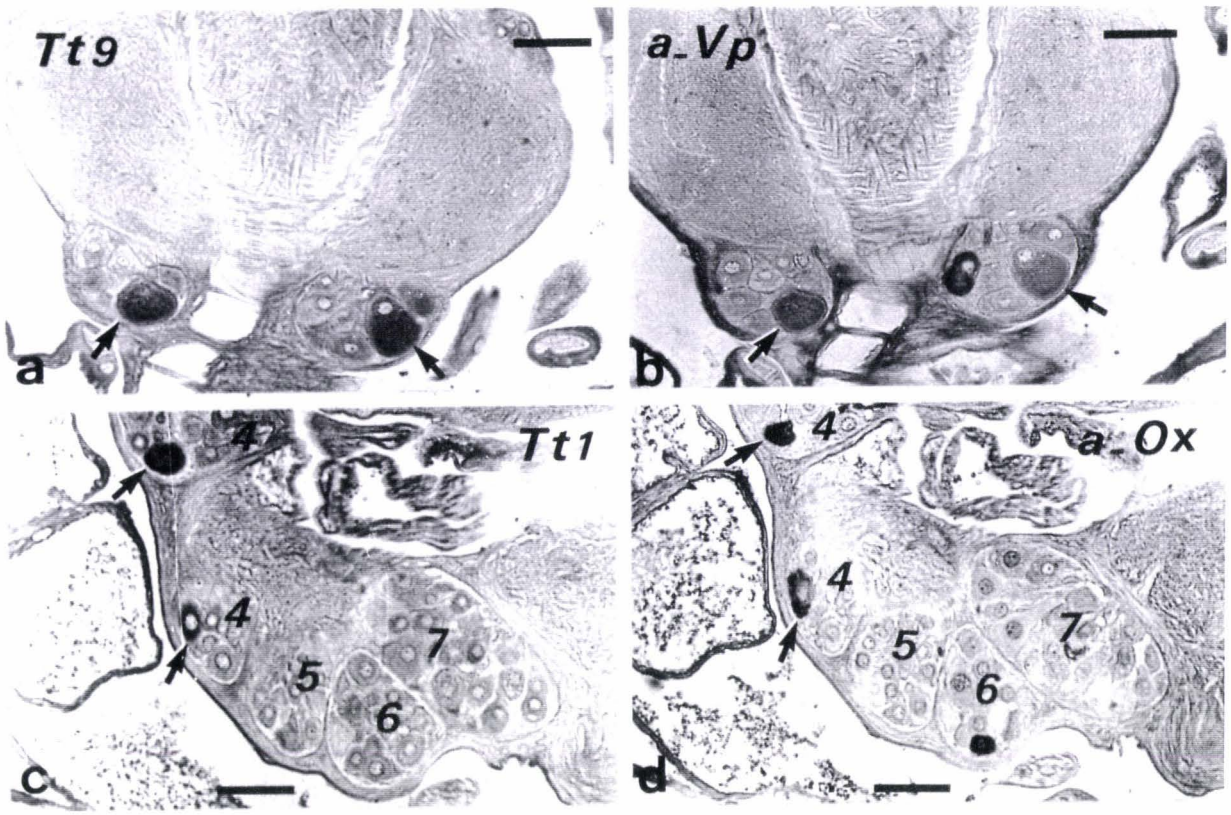

FIG. 6. (*a*, *b*) Consecutive sections through compartment 4 of the supraesophageal ganglion, showing (arrows) a cell of class IV immunoreactive with monoclonal antibody Tt-9 (*a*) and with anti-vasopressin (a-VP) (*b*). (*c*, *d*) Consecutive sections through the brain, revealing a class V cell (arrows) in compartment 4, immunoreactive with monoclonal antibody Tt-1 (*c*) and with anti-oxytocin (a-OX) (*d*). 4, compartment 4; 5, compartment 5; 6, compartment 6; 7, compartment 7. Scale bars = 50 μm.

or in compartment 5. The same observation has been made by Macagno (1980) at the level of the segmental ganglia of *Hirudo medicinalis*.

A variation in the number of immunoreactive cells depending on the physiological status of the animal was observed for the cells immunoreactive with anti-GRF, and especially for those immunoreactive with anti-motilin, in which the variability of immunoreactivity is correlated with sexual maturation (Malecha *et al.* 1989*a*). Unlike the anti-GRF and anti-motilin immunopositive cells, the variability in the number of AII-like cells (4–6 pairs) is not dependent on the physiological status of the animal. It is possible that, as with the segmental ganglia, the number of neurons produced in the brain during embryogenesis is more important than the number observed in the adult (Macagno and Stewart 1987; Stewart *et al.* 1987). The mechanism determining death or maintenance of some cells might be competition for a trophic factor (Macagno and Stewart 1987). Individual variations in the quantity of this factor could lead to the survival of more or fewer AII-like neurons.

Co-localization of antigens

In compartment 4, besides the vasopressin-like and the oxytocin-like immunoreactive cells (also immunoreactive with monoclonal antibodies Tt-9 and Tt-1, respectively), our attention has been focused on the cellular type immunoreactive with two polyclonal antibodies (anti-AII and anti-γ-MSH) and two monoclonal antibodies (Tt-7 and Tt-159).

To determine if the double staining (anti-AII and anti-γ-MSH) (Figs. 3*g*–3*j*) was caused by specific or cross-reacting IgG molecules present in the antisera, studies with sera preabsorbed with homologous or heterologous antigens were performed. From the data presented in Table 1, it is assumed that these cells contain two different antigenic determinants: an AII-like one and a γ-MSH-like one.

These neurons were also revealed by two monoclonal antibodies (Tt-7 and Tt-159) whose distribution of labelled cells in the other areas of the brain, although very close, seems to show that they constitute two distinct markers. A similar finding was reported by Schooneveld *et al.* (1989) in the Colorado potato beetle, *Leptinotarsa decemlineata*, in which neurons in the rostral and medial groups of the subesophageal ganglion are recognized by nine different monoclonal antibodies. Moreover, these neurons are also stained by several polyclonal antisera to different kinds of peptides (Veenstra and Schooneveld 1984; Veenstra *et al.* 1985).

With immunocytochemical techniques, seven subtypes of neurons characterized by the presence of one or more antigenic determinants have thus been identified in compartment 4. This step is necessary to demonstrate peptidergic communication pathways in the leech body. It could permit the selection of molecules of interest after a comparative study of the brains of different families of leeches to find neurons that react with the same antibodies. Molecules found in this way would appear to be conserved during evolution and thus likely to perform an important physiological function (Veenstra 1988). At

present, evidence for a physiological role exists for one of the mammalian peptide hormone-like substances detected at the level of compartment 4: the oxytocin-like substance. Indeed, passive immunization of *T. tessulatum* with an injection of anti-oxytocin leads to a significant decrease in the mass of the animal. The antidiuretic hormone of *T. tessulatum* could thus be structurally related to mammalian oxytocin; it is not identical, however, because the injection of this hormone into animals is ineffective (Malecha *et al.* 1989*b*).

Knowing the chemical nature of the different substances demonstrated is thus necessary for finding their physiological roles. The antibodies, both polyclonal and monoclonal, are essential either for assays (RIA, enzyme-linked immunosorbent assays, dot immunobinding assays) in the course of a purification procedure (Salzet *et al.* 1992; Verger-Bocquet *et al.* 1991) or for isolation of antigens by affinity chromatography with a view to a purification.

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Molécules immunoréactives à l'anti-ocytocine

Articles présentés

(2) Salzet, M., Wattez, C., Slomianny, M.C., Léü, B. and Siegert, K.J., Elisa for oxytocin. Highly sensitive tests and application for the titration of an oxytocin-like substance in the leech *Erpobdella octoculata, Comp. Biochem. Physiol.*, 102C (1992a) 483-487.

(3) Salzet, M., Wattez, C. and Slomianny, M.C., Epitope mapping with ELISA of an antibody against oxytocin used for the characterization of an oxytocin-like epitope in the sex segmental ganglia of the leech *Erpobdella octoculata, Comp. Biochem. Physiol.*, (1992b), sous presse.

(4) Salzet, M., Wattez, C., Verger-Bocquet, M., Beauvillain, J.C. and Malecha, J., Oxytocin-like peptide : a novel epitope colocalized with the FMRFamide-like peptide in the supernumerary neurons of the sex segmental ganglia of leeches. (Morphological and biochemical characterization ; putative anti-diuretic function), *Brain Research,* (1992c), sous presse.

(5) Salzet, M., Wattez, C., Verger-Bocquet, M. and Malecha, J., Cerebral anti-diuretic factor related to the C-terminal part of oxytocin (Prolyl-Leucyl-Glycinamide) in the Rhynchobdellid leech *Theromyzon tessulatum* (Morphological, biochemical and physiological characterizations), en préparation 1.

Chez*T. tessulatum* , il existe au niveau du ganglion supra-œsophagien, et vraisemblablement au niveau du follicule 4, un facteur régulateur de la balance hydrique (FRBH) ayant un effet anti-diurétique (Malecha, 1979, 1983). La cartographie de ce follicule (Verger-Bocquet *et al.*, 1992) ayant révélé l'existence de cellules immunoréactives à l'anti-ocytocine (a-OT), nous nous sommes intéressés à la molécule immunologiquement apparentée à l'ocytocine (OT-like), molécule susceptible d'être le FRBH.

La nécessité de posséder une méthode de quantification de l'OT-like à la fois rapide, très sensible et répétitive, nous a conduit à mettre au point trois types de tests ELISA (direct, d'inhibition et compétitif) répondant à cet ensemble de conditions (Salzet *et al.*, 1992a). Ces tests nous ont permis :

- De démontrer que l'anticorps a-OT est spécifique de la partie C-terminale de l'OT qui a pour séquence : Prolyl-X-Glycinamide (X : résidu non polaire) [PLGa] (Salzet *et al.*, 1992b).

- D'effectuer des dosages précis de l'OT-like au niveau du ganglion supra-œsophagien de *T. tessulatum* en fonction du stade physiologique des animaux (Salzet *et al.*, en préparation 1).

Compte tenu des faibles quantités d'OT-like cérébrale détectées, nous avons recherché une autre source que *T. tessulatum* pour l'isolement biochimique de cette substance. Des résultats immunohistochimiques ont montré l'existence d'un grand nombre de cellules immunoréactives à l'anti-OT dans les ganglions génitaux d'une autre sangsue, *Erpobdella octoculata* (Verger-Bocquet *et al.*, 1991). Les résultats de dosages ELISA effectués chez cette espèce, mettent en évidence au niveau du système nerveux central (SNC) 10 à 20 fois plus d'OT-like que chez *T. tessulatum.* Après vérification de l'effet anti-diurétique des extraits de ganglions génitaux, *E. octoculata* a été choisie comme "matériel biochimique" (Salzet *et al.*, 1992c).

Chez *E. octoculata*, la partie du SNC où le taux d'OT-like détecté est maximal se situe au niveau des ganglions génitaux (Salzet *et al.*, 1992a). Au niveau de ces ganglions, le taux d'OT-like varie en fonction de l'état physiologique des sangsues : il est 3 fois plus important chez les animaux immatures que chez les animaux matures (Salzet *et al.*, 1992c). Par contre, cette variation au cours du cycle vital du taux d'OT-like ne se retrouve pas au niveau du cerveau (Salzet *et al.*, inédit), ce qui diffère de ce que l'on observe pour *T. tessulatum.* En effet, chez *T. tessulatum.*, le taux d'OT-like cérébrale atteint son maximum au stade 3B (Salzet *et al.*, en préparation 1), stade correspondant à une phase de rétention d'eau et d'accumulation de la vitellogénine dans le liquide cœlomique (Baert *et al.*, 1991).

Les extractions biochimiques de l'OT-like ont été réalisées à partir de ganglions génitaux d'*E. octoculata.*

Dans les ganglions génitaux d'*E. octoculata* immatures, trois zones (Z1, Z2 et Z3) immunoréactives à l'a-OT, ont été détectées, Z3 présentant 80% de l'immunoréactivité totale (Salzet *et al.*, 1992c). Cependant, quand les animaux arrivent à maturité, Z3 disparaît et les 80 % de l'immunoréactivité sont dès lors portés par Z1. Ces résultats sont en accord avec les dosages ELISA réalisés à ces 2 stades, ainsi qu'avec la numération des cellules immunoréactives à l'a-OT dans les ganglions génitaux, qui ont montré un taux d'OT-like et un nombre de cellules plus élevé chez les animaux immatures que chez les animaux matures (Salzet *et al.*, 1992c). A partir de ces données, deux hypothèses peuvent être avancées : le peptide contenu dans Z3 est, soit un fragment issu d'une coupure aspécifique due aux techniques biochimiques d'extraction utilisées, soit un produit de clivage d'un précurseur de haut poids moléculaire. Les résultats récents obtenus en spectrométrie de masse après purification de ce produit ont révélé, parmi plusieurs masses, un pic majeur de 5776 Da (Salzet *et al.*, inédit). La purification finale de ce produit et son séquençage sont en cours afin de confirmer qu'il correspond bien au produit immunoréactif à l'a-OT.

L'étude des protéines du SNC nous a révélé la présence d'un homodimère de 34 kDa immunoréactif à l'a-OT. Au niveau des produits de traduction des ARNm extraits à partir des ganglions génitaux, une protéine de *ca* 19 kDa a été détectée en western blot avec l'a-OT. Aucune réaction croisée n'a été observée tant au niveau protéique qu'au niveau des produits de traduction, avec un anticorps anti-neurophysine (mélange de MSEL-neurophysine et de VLDV-neurophysine). Nous ne sommes donc pas en présence de la structure classique *i.e.* une OT associée à la neurophysine.

Le séquençage de Z1 a donné les résidus suivants : IPEPYVWD. Les résultats de spectrométrie de masse (1018, 6 ± 0,35) sont en accord avec la masse moyenne calculée (1017,8 Da). Ce peptide possède, comme son précurseur, une structure complètement différente de celle de l'OT. Il présente par contre, 100 % d'analogie avec un fragment de séquence du côté N-terminal de la myohémérythrine du Sipunculien *Themiste zostericola* (Klippenstein *et al.*, 1976) : GWD**IPEPYVWD**ESFRV...Deux hypothèses peuvent être avancées au sujet de ce peptide. Il s'agirait soit d'un nouveau neuropeptide des ganglions génitaux, soit d'un contaminant provenant de la dégradation d'une molécule de type hémérythrine dont la présence est connue chez la sangsue (Baert *et al.*, 1992). La contamination des ganglions génitaux pourrait provenir soit du liquide cœlomique, soit du sang

dans lequel ces ganglions baignent, la chaîne nerveuse étant contenue dans un vaisseau sanguin. De nombreux arguments plaident en faveur de la première hypothèse, c'est à dire celle d'un peptide localisé dans les cellules surnuméraires des ganglions génitaux. L'hypothèse d'une contamination n'est en effet pas en accord avec les faits suivants :

1- Le dosage de la substance OT-like donne des résultats différents pour les ganglions génitaux et pour les autres ganglions segmentaires, alors que ces deux types de ganglions sont dans les deux cas prélevés dans les mêmes conditions.

2- Les quantités de cette molécule varient en fonction du stade physiologique des animaux.

3- Les masses moléculaires de la pro-OT-like (*ca* 17 kDa) et de la prépro-OT-like (*ca* 19 kDa) diffèrent de celle des hémérythrines (14 kDa) isolées chez *T. tessulatum* (Baert *et al.*, 1992) et *E. octoculata* (Salzet *et al.*, inédit)

4- L'anticorps polyclonal a-hémérythrine (Baert *et al.*, 1992) ne reconnaît ni la pro-OT-like, ni la prépro-OT-like, ni les cellules surnuméraires des ganglions génitaux.

5- L'anticorps polyclonal a-OT ne reconnaît pas l'hémérythrine de *T. tessulatum* et d'*E. octoculata* caractérisées par Baert *et al.* (1992) et Salzet *et al.* (inédit).

6- L'utilisation d'anticorps polyclonaux a-OT et a-hémérythrine sur des produits de traduction issus d'un même pool d'ARN totaux extraits à partir de corps entiers d'*E. octoculata* a conduit à l'identification de 2 protéines différentes avec les 2 anticorps : une protéine d'une masse moléculaire de *ca* 19 kDa avec l'a-OT et une protéine d'une masse moléculaire de *ca* 14 kDa avec l'a-hémérythrine (Salzet *et al.*, inédit).

Le fait que la structure primaire du peptide OT-like soit différente de celle de l'OT n'a rien de surprenant. En effet, l'appellation d'OT-like ne préjuge pas de sa structure, mais rappelle simplement, comme il est d'usage en immunocytochimie, que cette molécule possède un déterminant antigénique reconnu par un anticorps a-OT. Il est bien connu qu'un tel épitope peut être très réduit. Maintenant que sa structure est connue, nous proposons de l'appeler peptide IPEP. Néanmoins, une confirmation de la présence de ce peptide dans les cellules surnuméraires des ganglions génitaux est nécessaire soit en immunocytochimie en utilisant un anticorps dirigé contre cette molécule, soit en hybridation *in situ* en utilisant une sonde oligonucléotidique réalisée à partir de la séquence obtenue. De plus, l'obtention du peptide de synthèse permettra de préciser son rôle physiologique.

La structure du peptide IPEP est différente de celle d'une OT classique mais permet de comprendre les résultats des injections d'OT faites *in vivo* par Malecha *et al*, (1989a). L'injection d'OT mammalienne est sans effet sur l'osmorégulation, alors que celle du PLGa (fragment C-terminal de l'OT) possède une action anti-diurétique et suit une loi de dose/réponse (Salzet *et al.*, en préparation 1). Chez les Vertébrés, le PLGa est considéré comme le métabolite actif de l'OT. Il se fixe sur les mêmes récepteurs que l'OT (Stancampiano *et al.*, 1991). Son clivage du côté C-terminal libère le dipeptide PL qui est actif (Burbach, 1986; Stancampiano *et al.*, 1991). On peut donc suggérer que l'activité biologique de la molécule isolée chez *T. tessulatum* est portée du côté N-terminal par le dipeptide PI (l'isoleucine étant un isomère de la leucine). L'action biologique du peptide IPEP pourrait donc être inhibée par la fixation de l'anticorps sur le domaine actif IP.

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ELISA FOR OXYTOCIN. HIGHLY SENSITIVE TESTS AND APPLICATION FOR THE TITRATION OF AN OXYTOCIN-LIKE SUBSTANCE IN THE LEECH *ERPOBDELLA OCTOCULATA*

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Abstract—1. Three different types of ELISA for oxytocin (OT) were developed: direct, inhibiting and competitive. The amounts of OT which could be detected varied with the type of test: 10 to 100 pmol in direct and inhibiting ELISA, 50 fmol to 10 pmol in competitive ELISA.

2. A comparison between the values of percentage of binding obtained with radioimmunoassay (RIA) and in competitive ELISA showed that this latter assay is as sensitive as the RIA but it offers the advantages of being cheaper and more reproducible.

3. The development of these sensitive ELISA provided a means to quantify OT-like molecules. Their usefulness has been first applied to the central nervous system of the leech, *Erpobdella octoculata*.

INTRODUCTION

Since their description in 1971 by Engvall and Perlmann, ELISA techniques have improved in sensitivity, both at the quantitative and the qualitative level. A great deal of research has been related to the quantification of peptides. These efforts have been aided by polystyrene treatment of microwells and amplification of the signal through chromogenic substrates such as streptavidin-biotin (Guesdon *et al.*, 1979) and peroxidase-anti-peroxidase (Hsu *et al.*, 1981) or fluorogenic substrates such as FITC–anti-FITC systems (Harmer and Samuel, 1989; Crowther *et al.*, 1990).

One way of modifying polystyrene microwells is to pre-coat the surface with polymers which have reactive groups, e.g. poly-L-lysine, a co-polymer of poly-L-lysine-arginine or poly-glutaraldehyde (Hobbs, 1989). Biomolecules can then be immobilized with linkers such as succinimidyl-esters or condensing agents like carbodiimide. These surface modifications, however, tend to cause increased background and a risk of peptide desorption (Rasmussen, 1990). To resolve these problems, a new generation of microtitre plates (e.g. CovaLink from NUNC) has been introduced. It is now possible to detect small amounts of peptides e.g. the titration window for angiotensin II can be as low as 1–100 pmol (Sondergard–Andersen *et al.*, 1990).

The aim of the present work was to set up three types of ELISA for oxytocin (OT). The direct ELISA (1) allows the detection of the entire (specific and non-specific) immunoreactivity present in a biological sample. The inhibiting ELISA (2), which can be considered as a negative control, measures only the non-specific immunoreactivity. The difference between the two values obtained respectively in direct and inhibiting ELISA gives an estimate for the amount of specific antigen. The competitive ELISA (3) determines directly only the specific amount of antigen.

Highly sensitive and specific ELISA for OT are required for developing studies on the hormonal regulation of water balance in leeches where an OT-like substance present in the central nervous system has an anti-diuretic effect (Malecha *et al.*, 1986, 1989; Verger-Bocquet *et al.*, in press). In a first test, these techniques have been used for the quantification of OT-like material in the segmental ganglia of the nerve cord of the leech, *Erpobdella octoculata*.

MATERIALS AND METHODS

Animals

For quantification of OT-like material in sex ganglia and non-sex ganglia, nerve cords were taken on leeches of the species *Erpobdella octoculata* collected in Harchies (Belgium) and maintained in aquaria.

Antiserum

An antiserum against OT generated in our laboratory was used both in ELISA and RIA. This serum has been well characterized, it is specific of the C-terminal part of OT (Verger-Bocquet *et al.*, in press). Its production was in two steps:

Coupling of OT. OT was coupled to thyroglobulin with glutaraldehyde by a one-step method: 4 mg OT (INTER-CHIM) were added to a solution of 10 mg bovine thyroglobulin (SIGMA) in 2 ml phosphate-buffered saline pH 7.4. Under constant stirring at 4°C, an equal volume of 0.5% aqueous glutaraldehyde solution was added dropwise. After 3 hr, the reaction was stopped by the addition of sodium metabisulfite (final concentration 2 mg/ml).

Immunization. A rabbit was immunized with OT-glutaraldehyde-thyroglobulin at forty different sites. Simultaneously, vaccines against tuberculosis and whooping

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cough were injected. Animals received a second and third injection one and three weeks after the primary immunization. The first bleeding was one week after the third injection. The fourth injection (intravenously) and the second bleeding took place five and six weeks after the primary immunization, respectively.

Preparation of sex ganglia

One hundred and twenty sex ganglia taken on *E. octoculata* were dissected, homogenized in 200 µl 1N acetic acid at 4°C, sonicated and then centrifuged 15 min at 4°C at 15,000 rpm. The pellet was re-extracted as above, the supernatants combined and immediately used.

ELISA procedure

Plates. Three types of immunoplates (NUNC) were tested: 1. CovaLink (catalogue number 478046 F16), with spacer 10^{14} groups/cm^2 (patent pending). 2. Maxisorp (catalogue number 469914 F16), maximum capacity 400 ng IgG/cm^2. 3. Polysorp (catalogue number 467679 F16), maximum capacity 100 ng IgG/cm^2.

Buffers. For all subsequent procedures the following buffers were used: Coating buffer (CB) 100 mM sodium carbonate pH 9.6. Blocking buffer (BB) 20 mM sodium phosphate, 50 mM NaCl, pH 7.4 (PBS); 2% bovine serum albumin (BSA). Washing buffer (WB) PBS, 0.05% Tween 20. Dilution buffer (DB) PBS, 0.05% Tween 20, 0.1% BSA.

Immunoassays. For direct and inhibiting ELISA plates were coated with 100 µl standard OT (CRB)/well at different concentrations (1.5, 3, 6, 12.5, 25, 50, 100 ng OT/ml CB) or with the equivalent of 20 sex ganglia of *E. octoculata* per well and at a single concentration of 25 ng OT/ml CB for the competitive ELISA. In each case coating was performed with agitation for 12 hr at 4°C or for 2 hr at 37°C. After being saturated with BB (200 µl/well), plates were rinsed twice with WB, then covered with parafilm and kept at 4°C. All subsequent steps were performed under continuous agitation. For the direct and inhibiting ELISA, the antiserum (diluted 1/1000) was incubated overnight with (inhibiting ELISA) or without (direct ELISA) the corresponding peptide (100 µg OT/ml pure antiserum) and 100 µl/well applied for 2 hr at 37°C. For the competitive ELISA, antigen (50 µl/well) at different concentrations (1.5, 3, 6, 12.5, 25, 50 and 100 ng OT/ml DB) or the equivalent of 20 sex ganglia of *E. octoculata* and specific antiserum (diluted 1/500 in DB; 50 µl/well) were applied simultaneously for 2 hr at 37°C. Wells were then rinsed three times with WB, followed by the addition of 100 µl peroxidase-conjugated goat anti-rabbit IgG (Diagnostics Pasteur; diluted 1/10000 in DB) to each well. After a 2 hr incubation, followed by two washes in WB and one in PBS, 100 µl of freshly prepared substrate (6 mg ortho-phenylenediamine/12 ml of 0.1 M citrate buffer pH 5.5 containing 0.08% H_2O_2) was added to each well. The reaction was stopped with 100 µl of 1N HCl and absorbance was measured at 490 nm on a MR 250 microplate reader (DYNATECH). All assays were conducted in duplicate.

Processing of ELISA data

Percentage of binding. Optical density readings were used to calculate the percentage of bound OT with the following formula:

$$\text{Percent of binding} = 100 \times ((B\text{-NSB})/(B_0\text{-NSB}))$$

with

NSB = blank value
B_0 = peroxidase activity in the absence of OT
B = peroxidase activity in the presence of OT

Affinity constants. A computer program for the calculation of antibody affinity constants (Ka) which avoids the limitations of graphical plotting of titration data using the Sips or Scatchard equations (Nelson and Griswold, 1986, 1988) was used.

RIA procedure

The RIA for OT used the polyclonal antibody anti-OT generated in our laboratory (see above) and ^{125}I OT as outlined by the manufacturer (NEN). Samples were counted with a γ-counter (LKB).

RESULTS

Standardization tests

Plates. Three types of microtitre plates were coated with OT and pre-treated with or without glutaraldehyde (Fig. 1). The best results were obtained with CovaLink plates. A pre-treatment with glutaraldehyde or disuccinimidyl suberate (data not shown) increased peptide coupling and sensitivity of the test. Polysorp plates gave the worst results (high background, low sensitivity). Maxisorp plates also gave good results (high sensitivity and reproducibility), but with this type of plates glutaraldehyde treatment gave a high background and a possible desorption of peptide from the solid phase after washing (data not shown). Based on this study, CovaLink plates pre-treated with glutaraldehyde were chosen for all subsequent assays.

Determination of the optimal dilution of the primary and secondary antisera. Firstly, the optimal dilution of the primary antiserum (AS1) was determined as a function of the peptide concentrations and with a secondary antiserum (AS2) at a given dilution (1/10000; Fig. 2A). Secondly, the AS2 dilution was used at two AS1 dilutions (1/1000 and 1/2000), to obtain both a minimal background and a linear curve from 10 to 100 pmol of OT (Fig. 2B). From the figures, it can be seen that the optimal dilutions are 1/1000 for AS1 and 1/10000 for AS2.

Determination of the optimal OT concentration required for coating in competitive ELISA. The optimal concentration of OT coated onto the solid phase was determined in order to get a linear curve, a minimal background and a high reproducibility. From Fig. 3 it is clear that with the lower concentration

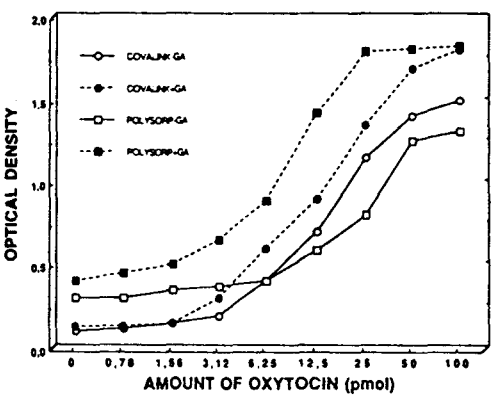

Fig. 1. Optical densities at 490 nm for direct ELISA performed with two types of microtitre plates (CovaLink and Polysorp) pretreated (+GA) or not (−GA) with glutaraldehyde and coated with different amounts of OT. Values obtained with a 1000-fold dilution of primary antiserum and a 10000-fold dilution of secondary antiserum.

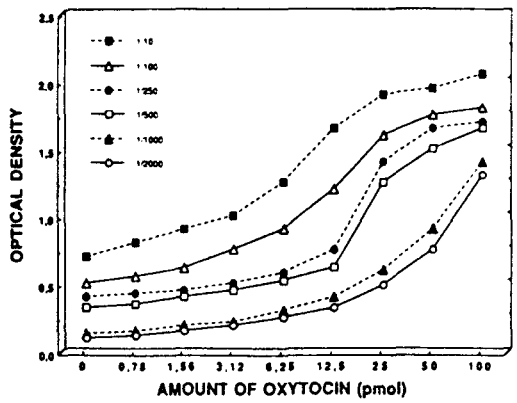

Fig. 2A. Optical densities at 490 nm obtained in direct ELISA at a given dilution of secondary antiserum (1/10000) and with different dilutions of primary antiserum (1/10, 1/100, 1/250, 1/500, 1/1000, 1/2000), as a function of the amount of OT fixed to the microtitre wells.

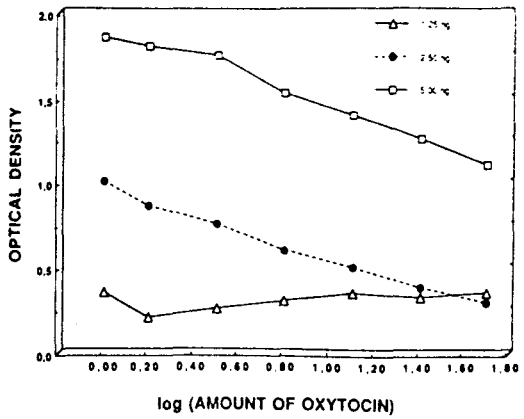

Fig. 3. Determination of the optimal amount of OT required for coating in competitive ELISA: optical density values at 490 nm as a function of the amount of OT fixed to the microtitre wells (1.25, 2.5 and 5 ng OT) and of the amount of OT (pmol) in competition expressed in log.

(1.25 ng/well), no linear curve was obtained. Moreover, tests at this concentration were not reproducible. The amount tested which was the best was 2.5 ng/well for with the higher amount tested (5 ng/well), background was high, reproducibility bad and artefactual immunoreactivities were found.

Determination of the minimal OT concentration required to block the primary antibody. A concentration of 0.1 nmol OT/ml of pure antiserum gave the same optical density reading as the antiserum not pre-adsorped with OT (Fig. 4). An 80% block, however, was achieved with 100 nmol OT/ml antiserum and this concentration was used in subsequent experiments for with concentrations inferior to 100 nmol OT/ml antiserum (e.g. 25 nmol OT/ml antiserum), block was insufficient. Concentrations in excess of 100 nmol OT/ml antiserum produced an even higher block (data not shown) but can produce artefactual steric block.

Sensitivity of the assays

Direct and inhibiting ELISA. From the titration curves obtained with the unblocked or the blocked antibody, the linear parts allowed to place the titration window between 10 and 100 pmol (Fig. 4). In the direct ELISA, the CV intra- and inter-assays ranged from 8.1–15.7% and from 10.1–26%, respectively (Table 1).

Competitive ELISA. A linear relationship was found between 0.1 and 100 pmol with 2.5 ng of pre-coated OT per well. Under these conditions, quantification was possible between 0.05 and 10 pmol (Fig. 5). The degree of inter- and intra-assay variation was evaluated (Table 1). For samples ranging in amounts from 0.15–5 pmol, the intra-assay CV was 1.7–2.2% and the inter-assay CV was 5.6–14.9%.

Comparison between competitive ELISA and RIA. The sensitivity of competitive ELISA was found to be similar to that of RIA (Fig. 6). The correlation coefficient for the two tests was $r = 0.97$ (Fig. 7). The Ka values were very close in the two tests: $1.038 \times 10^{10} \pm 8 \times 10^{8}\ M^{-1}$ in competitive ELISA against $1.307 \times 10^{10} \pm 6.7 \times 10^{9}\ M^{-1}$ in RIA.

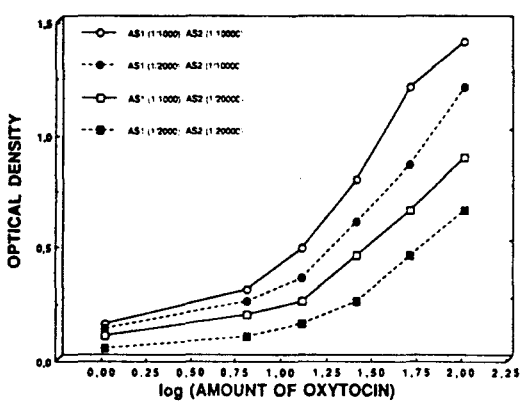

Fig. 2B. Optical densities at 490 nm from a direct ELISA at different dilutions of primary antiserum (AS1) and of secondary antiserum (AS2) as a function of the amount of OT (pmol) fixed to the microtitre wells and expressed in log.

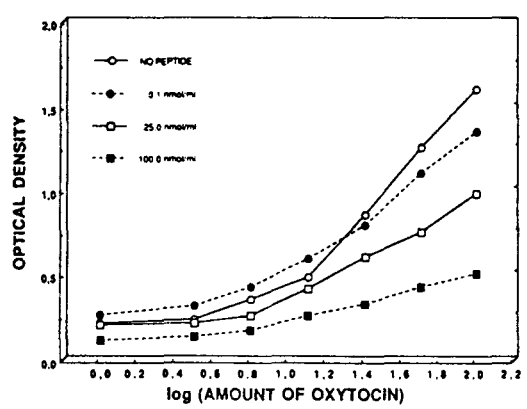

Fig. 4. Determination of the minimal OT concentration required to block the primary antiserum: optical density values at 490 nm as a function of the amount of OT (pmol) fixed to the microtitre wells (expressed in log) with primary antibody pre-adsorped or not with OT. --●-- —□— --■--: OT concentration/ml of pure antiserum.

Table 1. Inter- and intra-assay variation for direct and competitive ELISA at different amounts of OT. Means ± S.D. of four determinations

Type of ELISA	Amount of OT (pmol)	Intra-assay $\bar{X}$(%)	S.D.	CV(%)	Inter-assay $\bar{X}$(%)	S.D.	CV(%)
Direct	6.0	8.78	1.39	15.71	3.65	0.14	26.02
	25.0	28.39	2.31	8.13	21.38	4.10	10.14
Competitive	0.15	85.25	1.92	2.25	77.62	4.37	5.60
	5.00	41.12	0.73	1.79	39.56	4.56	14.92

$\bar{X}$: Mean of the absorbance percentage.
S.D.: Standard deviation.
CV: Coefficient of variation calculated as the Standard deviation/mean × 100.

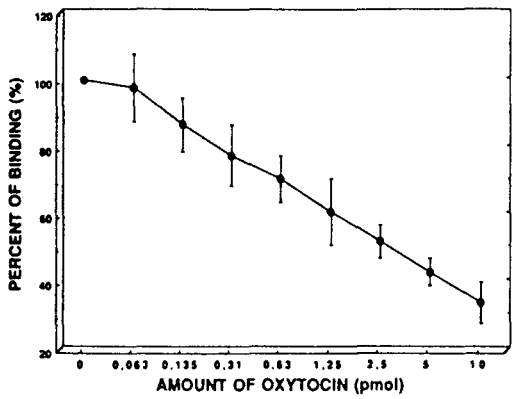

Fig. 5. Percentage of binding as a function of different amounts of OT in competitive ELISA. Each point represents the mean ± S.D. of four determinations.

Application of the ELISAs to the quantification of an OT-like molecule

The amount of OT-like material in the sex and non-sex segmental ganglia of the nerve cord of leeches (*Erpobdella octoculata*) in reproduction was determined with the competitive, inhibiting and direct ELISA (Table 2). The inhibiting ELISA estimates the non-specific part of the total OT-like material. A comparison between the values from the competitive ELISA with the difference between the values obtained in direct and inhibiting ELISA provided results which are in good agreement: the sex segmental ganglia contain *ca* 5 pmol of OT-like material, while only approx. 0.3 pmol were detected in the other segmental ganglia.

With regard to the high amounts of OT-like material found in the sex ganglia, this quantitative ELISA procedure is currently being applied to the purification of the OT-like substance of leeches in the view of a sequencing.

CONCLUSIONS

In the present contribution, three ELISA procedures sufficiently sensitive to allow the detection of small amounts of OT (50 fmol-10 pmol) with a new type of microtitre plate (CovaLink) are described. The significance of these different types of tests has been outlined above. They allow the characterization of an antiserum since they determine the non-specific recognition which is important for other neuropeptide studies e.g. immunocytochemistry, biochemical purification of related peptides or molecular biology. With the three procedures described, the specific and non-specific amount of antigen can be detected in a biological sample with a high sensitivity and reproducibility.

A new application for the CovaLink plates is described in the present investigation since they are used with a competitive ELISA technique. This technique is completely different from the classical technique based on an antibody sandwich (Deforge and Remick, 1991). The competitive ELISA allows the detection of 50 fmol–10 pmol OT with high reproducibility and minimal background. It is thus as sensitive as the RIA or EIA technique for OT

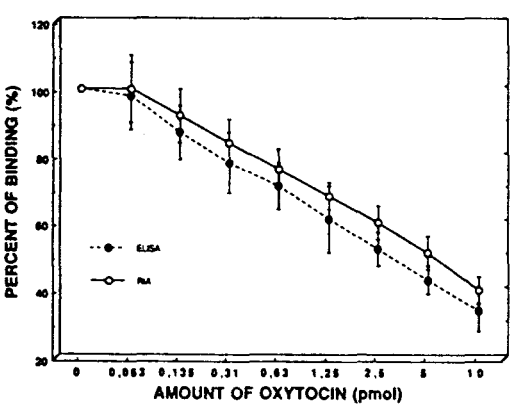

Fig. 6. Percentage of binding as a function of different amounts of OT in two types of assay, competitive ELISA and RIA. Each point represents the mean ± S.D. of four determinations.

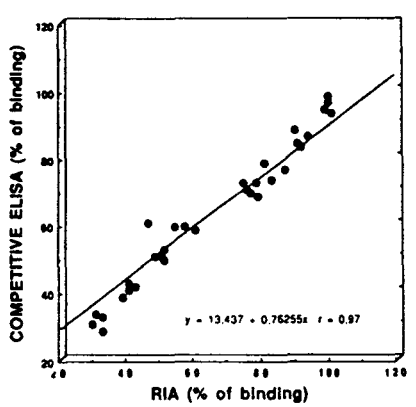

Fig. 7. Correlation between the percentage of binding of OT and different amounts of OT determined in competitive ELISA and RIA. Four pairs of samples were used for each concentration of OT.

Table 2. Amounts of OT-like material in segmental ganglia of the nerve cord of the leech, *E. octoculata*, determined with ELISA. Means ± S.D. of four determinations

Type of segmental ganglia (from reproductive animals)	Amount of OT-like material per ganglion (pmol) Type of ELISA		
	Direct	Inhibiting	Competitive
Sex ganglia	5.04 ± 1.35	0.35 ± 0.15	4.35 ± 1.04
Non-sex ganglia	0.60 ± 0.45	0.45 ± 0.18	0.28 ± 0.35

developed by Yamaji *et al.* (1981) or Yasuda *et al.* (1989), but we obtained a much better coefficient of correlation between competitive ELISA and RIA ($r = 0.97$) than the one obtained by these last authors between RIA and EIA ($r = 0.90$).

The covalent coupling and the competitive nature of the procedure allow the detection of very small amounts of OT. The technique is also applicable to other peptides e.g. FMRFamide, angiotensin II and III, N-terminal part of angiotensin II and C-terminal part of angiotensin II (Salzet, unpublished data).

The direct ELISA for OT is less sensitive than the competitive test (an efficient titration window was found between 10 and 100 pmol) which is comparable to results obtained for angiotensin II in direct ELISA by Sondergard-Andersen *et al.* (1990) with also CovaLink plates.

From the characterization of the antiserum used in the present study (Verger-Bocquet *et al.*, in press), it becomes clear that the antibody recognized the linear part of vertebrate OT and this part of the leech OT-like material therefore must be similar to vertebrate OT. This view is supported by the finding that all the invertebrate peptides of the OT family characterized so far have the general C-terminal sequence Cys-Pro-X-GlyNH_2 where X is represented by a non-polar amino acid (Mühlethaler *et al.*, 1984; Acher *et al.*, 1985).

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EPITOPE MAPPING WITH ELISA OF AN ANTIBODY AGAINST OXYTOCIN USED FOR THE CHARACTERIZATION OF AN OXYTOCIN-LIKE EPITOPE IN THE SEX SEGMENTAL GANGLIA OF THE LEECH *ERPOBDELLA OCTOCULATA*

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Abstract—1. Using direct, inhibiting and competitive enzyme-linked immunosorbent assay (ELISA), two steps were involved in the mapping of the recognition site of a polyclonal antibody against oxytocin (OT).

2. The percentage of cross-reactivity between OT and the N-terminal or the C-terminal fragment of OT demonstrated that the C-terminal fragment is the antigenic part of OT.

3. The percentage of cross-reactivity between OT and other molecules of the OT family indicated that the amino acid in the 8-position and the C-terminal amide of the OT molecule contribute to the recognition.

4. In the two sex segmental ganglia of the leech *Erpobdella octoculata*, where cells immunoreactive to the anti-OT are detected, the antibody has allowed to characterize an epitope close to the mammalian OT by its C-terminal part.

INTRODUCTION

One of the difficulties in the production of antisera against short peptides is that they are not by themselves immunogenic. They have to be coupled to a carrier protein such as serum albumin or hemocyanin (Gullick, 1988). On the other hand, immunizations with synthetic peptides or peptide fragments are advantageous since the resulting antibodies are "site-directed": their primary structure conditions the recognition of the antibody (Hopp and Woods, 1981; Thornton *et al.*, 1986).

The knowledge of the antigenic part of a peptidergic molecule is very important for the understanding of the recognition between an antibody and a peptide, especially when a characterization of an epitope close to a given peptide is investigated.

A large number of antibodies against short neurohypophyseal peptides have been produced which are either monoclonal e.g. anti-arginine–vasopressin (Hou-Yu *et al.*, 1982; Robert *et al.*, 1985) and anti-oxytocin (Burgeon *et al.*, 1991) or polyclonal e.g. anti-arginine–vasopressin (North *et al.*, 1978). Characterization of the above antibodies was mostly done by radioimmunoassay (RIA). In the present paper, a new alternative, based on the use of enzyme-linked immunosorbent assay (ELISA) techniques is proposed to characterize an antibody and to map its recognition site.

Knowing the epitope recognized by an antibody anti-oxytocin that we produced, this antibody was used for the characterization in an invertebrate, the leech *Erpobdella octoculata*, of an oxytocin (OT)-like epitope known to exert an antidiuretic effect in another leech *Theromyzon tessulatum* (Malecha *et al.*, 1989). The knowledge of the theoretical sequence (part of OT recognized by the antibody) is a necessary step which will greatly facilitate the biochemical purification of the OT-like substance in avoiding artefactual immunoreactivities and will permit to obtain the true sequence recognized in neurons.

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MATERIALS AND METHODS

Antibody production

Coupling of OT. OT was coupled to thyroglobulin with glutaraldehyde by a one-step method: 4 mg OT (Interchim) were added to a solution of 10 mg bovine thyroglobulin (Sigma, St Louis, MO, U.S.A.) in 2 ml phosphate-buffered saline pH 7.4. Under constant stirring at 4°C, an equal volume of 0.5% aqueous glutaraldehyde solution was added dropwise. After 3 hr, the reaction was stopped by the addition of sodium metabisulfite (final concentration 2 mg/ml).

Immunization. A rabbit was immunized with OT-glutaraldehyde-thyroglobulin at 40 different sites. Simultaneously, vaccines against tuberculosis and whooping cough were injected. Animals received a second and third injection 1 and 3 weeks after the primary immunization. The first bleeding was 1 week after the third injection. The fourth injection (intravenously) and the second bleeding took place 5 and 6 weeks after the primary immunization, respectively.

Purification of the antibody

Ammonium sulfate precipitation. The 2 ml of antibodies (from the second bleeding) were added to 2 ml of physiological buffer (9‰ NaCl in deionized water pH 7.2), and to 4 ml of 40% ammonium sulfate (0.76 g/ml). The mixture was shaken, and left for 10 min at room temperature, before being centrifuged for 15 min at 4000 rpm. The supernatant

was dissociated from the pellet and discarded. The pellet was subjected three times to the same procedure as above. Then, the purified pellet was resuspended with 1 ml of deionized water and subjected for 48 hr to a dialysis in physiological buffer.

Affinity chromatography. This was performed in 0.1 M sodium carbonate pH 9.0, 0.5 M sodium chloride with BrCN Sepharose 4B (Pharmacia Uppsala, Sweden) to which OT was linked as described by the manufacturer. Antibodies were eluted from the column with 1 M propionic acid pH 3.0 and neutralized with 0.5 M Tris.

ELISA procedures

Buffers. For all subsequent procedures (previously established in Salzet *et al.*, 1992), the following buffers were used:

Coating buffer (CB):	100 mM sodium carbonate pH 9.6.
Blocking buffer (BB):	20 mM sodium phosphate, 50 mM NaCl, pH 7.4 (PBS); 2% bovine serum albumin (BSA).
Washing buffer (WB):	PBS; 0.05% Tween 20.
Dilution buffer (DB):	PBS; 0.05% Tween 20; 0.1% BSA.

Immunoassays. (a) Direct and inhibiting ELISA. All steps were performed under continuous agitation.

CovaLink immunoplates (NUNC, catalogue number 478046 F16) were coated with 100 μl standard OT (CRB)/well at different concentrations (1.5, 3, 6, 12.5, 25, 50, 100 ng OT/ml in CB), 12 hr at 4°C or 2 hr at 37°C. After saturation with BB (200 μl/well), 2 hr at 37°C, plates were rinsed twice with WB, then covered with parafilm and kept at 4°C.

The antiserum (diluted 1/1000 in DB) was incubated (inhibiting ELISA) or not incubated (direct ELISA) overnight at 4°C with the corresponding peptide (100 μg OT/ml pure serum), and 100 μl/well applied for 2 hr at 37°C. Wells were rinsed three times with WB, and 100 μl of peroxidase-conjugated goat anti-rabbit IgG (Diagnostics Pasteur) diluted 1/10,000 in DB then were added to each well. After a 2 hr incubation, followed by two washes in WB and one in PBS, 100 μl of freshly prepared substrate (*ortho*-phenylenediamine 0.5 mg/ml in 0.1 M citrate buffer pH 5.5 containing 0.08% H_2O_2) were added to each well. The reaction was stopped with 1 N HCl (100 μl/well) and absorbance was measured at 490 nm on a MR 250 microplate reader (Dynatech).

(b) Competitive ELISA. Microtiter plates were coated for 2 hr at 37°C with a single concentration (2.5 ng OT/ml in CB). Blocking and washing procedures were the same as for direct and inhibiting ELISA. The primary antibody (diluted 1/500 in DB, 50 μl well) was added simultaneously with the different concentrations of OT: 1.5, 3, 6, 12.5, 25, 50, 100 ng OT/ml (50 μl/well). Incubation, washings, secondary antibody application, color development and stoppage of the reaction were as for indirect and inhibiting ELISA.

In each type of ELISA, all assays were performed in duplicate.

Processing of ELISA data

Percentage of binding. Optical density (OD) readings were used to calculate the percentage of bound OT with the following formula:

$$\text{Percentage binding} = 100 \times [(B - \text{NSB})/(B_0 - \text{NSB})]$$

where NSB, blank value; B_0, peroxidase activity in absence of OT; B, peroxidase activity in presence of OT.

Affinity constants. A computer program for the calculation of antibody affinity constants (K_a) which avoids the limitations of graphical plotting of titration data using the Sips or Scatchard equations (Nelson and Griswold, 1986, 1988).

Percentage of cross-reaction. It was calculated, according to the method of Wakabayashi and Tanaka (1988), from the curve:

$$B/B_0(\%) = f[\text{Log(concentration(OT)}] \text{ at } 50\% \text{ of inhibition.}$$

Peptide treatments

Reduction. For reduction 200 μl of a solution of 1 μmol of OT (CRB) were treated with 600 μl 0.75 M Tris–HCl pH 8.5 containing 0.6 g of 6 M guanidine–HCl. Then, 100 μl of 1 M dithiothreitol were added to the mixture for 1 hr (Proux *et al.*, 1987). The reduced OT was separated in reverse phase HPLC (RP-HPLC) on a 25 × 0.46 cm C18 column (VYDAC 218TP4, 300 Å).

Oxidation. For oxidation 200 μl of a solution of 1 μmol of OT (CRB) were oxidized in 400 μl of pure H_2O_2 for 1 hr. Then, the oxidized OT was separated on RP-HPLC as previously described for reduction.

Imidation. Imidation was achieved by treating 200 μl of a solution of 1 μmol of OT (CRB) or MIF (Sigma) 400 μl of pure acetone for 30 min. Then, fractions were separated on RP-HPLC as described previously.

Immunocytochemical procedure

Parts of *Erpobdella octoculata* (Hirudinae) taken at the level of the genital pores and thus including the two sex ganglia, were fixed overnight at 4°C in Bouin Hollande fixative supplemented with 10% mercuric chloride. They were subsequently embedded in paraffin and serially sectioned at 7 μm. After removal of paraffin with toluene, alternate sections were treated by indirect immunofluorescence. Sections were incubated overnight at room temperature with the primary antibody (anti-OT, 1/200) preadsorbed or not overnight at 4°C with the homologous peptide (OT: 100 μg/ml pure antiserum) or a fragment of this peptide [200 μg/ml pure antiserum of melanocyte stimulating hormone releasing factor (MIF) or Tocinoic Acid: TA (Sigma)]. After rinsing in PBS, pH 7.4, sections were incubated in the dark with FITC-labelled anti-rabbit IgG, diluted at 1/100 for 2 hr at room temperature. Preparations were observed with a fluorescence microscope (Zeiss Axioskop).

RESULTS

Determination of the titre and quality of the anti-OT at different stages of its production (Fig. 1)

The anti-OT titre was investigated in direct ELISA in the preimmune serum (PIS) and after each bleeding. A very low level of anti-OT was detected in the PIS. The best results concerning the anti-OT titre were found after the second bleeding. At this level and in order to determine the quality of the antibody, a comparison between the non-purified antiserum and the antiserum subjected to an IgGs purification was performed at the same dilution (1/1000). Then, a determination of the proportion of anti-carrier and anti-crosslinker was undertaken by titration in direct ELISA of the fraction non retained after filtration of the purified IgGs throughout an OT affinity column. Results indicated a slight difference of quality between the purified and the non-purified antibody and a very low level of anti-crosslinker and anti-carrier in the anti-OT after the second bleeding.

Recognition site mapping

Intrinsic characterization. (a) Direct ELISA. In order to know if the integrity of the OT molecule was necessary for the antibody recognition, an opening of

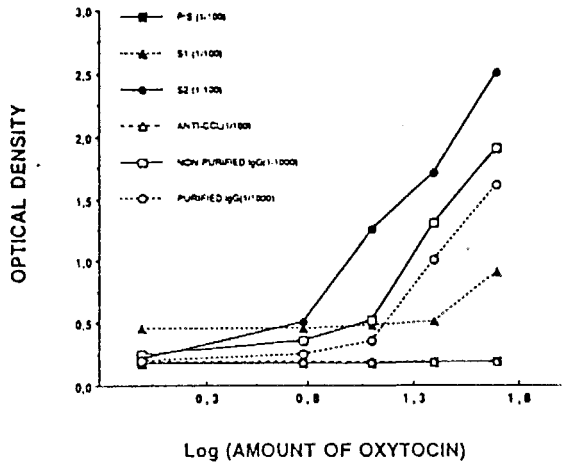

Fig. 1. OD values obtained at 490 nm by direct ELISA, with pre-immune serum (PIS) and with sera (S1, S2) at two steps of the production of the anti-OT, as a function of the OT amount (pmol) fixed to the microtitre wells. S1: serum from the first bleeding; S2: serum from the second bleeding; ANTI-CCL: anti-carrier and anti-cross-linker; non purified IgG and purified IgG are from S2. Dilutions of the antisera are given in parentheses.

the disulfide bridge was performed either by oxidation or reduction. In these conditions, results did not indicate any change in the antibody recognition (Fig. 2A).

With the same aim, the two parts of OT [the N-terminal fragment (TA) and the C-terminal fragment (MIF)] were tested. Very low optical density (OD) readings, similar to background readings were obtained with TA, indicating non recognition by the antibody of TA (Fig. 2B). On the other hand, MIF was as well recognized by the anti-OT as OT (Fig. 2B).

In order to know if the C-terminal amidation was fundamental for the antibody recognition, a treatment of OT or MIF with 100% of acetone which transforms the amide into an imide was performed. In the two cases, a decrease of 50% of the recognition by the antibody was registered (Fig. 2A).

(b) Inhibiting ELISA. The effect of the pre-adsorption of the anti-OT with native, oxidized or reduced OT was estimated by a decrease in the OD values. A strong OD decrease was observed when the antibody was pre-adsorbed with either native or oxidized or reduced OT (Fig. 3A). Similar results were obtained when the anti-serum was adsorbed with oxidized or

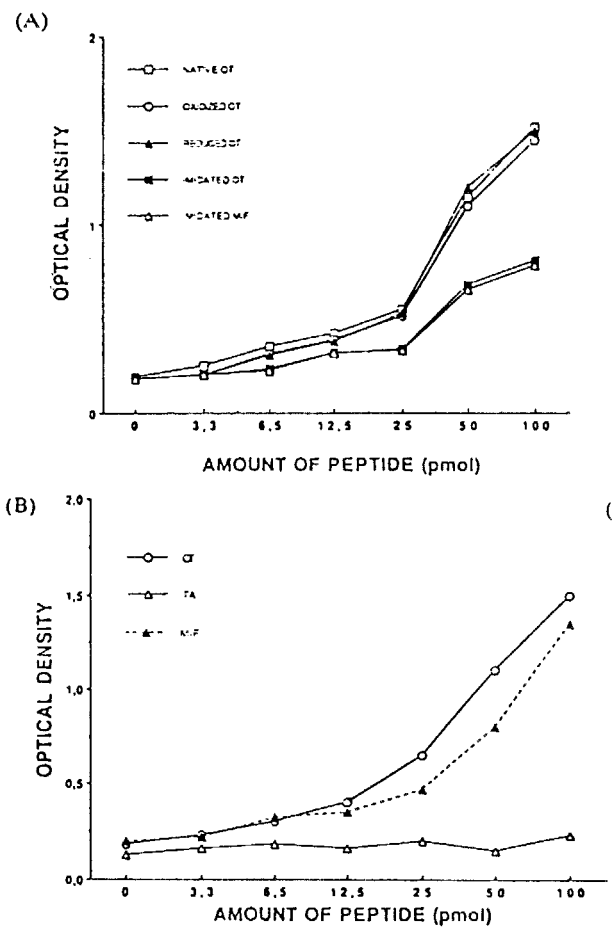

Fig. 2. OD values obtained at 490 nm by direct ELISA as a function of the amounts (pmol) fixed to the microtitre wells of either native, oxidized, reduced and imidated OT, imidated MIF (A) or native OT fragments: TA and MIF (B).

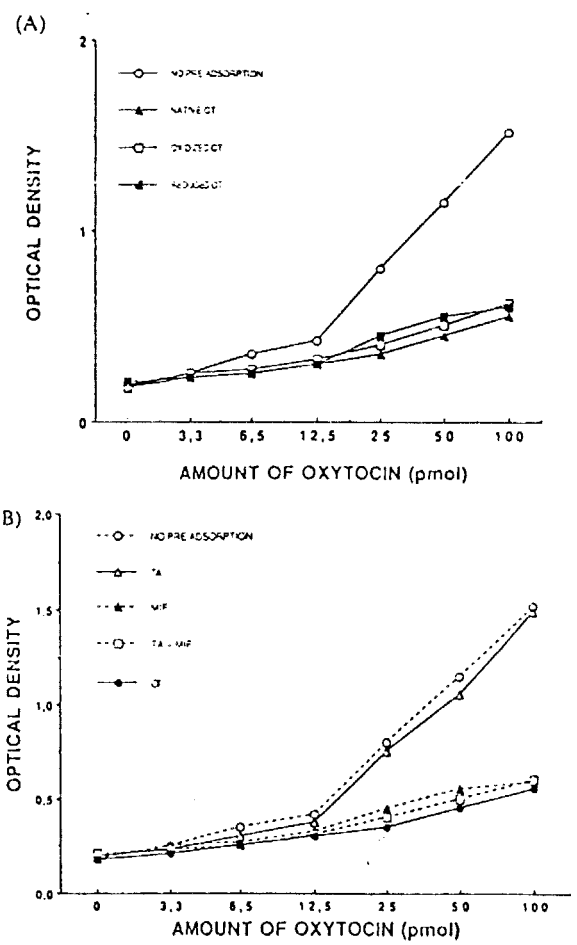

Fig. 3. OD values obtained at 490 nm by direct ELISA after pre-adsorption of the OT antiserum with native or oxidized or reduced OT (A) or OT fragments: TA, MIF, TA + MIF (B) as a function of the different amounts of OT (pmol) fixed to the microtitre wells.

Table 1. Determination of the percentage of cross-reactivity of different peptides (AVP, FMRFamide, ISO, LVP, MIF, OT, oxidized OT, reduced OT, TA) in competition with OT

Peptide	Sequence	Cross-reactivity (%)
OT	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH$_2$	100
Reduced OT	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH$_2$ (SH, SH)	98
Oxidized OT	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH$_2$ (SO_3^-, SO_3^-)	98
TA	Cys-Tyr-Ile-Gln-Asn-Cys	<0.06
MIF	Pro-Leu-Gly-NH$_2$	80.7
ISO	Cys-Tyr-Ile-Ser-Asn-Cys-Pro-Ile-GlyNH$_2$	40
AVP	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH$_2$	<0.8
LVP	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH$_2$	<0.7
FMRFamide	Phe-Met-Arg-PheNH$_2$	<0.7

AVP, arginine vasopressin; FMRFamide, phenylalanyl-methionyl-arginyl-phenylalanine-amide; ISO, isotocin; LVP, lysine vasopressin; MIF, melanocyte stimulating hormone releasing factor; OT, oxytocin; TA, tocinoic acid.

reduced OT (Fig. 3A) or with MIF (Fig. 3B). By contrast, no decrease in the registered OD values was found after pre-adsorption of anti-OT with TA (Fig. 3B).

(c) Competitive ELISA. Oxidized or reduced OT gave 98% cross-reactivity (Table 1) with OT and an affinity constant very close to the one of OT (K_a, *ca* $10^{10}\,M^{-1}$). Close values were obtained with MIF, that gave 80.7% cross-reactivity with OT (Table 1), and had a slightly higher affinity constant ($K_a = 1.85 \times 10^{10} \pm 5.30 \times 10^8\,M^{-1}$) than OT ($1.04 \times 10^{10} \pm 4.70 \times 10^8\,M^{-1}$). No cross-reactivity and no affinity constant, however, were obtained with TA (Fig. 4 and Table 1).

Extrinsic characterization. An oxidation or a reduction of the disulfide bridge of OT had no effect on the recognition of the antibody. By contrast, no recognition was obtained with the N-terminal fragment of OT (TA). Moreover, the C-terminal amidation was important for the antibody recognition. These results were in favour of the existence of an antigenic site on the OT borne by the C-terminal part (MIF). In another step, we investigated whether molecules with a C-terminal amidation, such as FMRFamide, could be recognized by the antibody. Moreover, molecules of the OT family [arginine–vasopressin (AVP); lysine–vasopressin (LVP) and isotocin (ISO)], which differ between each other only by the amino acid in position 8 (leucine) on the linear part, were also investigated, to determine whether a substitution of leucine by arginine or lysine in the suggested antigenic sites could be important for recognition.

(a) Direct ELISA (Fig. 5). Very low OD values were obtained with AVP, LVP and FMRFamide, which indicated a very low level of recognition by the anti-OT. On the other hand, a high level of recognition was found with ISO.

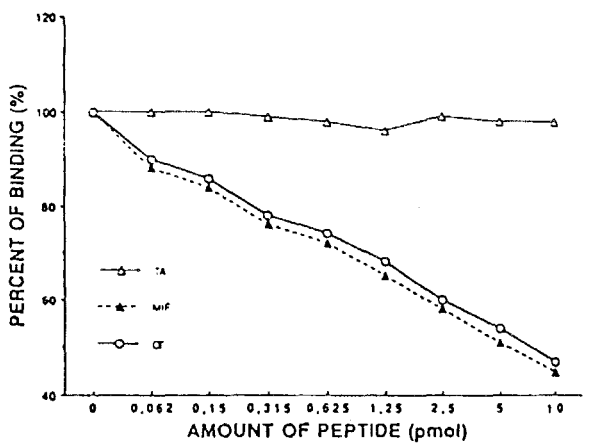

Fig. 4. Percentage of binding in competitive ELISA as a function of different concentrations of OT or OT fragments (TA, MIF).

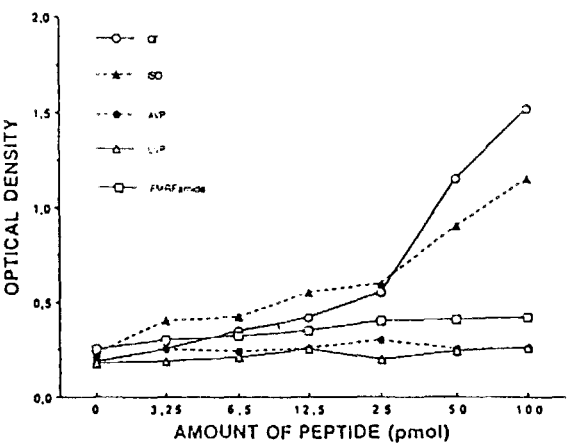

Fig. 5. OD values obtained at 490 nm by direct ELISA for different amounts (pmol) of peptides (AVP, FMRFamide, ISO, LVP, OT) fixed to the microtitre wells.

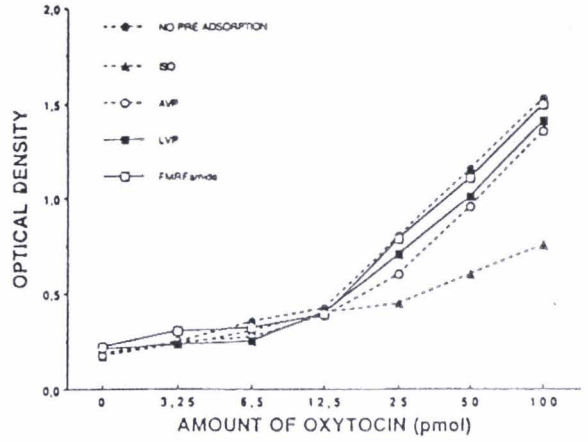

Fig. 6. OD values obtained at 490 nm by direct ELISA after or not pre-adsorption of the OT antiserum with various peptides (AVP, FMRFamide, ISO, LVP) at different concentrations of OT (pmol) fixed to the microtitre wells.

(b) Inhibiting ELISA (Fig. 6). AVP, LVP and FMRFamide did not block the anti-OT. Only ISO produced a decrease of OD.

(c) Competitive ELISA (Fig. 7 and Table 1). Forty per cent of cross-reactivity was obtained when the competitive ELISA was used with ISO. The affinity constant obtained with ISO was $K_a = 1.8 \times 10^8 \pm 7.54 \times 10^6\ M^{-1}$. On the other hand, a very low level of cross-reactivity was detected with AVP, LVP and FMRFamide.

No recognition was found after substitution of the non-polar amino acid (leucine) by a polar amino acid (arginine or lysine) in position 8. By contrast, a substitution of leucine by another non-polar amino acid such as isoleucine did not suppress the recognition but provoked an approximate 60% decrease in recognition.

Evidence for an OT-like epitope in the segmental sex ganglia of the leech Erpobdella octoculata

The anti-OT allows the characterization of an OT-like epitope as demonstrated in the sex segmental ganglia of *E. octoculata* by Verger-Bocquet *et al.* (1991). About 150 cells per sex ganglion were stained with this antibody (Fig. 8a,c,e). Staining was abolished (Fig. 8b,f) after preadsorption of anti-OT with the homologous antigen (OT) or with the C-terminal fragment of OT (MIF). Conversely, the staining capability of anti-OT was not affected after preadsorption with the N-terminal fragment of OT: TA (Fig. 8d).

These results strongly suggest the existence of an OT-like epitope close to the mammalian OT by the C-terminal part (MIF) whose sequence would be: PRO-X-GLY-NH_2 (with X = non-polar amino acid.

DISCUSSION

The present results demonstrate that it is possible to characterize an antiserum by precisely mapping its recognition site using ELISA. This technique offers the advantages of being cheaper and more reproducible than the radioimmunoassay technique. The antibody used in the present work was directed against the C-terminal fragment (MIF), linear part of the OT molecule; it did not recognize the N-terminal part (TA), cyclic moiety of the OT molecule. At the level of the C-terminal part, it was shown that the C-terminal amide contributes to the recognition; treatment of the MIF with 100% acetone which transforms the amide into imide, provoked a decrease of recognition. Nevertheless, this C-terminal amide is not itself responsible for recognition but only contributes to it. For example, the antibdy did not recognize another peptide with a C-terminal amidation e.g. FMRFamide. Moreover, the amino acid in the 8-position (leucine) in OT is fundamental for antibody recognition, a substitution of this amino acid by a polar amino acid (arginine or lysine) provoked non-recognition of the antibody. By contrast, a substitution of leucine by another non-polar amino acid (isoleucine) led to a lower recognition of the antibody. So, leucine is important in determining recognition and its place in the sequence of the OT molecule, i.e. in position two in the tripeptide PRO-LEU-GLYNH$_2$ is also important. We are as yet not able to know if glycine or proline are important for recognition. We can only suggest that proline could participate by giving the tripeptide a bow structure which would be more flexible, accessible in three dimensional (3D) space and perhaps more immunogenic. Glycine, which is the smallest amino acid that exists, could also contribute to the antibody recognition by its simple structure, in giving a better accessibility to leucine.

Previous studies on OT in 3D spacial conformation have led to the proposition of two antigenic domains (Walter, 1977). The first would be on the linear part of the molecule. The second would constitute the hydroxyl group of the tyrosine side chain, folded over the 20-membered ring of OT and by the asparagine carboxamide group (Burgeon *et al.*, 1991).

The antigenicity borne by the linear part could be explained by the site of coupling of OT to the carrier. Glutaraldehyde may interact with amino acids contained in the cyclic part of OT (e.g. TYR and ASN) leaving the linear part free, and thereby favouring the production of antibodies against this moiety and

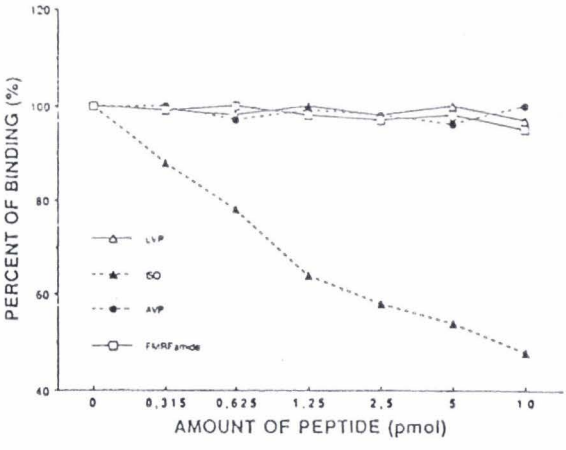

Fig. 7. Percentage of binding in competitive ELISA as a function of different concentrations of various peptides (AVP, FMRFa, ISO, LVP).

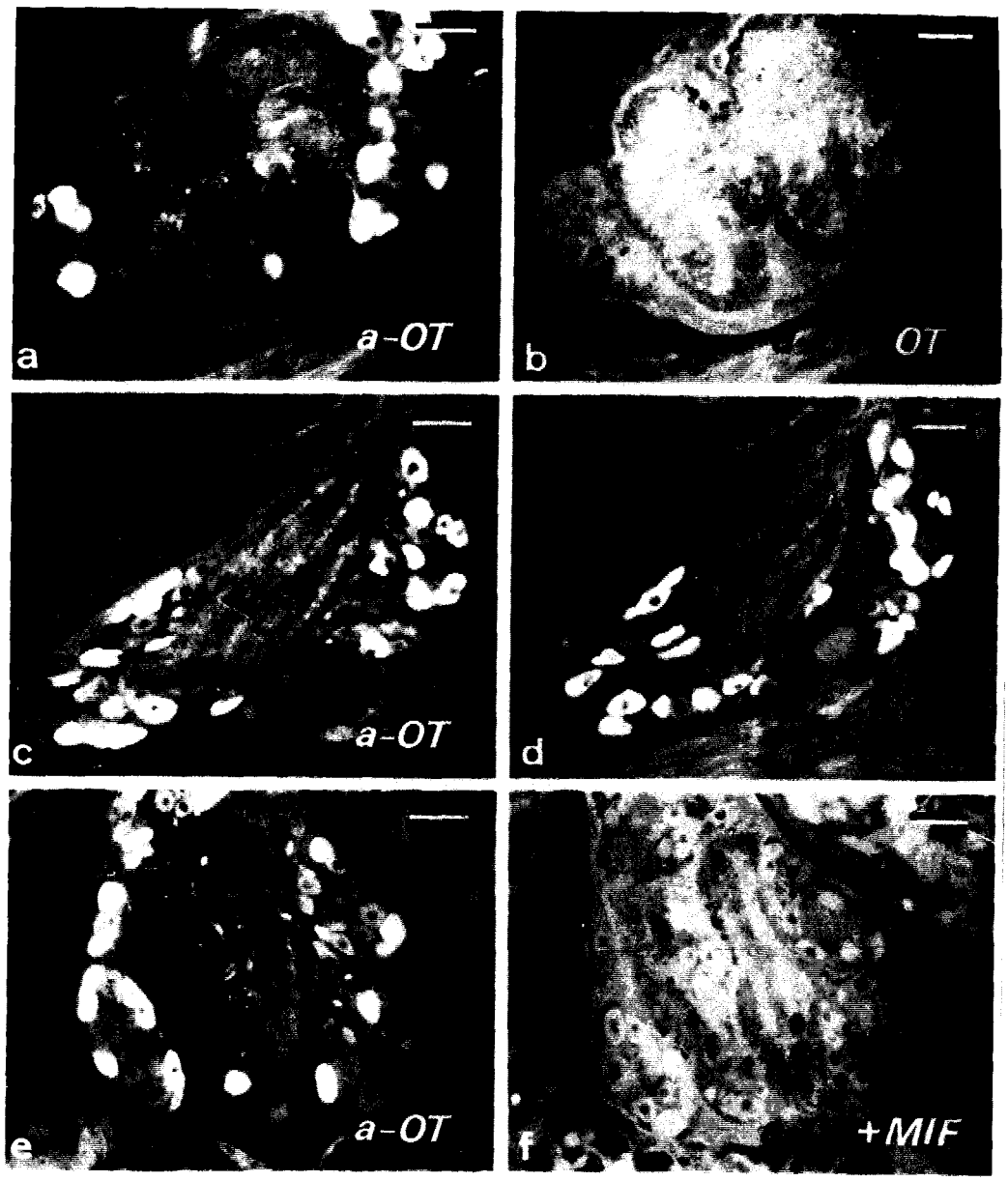

Fig. 8. Photomicrographs of OT-related immunofluorescence in sex segmental ganglia of *E. octoculata* in reproduction. (a,b) Adjacent sections incubated either with anti-oxytocin: a-OT (a) or anti-OT preadsorbed with oxytocin: a-OT + OT (b). Adsorption with OT completely abolished the immunostaining. (c,d) Adjacent sections incubated either with anti-oxytocin: a-OT (c) or anti-OT preadsorbed with N-terminal part of OT: a-OT + TA (d). Cells immunoreact in both cases. (e,f) Adjacent sections incubated either with anti-oxytocin: a-OT (e) or anti-OT preadsorbed with C-terminal part of OT: a-OT + MIF (f). The staining capability of the antiserum is lost after adsorption with MIF. Bars equal 30 μm.

allowing to differentiate mammalian OT from other vertebrate OT or from other molecules of the OT family, by the non-polar amino acid in the 8-position (Acher *et al.*, 1985, Mühlethaler *et al.*, 1984). The use of this characterized antibody will allow better characterization of epitopes to OT in vertebrates and invertebrates. In the leech *E. octoculata*, where numerous cells immunoreactive to this antibody were detected in the two sex segmental ganglia, it can be stated that the epitope recognized is close to the mammalian OT by virtue of its C-terminal part. In this invertebrate, the knowledge of the part recognized by the antibody will be useful for the biochemical purification of the OT-like substance, in view of a sequencing, by eliminating artefactual immunoreactivities.

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Oxytocin-like peptide: a novel epitope colocalized with the FMRFamide-like peptide in the supernumerary neurons of the sex segmental ganglia of leeches — (Morphological and biochemical characterization; putative anti-diuretic function)

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Key words: Oxytocin-like; FMRFamide-like; Sex segmental ganglia; Leech; Immunocytochemistry; Immunoassay; Reverse phase HPLC; Anti-diuretic effect

A large number of oxytocin (OT)-like neurons were detected in the sex segmental ganglia (SG5, SG6) of three species of leeches belonging to different orders: *Theromyzon tessulatum*; *Hirudo medicinalis* and *Erpobdella octoculata*. In this latter species, an epitope close to the vertebrate OT by its C-terminal part (MSH release inhibiting factor: MIF), localized in granules of a size diameter of *ca* 120 nm and colocalized with FMRFamide(FMRFa)-like material was demonstrated. With reverse phase-high performance liquid chromatography, evidence was given that the two epitopes (OT and FMRFa) colocalized in the same neurons were biochemically different. A titration of OT per SG indicated that the OT-like amount was considerably higher in sex SG than in non-sex SG (ca. 5 pmol vs. ca. 0.5 pmol). Moreover, at the level of sex SG, this amount was ca. 3-fold higher in immature leeches than in mature specimens. Injections of extracts of SG of *E. octoculata* and of fragments of OT (Tocinoic acid or MIF) to *T. tessulatum*, indicated that MIF (the epitope found in the sex SG) and sex SG have the same anti-diuretic effect on the leeches injected. These results pointed to an anti-diuretic role of the leech OT-like substance.

INTRODUCTION

Hormones of the vasopressin-oxytocin family are neuropeptides structurally conserved and extensively distributed in the animal kingdom. More than ten hormones of this family are known in vertebrates[1]. Homologous substances have been isolated in Molluscs and Insects[8]. They are cyclic nonapeptides differing only by an amino acid substitution in position 2, 3, 4 or 8. The cycle consists in a disulfure bridge between the cysteins situated in position 1 and 6. In mammals, they play an important role in the control of reproduction and of metabolism of water and salts. In *Locusta migratoria*, a molecule showing 70% sequence identity with arginine-vasotocin has been isolated. It comes under two forms: one is monomeric, the other is its antiparallel dimer which possesses a diuretic activity[23,24]. Conversely, the function of the substances isolated in Molluscs[7,25,34] is largely unknown. In Hirudinae, arguments are in favour of a role of an OT-like substance on osmoregulation. The works of Rosca[26] pointed to a role of mammalian OT on water exchanges of *Hirudo medicinalis*. In the semelparous leech, *Theromyzon tessulatum*, the period between the third and last blood meal and egg-laying (stage 3)[20] is characterized by a considerable increase of mass due to an important water retention[16,33]. Among the molecules immunocytochemically detected in the central nervous system (CNS) of Hirudinae[18,27], three could be candidates, with regard to their known role in vertebrates, for a role in osmoregulation i.e. those immunologically related to vasopressin (VP), oxytocin (OT) and angiotensin II (AII). In *T. tessulatum* at stage 3, the administration of AII resulted in a decrease of mass

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expressing a diuretic effect of this molecule[27]. Injection of mammalian OT or of VP (lysine-VP or arginine-VP) did not affect osmoregulation[19,38]. On the other hand, injection of anti-OT exerted a diuretic effect on leeches[19]. Thus, the anti-diuretic hormone of *T. tessulatum* could be structurally related to mammalian OT without nevertheless being identical.

Immunohistologically, the OT-like substance was detected in neurons of the CNS[18]. The CNS of leeches is composed of a supraesophageal ganglion and of a ventral nerve cord constituted by 32 ganglia. The first four ganglia are fused to form the subesophageal nervous mass. The last seven ganglia are also fused and constitute the caudal ganglion. The twenty one others named segmental ganglia (SG1–SG21) are distributed at the rate of one per metamer. These ganglia contain about 400 neurons except those innervating the two segments bearing the genital pores. These latter (SG5 and SG6), also designated sex SG, have about 350 additional neurons in gnathobdellids[15]. These additional cells appear during the postembryonic development[4], under the influence of a signal produced by the male genitalia primordia[3] and are immunolabelled with an antibody raised against FMRFamide (FMRFa)[4,9,12].

In a preliminary work on the leech *Erpobdella octoculata*, we suggested the coexistence in these cells of an antigen related to FMRFa and of another one related to OT[36].

The aim of the present work was to demonstrate and to characterize a novel epitope (OT-like) colocalized with FMRFa in the supernumerary cells of the sex segmental ganglia of *Erpobdella octoculata*. Moreover, in vivo experiments were conducted in order to investigate its eventual role on osmoregulation.

MATERIALS AND METHODS

Animals

Leeches of three species were used in this study: (i) *Erpobdella octoculata*, collected at Harchies (Belgium); (ii) *Hirudo medicinalis*, purchased from Ricarimpex (France); and (iii) *Theromyzon tessulatum* reared in our laboratory and whose life cycle is subdivided in stages defined by taking as indicators the three blood meals and sexual maturation[20].

Antibodies

Anti-oxytocin (a-OT)

This polyclonal antiserum used in immunocytochemical, ELISA and RIA procedures was raised in our laboratory by immunizing rabbits with synthetic oxytocin (Interchim) coupled to thyroglobulin with glutaraldehyde. It was characterized by Salzet et al.[28]. In short, it did not cross-react with arginine-vasopressin and lysine-vasopressin but presented in ELISA 40% of cross-reactivity with isotocin. As regards to the molecule of oxytocin (OT), it was only directed against its C-terminal fragment (MSH release inhibiting factor = MIF): 80.7% of cross-reactivity with MIF in competitive ELISA, 78.8% of cross-reactivity with MIF in RIA. It did not recognize the N-terminal fragment (tocinoic acid: TA) of OT: 0.06% of cross reactivity with TA in competitive ELISA and in RIA.

Anti-FMRFamide (646) (a-FMRFa)

This polyclonal antibody raised in rabbit and kindly provided by Dr J. Van Minnen (Free University, Amsterdam, the Netherlands) was used in immunocytochemical and ELISA procedures. Its specificity has been described elsewhere[31].

Immunocytochemical procedures

Both sectioned material and whole-mounts were employed.

Whole-mounts

Fragments of ventral nerve cords from *T. tessulatum*, *E. octoculata* and *H. medicinalis* were treated according to the method of Salzet et al.[27]. Primary antibody (a-OT) was diluted at 1:500; FITC-labeled goat anti-rabbit IgG (Sigma) was used at a dilution of 1:100. Whole-mounts were examined with a Zeiss Axioskop fluorescence microscope.

Sections

Light microscopy. Parts of *E. octoculata* taken at the level of the genital pores, and thus including the two sex segmental ganglia (SG) were fixed overnight at 4°C in Bouin-Hollande fixative (+10% $HgCl_2$ saturated solution), they were then embedded in paraffin and serially sectioned at 7 μm. After removal of paraffin with toluene, the sections were successively treated with the primary antibody (a-OT or a-FMRFa) diluted 1:200 and with goat anti-rabbit IgG conjugated either to horseradish peroxidase or to fluorescein isothiocyanate as described elsewhere[35,36].

The specificity of the antisera (a-OT and a-FMRFa) was tested on consecutive sections mounted on different slides by preadsorbing these antisera overnight at 4°C with the homologous or an heterologous antigen (synthetic OT (CRB) or synthetic FMRF-amide (CRB)) at a concentration of 100 μg/ml of pure antiserum. The anti-OT was also preadsorbed with different fragments of OT (MIF (Interchim); TA (Interchim)) at a concentration of 200 μg/ml of pure antiserum.

Electron microscopy. Sex SG were fixed for 2 h at 4°C in a mixture of 4% paraformaldehyde, 0.2% picric acid and 0.1% glutaraldehyde in 0.1 M phosphate buffer and embedded in Epon.

Immunostaining was performed directly on ultrathin sections of SG collected on nickel grids and treated according to the following procedure: (i) 10% H_2O_2, 8 min; (ii) distilled water, 10 min; (iii) Coons buffer, pH 7.4, containing 1% normal goat serum (NGS), 10 min; (iv) a-OT diluted 1:1200 in Coons buffer containing 1% NGS and 1% bovine serum albumin (BSA), 24 h, 4°C; (v) Tris 0.1 M, pH 7.6 containing 0.5 M NaCl, 1% BSA and 1% NGS, 2×10 min; (vi) 5 nm colloidal gold-labeled goat anti-rabbit IgG (Janssen, Belgium) diluted 1:50 in the precedent buffer, 1.5 h; (vii) washings in the buffer (2×10 min) and distilled water (2×10 min).

Finally, the sections were stained with uranyl acetate and lead citrate and then examined with a Jeol JEM 100 CX electron microscope.

ELISA procedures

Covalink immunoplates (NUNC) pretreated with 1% glutaraldehyde were used. They were coated (12 h at 4°C or 2 h at 37°C) with the equivalent of 10 pairs of sex SG of *E. octoculata* per well (direct and inhibiting ELISA) or at a single concentration of 25 ng synthetic OT (CRB)/ml coating buffer (competitive ELISA). For the immunoassays, these coated plates were treated according to Salzet et al.[29].

OT-like substance in sex SG of *E. octoculata* was quantified with competitive ELISA or with direct and inhibiting ELISA. In the latter case, the difference between the two values registered gave the

specific amount (expressed in pmol) of OT-like substance per ganglion.

The immunoreactivity with a-OT and a-FMRFa of the fractions obtained after fractionation in reverse phase HPLC of an extract of sex SG from *E. octoculata* was expressed by the amount (ng) of peptide (OT or FMRFa) per fraction. This value was obtained from OD readings both in direct and inhibiting ELISA reported on the standard curve. Immunoreactivity per fraction was expressed in percentage obtained with the formula:

$$[(B - B_0)/(B_{max} - B_0)] \times 100$$

with B = O.D. reading per fraction in the presence of peptide (OT or FMRFa); B_0 = O.D. reading per fraction in the absence of peptide (OT or FMRFa); and B_{max} = O.D. reading at the maximum of the standard curve.

RIA procedure

Sex SG were quickly excised, frozen at − 180°C in liquid nitrogen and stored at −20°C until assay. The ganglia were homogenized in 1 M acetic acid with a Dounce homogenizer, the homogenate sonicated 30 sec twice and centrifuged (10,000 rpm, 10 min, 0°C). The supernatant was removed and neutralized with 2 N NaOH. Samples were subjected in duplicate to radioimmunoassay for OT. At a final dilution of 1:300, the antiserum a-OT bound 20±3.4% of ^{125}I-OT (Amersham). Assay volume was 300 μl comprising 100 μl of a-OT (1:300), 100 μl of standard OT (CRB) or sample and 100 μl of ^{125}IOT (10,000 cpm). Standard and sample were reconstituted in assay buffer (0.1 M carbonate buffer pH 9.5 containing 0.2% of BSA). Antiserum was incubated with standard or sample overnight at 4°C prior to addition of label and then for a further 24 h at 4°C. Separation of bound from free tracer was performed by the addition of 400 μl of dextran-coated suspension (0.5 g of Norit EXW charcoal (Sigma) and 0.05 g dextran (BDH) suspended in 50 ml of assay buffer) to all tubes apart from total counts. Tubes were centrifuged at 6,000 rpm for 15 min at 4°C and the supernatants decanted.

Processing of RIA data

Samples were counted with a γ-counter (LKB). The assay was used to quantify OT-like material contained in sex SG of *E. octoculata*. The averages of the measures were expressed in pmol of OT per SG. Values obtained derived from samples counts reported to a standard curve drawn with the following formula:

$$B - B_0 = f(\log[OT])$$

with B_0 = number of counts (cpm) given by the solution comprising the 0.1 M carbonate buffer, the ^{125}I-tracer (10000 cpm/100 μl) and the antibody a-OT (1:300); and B = number of counts (cpm) of tracer in competition with different dilutions of samples.

Biochemical procedures

Extraction of tissues and purification

Fragments of nerve cord, including the two sex SG of mature or immature *E. octoculata* were dissected in leech Ringer[21] then frozen at − 180°C in liquid nitrogen and finally stored at −20°C. Sex SG in batches of 200–400 were homogenized in 200–400 μl of 1 M acetic acid with a Dounce homogenizer and then sonicated twice (30 s). Each homogenate was centrifuged at 12,000 rpm for 30 min at 0°C. The pellet was dissociated from the supernatant and reextracted as before and the two supernatants were combined. These supernatants were subjected to a preliminary purification using small C_{18} reverse-phase cartridges (Sep-Pak, Waters Associates). The Sep-Pak matrix was activated with 10 ml pure methanol and washed with 10 ml of 1 M acetic acid. After application of the extract to the matrix, the Sep-Pak was washed with 5 ml of 1 M acetic acid and elution was then performed with 2 ml of 50% acetonitrile. The eluant (2 ml) was concentrated 10-fold in a SVC 200H Speed-Vac concentrator (Savant) before being separated on reverse-phase HPLC (RP-HPLC) with a 25×0.46 cm C_{18} column (Vydac, 218 TP4, 5 μm, 300 Å) associated to a RP 18 precolumn (Merck). The solvent program consisted of an initial hold with 100% of solvent A (0.1% trifluoroacetic acid (TFA) in water), then gradients of solvent B (0.1% TFA in 100% acetonitrile) as mentioned in the Fig. 5 heading. The flow rate was 1

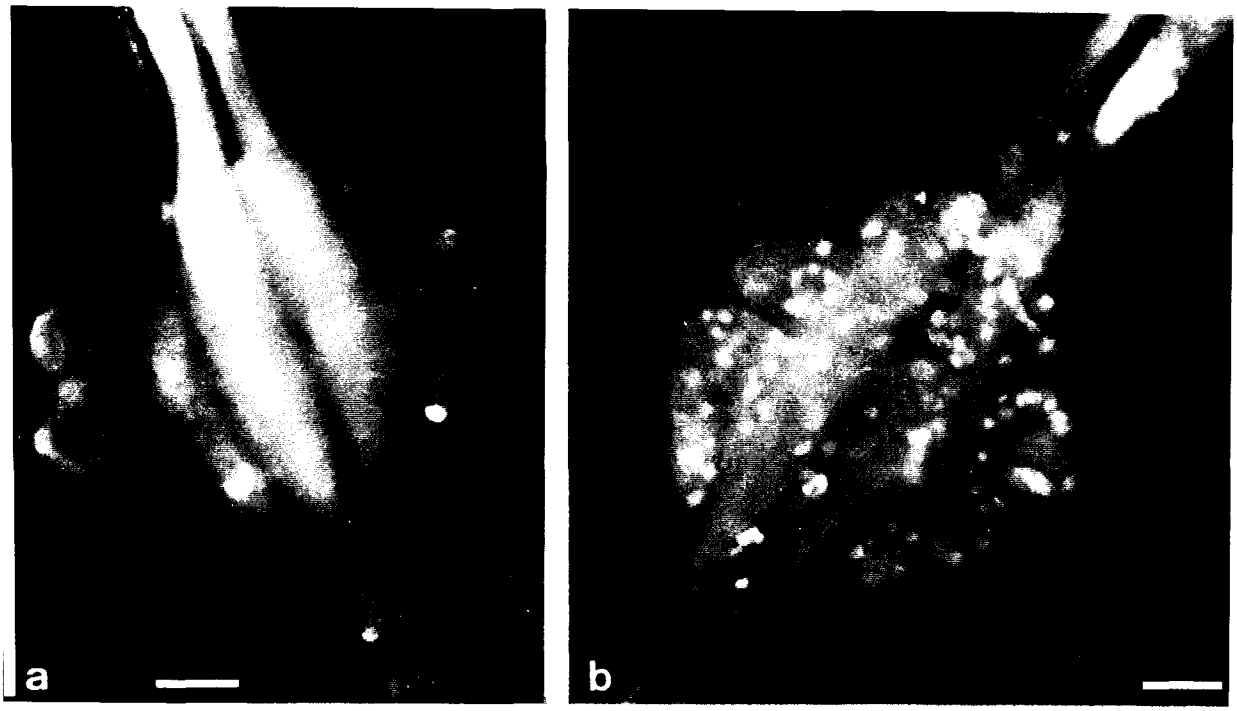

Fig. 1. Immunohistochemical fluorescent micrographs of whole-mount preparations of a nerve cord of *E. octoculata* treated with anti-oxytocin. Posterior is to the top of figure. a: non-sex ganglion (SG8) focused dorsally at the level of two imunoreactive cells located in the lateral compartments. Immunoreactive cells are in low number. b: sex ganglion (SG6) viewed ventrally. Numerous immunoreactive neurons are detected. Bars = 50 μm.

ml/min. Peptide peaks were detected by monitoring absorbance at 226 nm. Fractions were collected at 1 min interval and concentrated 5-fold in the Speed-Vac concentrator. The resulting 200 μl were distributed in the immunoplates (100 μl/well) and assayed in direct and inhibiting ELISA for OT and FMRFa.

Peptides treatment

Reduction. Two hundred μl of a solution at 1 μM of OT (CRB) were treated with 600 μl 0.75 M Tris/HCl pH 8.5 containing 0.6 g of 6 M guanidine/HCl. Then, 100 μl of 1 M dithiothreitol (DTT) were added to the mixture for 1 h[24]. Reduced OT was separated on RP-HPLC on a 25×0.46 cm C_{18} column (Vydac 218 TP4, 5 μm, 300 Å).

Oxidation. Two hundred μl of a solution of OT or FMRFa (CRB) at 1 μM were oxidized in 400 μl of pure H_2O_2 for 1 h. Then, the oxidized peptide was separated on RP-HPLC as precedently.

In vivo experiments

The bioassay[17] was conducted on leeches of the species *T. tessulatum* which had taken their third meal on the same day and in which water retention is known to be high (stage 3B). Products (extracts of sex SG and non-sex SG, MIF and TA) were dissolved in a 0.05 M PBS and injected in the subepidermal connective tissue.

Each animal received 10 μl of a 1 nmol solution of peptide or 20 μl of a 5 SG-equivalent solution. In the latter case, 100 SG (sex or non-sex SG) of the nerve cord of *E. octoculata* were homogenized in 200 μl of 0.05 M PBS at 0°C with a Dounce homogenizer. The homogenate was boiled 5 min then centrifuged at 6,000 rpm for 5 min. The supernatant was taken and complemented to 400 μl with milliQ water. Control animals received 0.05 M PBS (20 μl).

The effect of each treatment was estimated by measuring the change of body mass of the animals between the beginning (injection) and the end of the experiment (2, 4, 8 and 24 h after injection). The results are shown as mean (±S.D.), with a minimum of 17 animals at each point. Statistical analysis of results was performed by calculating the mass variation of each leech at a t instant compared to the initial mass (at the time of injection). Statistical comparisons were made with Student's t-test. Confidence interval (with $\alpha = 0.5$) of the mean of the relative variations of mass was obtained according to Scherrer[30].

RESULTS

Immunocytological investigations

In the Pharyngobdellid *E. octoculata* (Fig. 1), all SG contained cells immunoreactive to a-OT. A higher number of OT-like immunoreactive cells was found in the two sex SG (SG5 and SG6), compared to the non-sex SG (100–200 vs. ca. 10). At the ultrastructural level, the gold label was found in the OT-like immunoreactive cells of the sex SG over a single type of electron-dense secretory granules of a size ca. 120 nm (Fig. 2). Two leeches belonging to two different orders were also investigated immunocytochemically: *T. tessulatum* (Rhynchobdellae), animal on which the biological test for osmoregulation is conducted, and *H. medicinalis* (Gnathobdellae). Results indicated that in these two leeches, OT-like cells were, as in *E. octoculata*, in higher number in the two sex SG than in the non-sex SG (Fig. 3a–f).

In the sex SG of *E. octoculata*, numerous FMRFa-like immunoreactive cells were also detected. Using adjacent sections of sex SG, it was demonstrated that in sex SG, FMRFa-like immunoreactivity is colocalized with OT-like immunoreactivity (Fig. 4).

A more detailed study of the sex SG of *E. octoculata* (Table I) demonstrated that the number of OT-like immunoreactive cells varied according to both the SG and the stage considered. Indeed, at the two physiological stages investigated, it was higher in SG6 which innervates both male and female genital organs than in

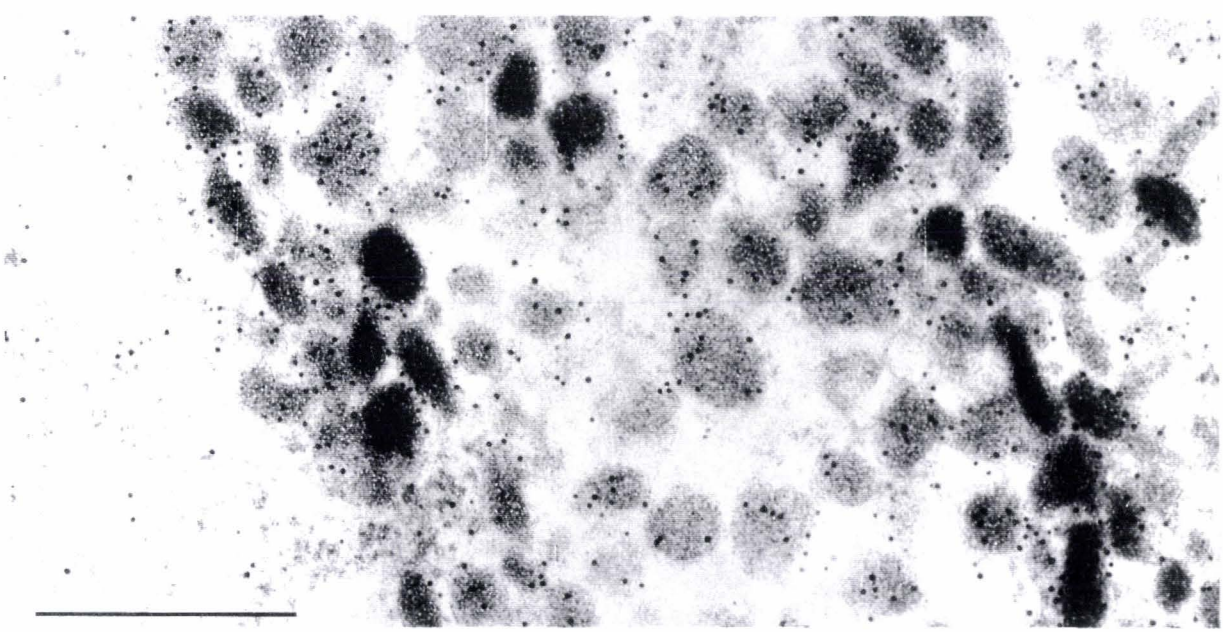

Fig. 2. Electron micrograph at the level of the cell body of an oxytocin-like immunoreactive cell in a sex segmental ganglion (SG5) of *E. octoculata* showing immunogold-labelled secretory granules. A certain variability of the density of these granules is observed. Bar = 0.5 μm.

SG5 which innervates only the male organs (Fig. 5a). Moreover, for a given ganglion, this number was higher in the immature specimens than in mature specimens. In the same species, preadsorption of a-OT (see Fig. 5b–d) with the homologous antigen abolished the staining capacity of this antibody (Table II). In contrast, preadsorption of a-OT with FMRFa (localized in the supernumerary neurons of the sex SG, see Introduction) did not affect the immunopositivity of the cells in the sex SG (Table II). Moreover, the staining capacity of a-FMRFa was abolished after preadsorp-

TABLE I

Number of cells immunoreactive with a-OT in the sex segmental ganglia (SG5, SG6) of E. octoculata at two different physiological stages

Numerical data represent the mean ±S.D. (*n*). S.D. is the standard deviation; n is the number of specimens analyzed. All the cell counts were done from serial sections stained with immunoperoxidase.

Physiological condition of the leeches	*Number of OT-like immunoreactive cells in SG*	
	5	*6*
Immature	133.5 ± 6.3 (6)	193.5 ± 23.7 (6)
Mature	116.6 ± 9.9 (6)	153.3 ± 16.3 (6)

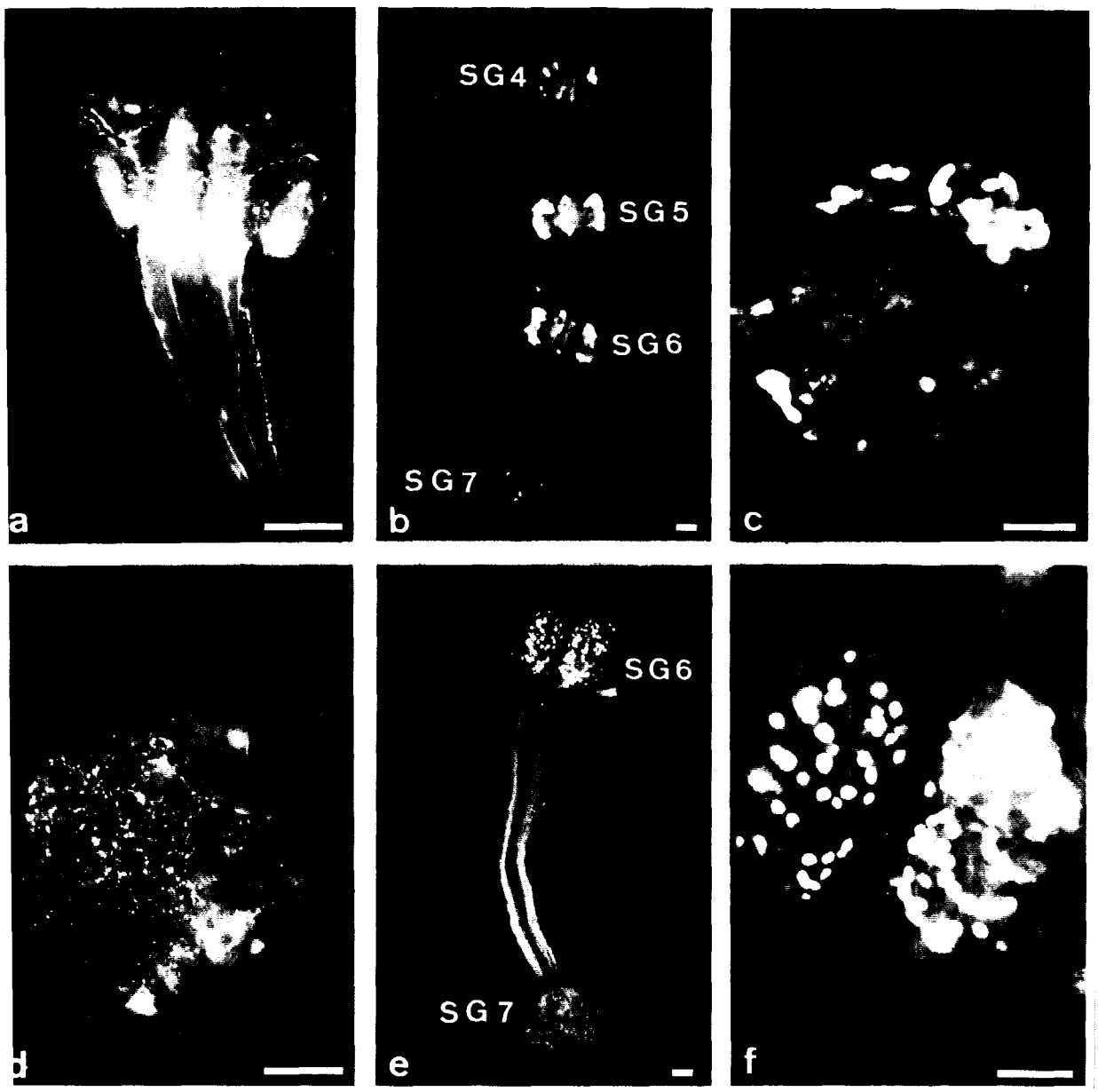

Fig. 3. Immunohistochemical fluorescent micrographs of whole-mount preparations of nerve cords of stage 3B *T. tessulatum* (a–c) and of *H. medicinalis* (d–f) treated with anti-oxytocin. a,d: dorsal views of non-sex ganglia (SG4 for a, SG7 for d). Anterior is to the bottom (a) and to the top (d) of figure. Focal plan is at the level of the neuropile. Only a few immunoreactive cells are present. Immunoreactive processes are visible within the neuropile. b: ventral view of a portion of nerve cord featuring two non-sex segmental ganglia (SG4, SG7) and two sex segmental ganglia (SG5, SG6). Anterior is to the top of figure. Sex SG are strongly labelled. c,f: dorsal views of sex ganglia (SG6) presenting numerous immunoreactive neurons. Anterior is to the left (c) or to the top (f) of figure. e: dorsal view of a portion of nerve cord featuring a non-sex segmental ganglion (SG7) and a sex segmental ganglion (SG6). The latter contains more immunoreactive neurons than a non-sex SG. Anterior is towards the top. Bars = 100 μm.

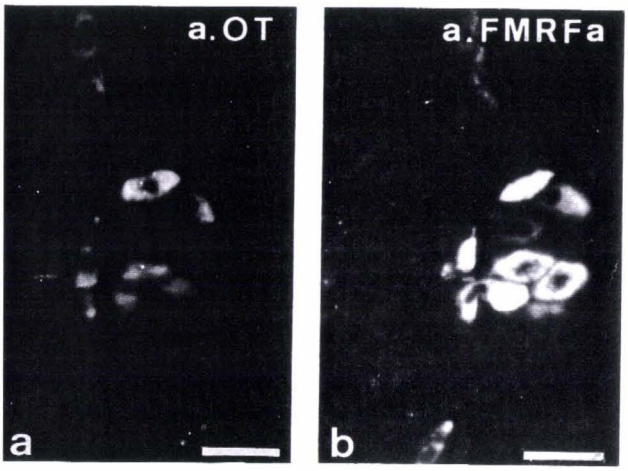

Fig. 4. Immunohistochemical fluorescent micrographs of adjacent frontal sections at the level of a lateral posterior compartment of a sex segmental ganglion (SG6) treated either with anti-oxytocin (a-OT; a) or with anti-FMRFamide (a-FMRFa; b). Bars = 20 μm.

tion with FMRFa but not after preadsorption with OT (Table II). These results strongly suggested the localization of two distinct epitopes (OT-like and FMRFa-like) in the same cells.

Concerning the characterization of the OT-like epitope demons-trated in *E. octoculata*, the fact (Table II) that the immunostaining was abolished after preadsorption of a-OT with the C-terminal fragment of OT (MIF) but not after preadsorption of a-OT with the N-terminal fragment of OT (TA), supported the idea that the C-terminal part of OT was recognized. As for the N-terminal part, the use of another a-OT characterized by Tramu[32] and which recognizes both the N- and the C-terminal parts of OT, indicated (unpublished data) that TA was not recognized. All these results were in favour of the existence of an OT-like epitope close to the mammalian OT by its C-terminal part.

Biochemical investigations

Evidence for distinct OT-like and FMRFa-like epitopes in the sex SG of the leech E. octoculata

To confirm the coexistence of distinct antigens related to FMRFa and OT, an extract of sex SG from mature *E. octoculata* was fractionated in RP-HPLC and each eluted fraction was assayed in ELISA for OT and FMRFa-immunoreactivity (Fig. 6).

Assays with a-OT indicated that three zones z1, z2 and z3 exhibited an OT-like immunoreactivity. After preadsorption of the a-OT with synthetic OT peptide, the immunoreactive zones (z1 and z3) disappeared, in contrast to z2 which subsisted. So, only z1 and z3 were considered as specific zones immunoreactive to a-OT. These two zones had retention times (30–31 min for z1 and 36–37 min for z3) which did not correspond to the ones found in this system for native OT (21.7 min), reduced OT (19.2 min) or oxidized OT (23.2 min). Assays with a-FMRFa preadsorbed or not with FMRFa demonstrated that among three immunoreactive zones (z4, z5 and z6), only z4 and z5 reacted specifically with a-FMRFa. These two zones (z4 (retention time: 16–19 min) and z5 (retention time: 23–24 min)) were different from the ones immunoreacting with a-OT. However, in the same conditions FMRFamide and oxidized FMRFa eluted in these two immunoreactive zones with retention times of 17 min for the oxidized FMRFa and 23.6 min for the native FMRFamide.

Amounts of oxytocin-like material in the segmental ganglia of E. octoculata

The amounts of OT-like material in the segmental ganglia were determined with RIA and with ELISA (competitive and both direct and inhibiting ELISA). These methods gave results in good agreement (Table III). They allowed to state that in mature leeches, the amount of OT-like material per sex SG (ca. 5 pmol) was considerably higher than the one found in a non-sex SG (ca. 0.5 pmol).

A comparison of the RIA titration values in the sex SG of leeches at two different physiological stages showed an amount of OT-like material 3-fold higher in immature animals than in mature animals (17.5 ± 2.9 pmol/sex SG vs.. 5.82 ± 0.8 pmol/non-sex SG, $n = 4$).

Comparative study of the OT-like immunoreactive zones detected in mature and immature animals

Since results obtained with RIA indicated a variation in the OT-like substance content in the sex SG of *E. octoculata* at different physiological stages, a comparison of the immunoreactive zones obtained with a-OT after separation of a sex SG extract in RP-HPLC was undertaken with sex SG from either mature or immature *E. octoculata*.

Results showed that the two specific immunoreactive zones detected in mature animals (z1, retention time: 30–31 min and z3, retention time: 36–37 min) were present in sex SG of immature animals. However,a third zone (z7, retention time: 12–13 min) was detected only in immature leeches. From Table IV, it can be stated that in mature animals, the amount of OT-like material per sex SG in the OT-like immunoreactive zones was ca. 3-fold higher in z1 than in z3. On the other hand, in immature leeches (see Table IV), this amount was greatly higher in z7 than in z1 and z3 (ca. 8-fold higher compared to z1, ca. 10-fold higher compared to z3).

TABLE II

Immunostaining in sex segmental ganglia of E. octoculata with antibodies (a-OT or a-FMRFa) preadsorbed with various antigens

OT, oxytocin; FMRFa, FMRFamide; MIF, MSH release-inhibiting factor; TA, tocinoic acid.

Antibody	*Staining reaction*	
	Blocked by	*Unaffected by*
a-OT (1:200)	OT; MIF	FMRFa; TA
a-FMRFa (1:200)	FMRFa	OT

Interrelation between the OT-like immunoreactive zones

Reduction with DTT of z1 and z3 generated a single immuno-reactive peak (retention time: 21 min) in both mature and immature animals. However, in immature animals reduction with DTT shifted the retention time of the z7 peak to 9.5 min.

Physiological investigations

Our observations in *E. octoculata* of the existence in the sex SG of numerous cells immunoreacting with a-OT have led us to investigate if the SG of *E. octoculata* could contain a substance acting on osmoregulation. Specimens of *T. tessulatum* were thus injected (Fig. 7) with extracts of two types of SG (sex and non-sex SG) from *E. octoculata*. The mass variations of

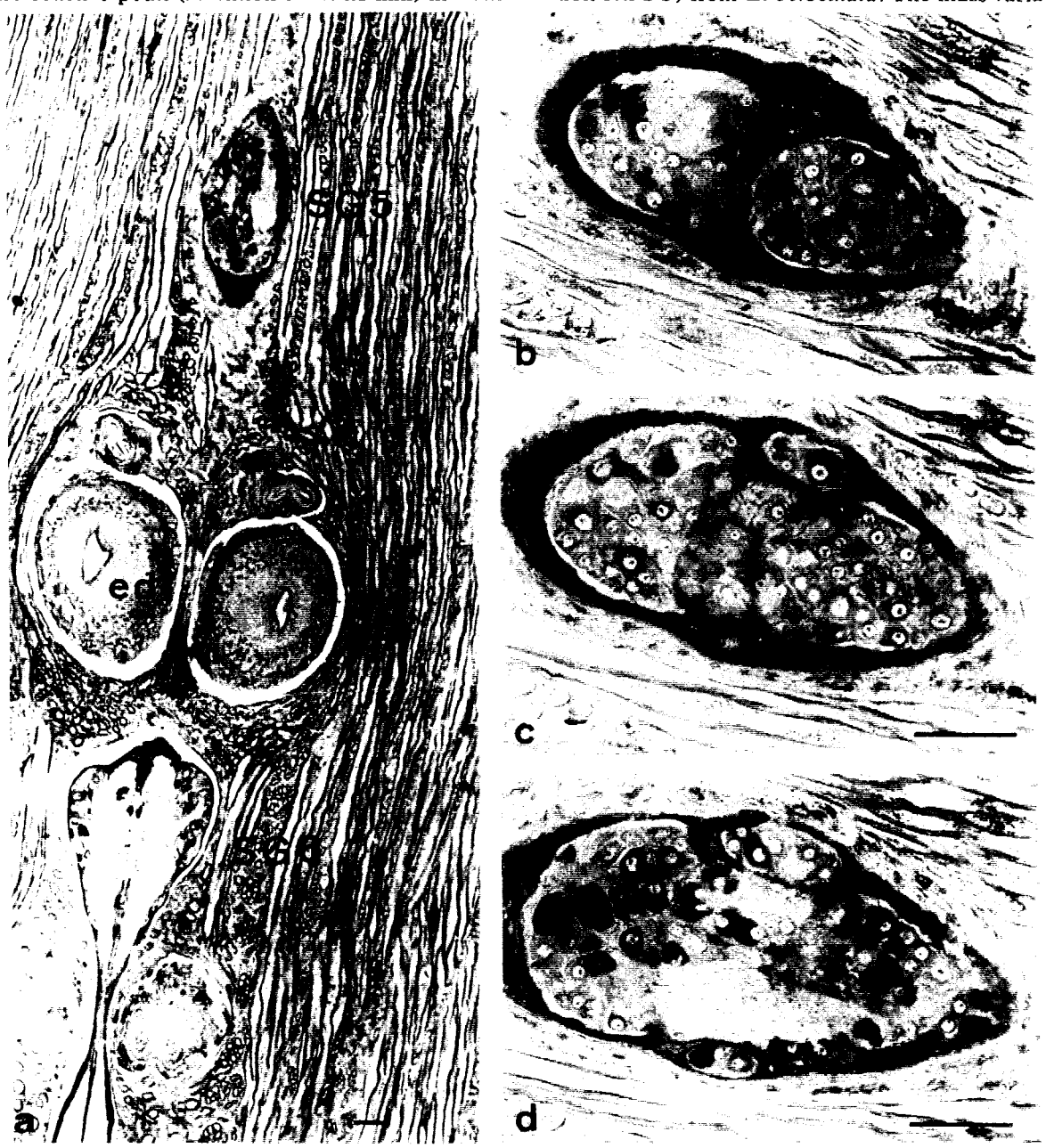

Fig. 5. Frontal sections through the sex segmental ganglia (SG5, SG6) of *E. octoculata* treated with anti-oxytocin preadsorbed (c) or not (a,b,d) with the homologous peptide (indirect immunoperoxidase). Preadsorption of the antibody with OT abolished the immunostaining (see consecutive sections b–d). e,d: section of the ejaculatory duct. Bars = 50 μm.

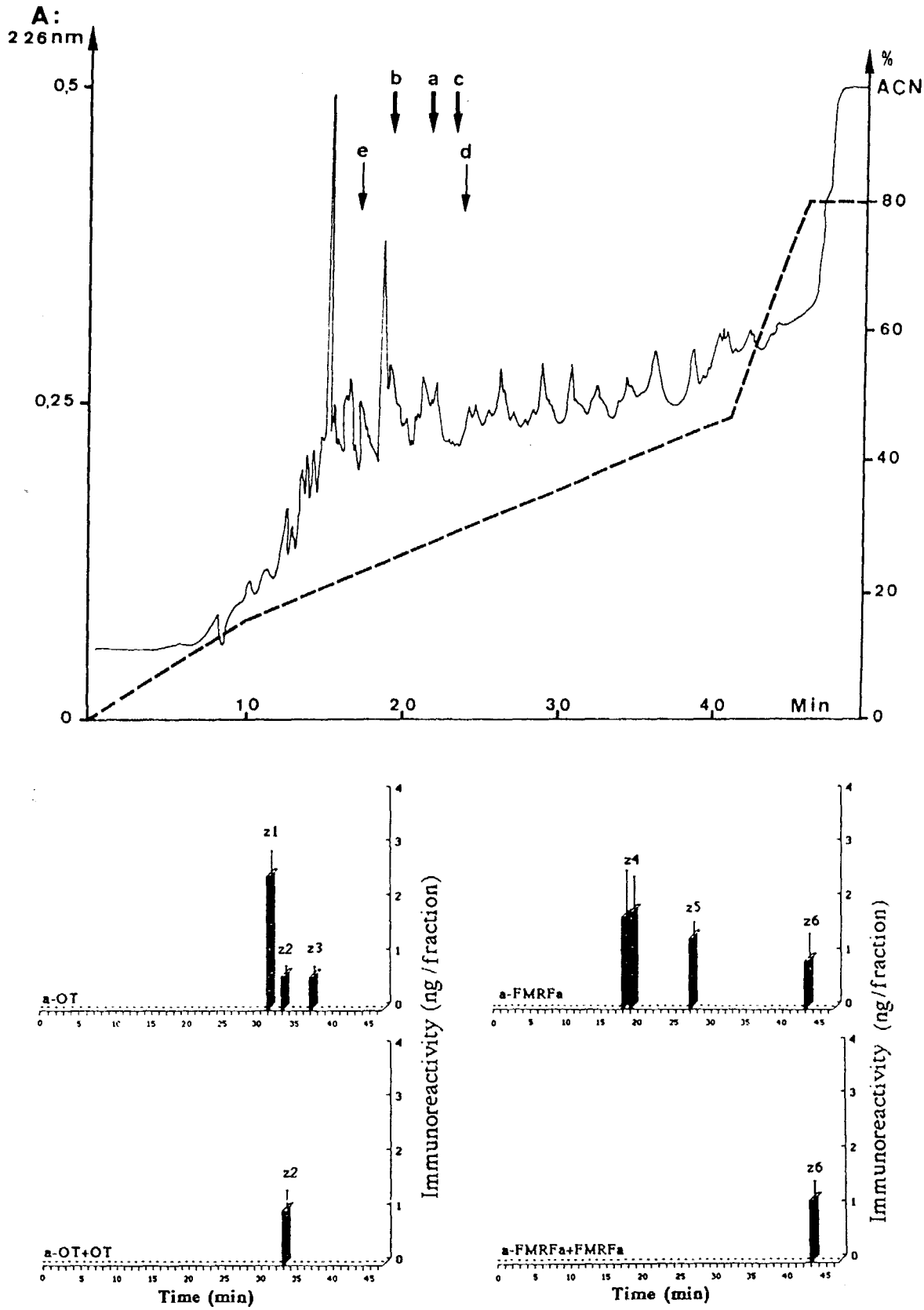

Fig. 6. Reverse phase HPLC elution profile of an extract of 400 sex SG from mature *E. octoculata*. Elution rate: 1 ml/min; solvent A: 0.1% TFA in water; solvent B: 0.1% TFA in 100% CH_3CN; solvent program: gradients of B at 1.5%/min from 0 to 15% followed by 1%/min from 15 to 45% and by 7%/min from 45 to 80%. The solid line indicates absorbance; the dotted line indicates the gradient. Arrows show the respective elution positions of native (a), reduced (b) or oxidized oxytocin (c) and native (d) or oxidized FMRFamide (e). Fractions were assayed for OT-like and FMRFa-like immunoreactive material (diagrams) with antibodies (a-oxytocin, a-OT; FMRFamide, a-FMRFa) preadsorbed (a-OT + OT, a-FMRFa + FMRFa) or not with the homologous antigen. Values given for each assayed fraction correspond to aliquots of fractions of 1 μl.

TABLE III

Amounts of OT-like material in segmental ganglia (SG) of mature E. octoculata determined with different assays

Each value is a mean ± S.D. of 4 determinations. n.d., not determined.

Type of SG	*Amount of OT-like material (pmol / ganglion)*		
	Direct and inhibiting ELISA	*Competitive ELISA*	*RIA*
Sex SG	4.69 ± 1.36	4.35 ± 1.04	5.82 ± 0.78
Non-sex SG	0.15 ± 0.48	0.28 ± 0.35	n.d.

TABLE IV

Amounts of OT-like material per sex SG in OT-like specific immunoreactive zones detected after RP-HPLC separation of an extract of sex SG from immature and mature E. octoculata

Each value is the mean ± S.D. of 4 determinations.

Specific immunoreactive zones to a-OT	*Amount of OT-like material per sex SG (pmol)*	
	Immature leeches	*Mature leeches*
z1	0.48 ± 0.2	1.24 ± 0.69
z3	0.30 ± 0.13	0.48 ± 0.12
z7	3.84 ± 0.57	-

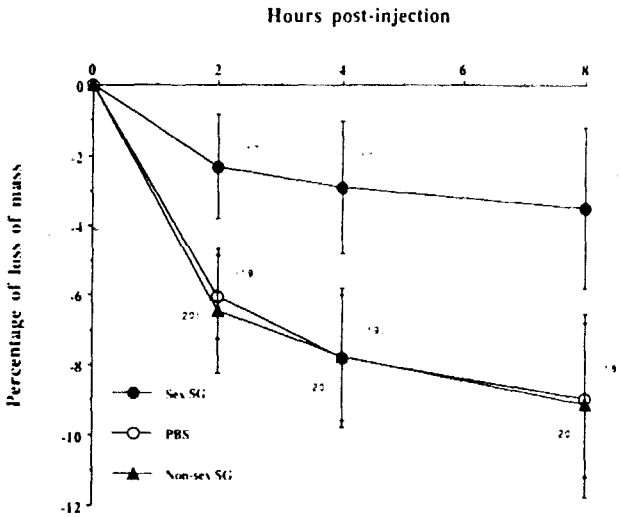

Fig. 7. Effect of the injection of extracts of sex and non-sex segmental ganglia (SG) of *E. octoculata* on the body mass of stage 3B *T. tessulatum*:: mean percentual loss of mass at different times after injection. Each mean value and standard deviation is based on a number of animals figured in parentheses. Dose injected: 5 ganglia-equivalent.

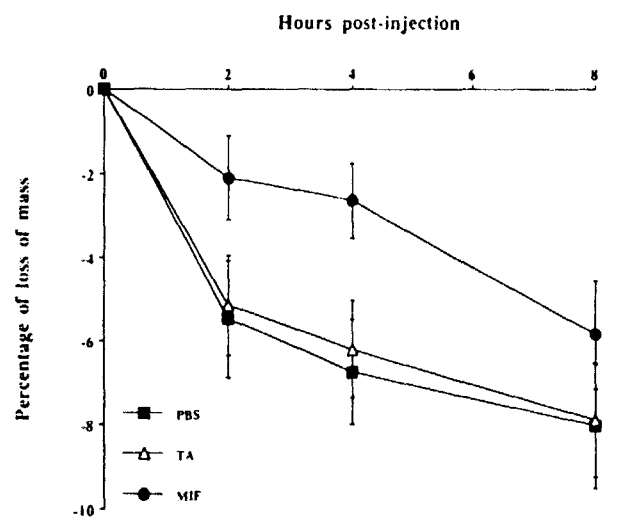

Fig. 8. Effect of the injection of 1 nmol per leech of either C-terminal fragment of oxytocin (MSH release inhibiting factor, MIF) or N-terminal fragment of oxytocin (tocinoic acid, TA) on the body mass of stage 3B *T. tessulatum*:: mean percentual loss of mass at different times after injection. Each mean value and standard deviation is based on 20 injected animals. PBS, phosphate-buffered saline.

the leeches treated with extracts of non-sex SG were very close to the one of the controls receiving PBS. In contrast, the animals injected with extracts of sex SG (SG5 and SG6) presented a decrease of mass significantly lower than the one registered both in controls and in leeches treated with extracts of non-sex SG. Moreover, knowing that the epitope recognized by the a-OT was MIF, the C-terminal (MIF) and the N-terminal (TA) fragment of OT were respectively administered to *T. tessulatum* at stage 3B. From Fig. 8, it can be concluded that only MIF provoked an effect in the injected animals similar to the one registered in animals injected with sex SG.

DISCUSSION

In leeches, different substances have been colocalized with FMRFa-like substance at the level of the nerve cord: GABA and proctolin-like substance in neurons of non-sex SG[6,14], small cardioactive peptides in neurons of sex and non-sex SG[9]. In this paper, we have demonstrated with immunocytochemistry, that an OT-like substance is present in large amounts and colocalized with a FMRFa-like substance in most of the supernumerary cells of the sex SG of the Pharyngobdellid *E. octoculata*. This OT-like substance was also found in the sex SG of two leeches belonging to two other different orders: the Gnathobdellid *Hirudo medicinalis* and the Rhynchobdellid *Theromyzon tessulatum*. Nevertheless, it is known that unexpected interactions with structurally non-related compounds can be observed in immunocytochemistry[5]. For this reason, in order to confirm the colocalization of OT- and FMRFa-like substances in the sex SG of *E. octoculata*, a biochemical investigation was undertaken. In reverse-phase HPLC with extracts of SG from mature *E. octoculata*, the presence of two specific zones immunoreactive to a-OT and of two specific zones immunoreactive to a-FMRFa was demonstrated. No over-

lapping between the OT-like zones and the FMRFa-like zones was observed. Moreover, no overlapping of the OT-like zones was found with either native, reduced or oxidized OT or fragments of OT. On the other hand, native and oxidized FMRFa eluted in the two zones immunoreactive to a-FMRFa. This is in good agreement with the results of Evans[10] who extracted from the nerve cord of *H. medicinalis* a native FMRFa. All these biochemical results confirm that two substances (OT- and FMRFa-like) are colocalized in the supernumerary neurons of the sex SG of *E. octoculata* and that the OT-like substance has a physicochemical behavior different from that of vertebrate OT.

Besides those belonging to the supernumerary cells category of SG5 and SG6, neurons immunoreactive for FMRFa are also present in the other segmental ganglia of the ventral nerve cord, e.g. in the neurons innervating the heart tubes: the heart accessory modulating neurons and the heart excitatory motor neurons[12]. The physiological role of FMRFa is known in Hirudinae. This peptide appears to play a significant role in the control of heartbeat[12,13] and in the contraction of longitudinal muscles[22]. In contrast, nothing was known about the OT-like peptide present in the SG of leeches and more specially in the sex SG.

Our results show that this substance is related to vertebrate OT by its C-terminal part (MIF) and is localized in granules of one type, with a size ca. 120 nm diameter, in the supernumerary cells of the sex SG. In *E. octoculata*, the number of cells immunoreactive to a-OT (ca. 150), was found to be higher in SG6 which innervate both the male and female atria than in SG5 which innervates only the male one. Only further experiments could determine if these observations really correspond to a difference in the number of supernumerary OT-like neurons in SG5 and SG6 or to a difference in the expression of the OT-like substance.

In *E. octoculata*, cell counts of OT-like cells in immature and mature animals indicated that the number of immunoreactive cells was higher in immature specimens. Moreover, radioimmunoassays showed an amount of OT-like material 3-fold higher in immature than in mature leeches. A biochemical study of an extract of sex SG of mature *E. octoculata* demonstrated the presence of two zones immunoreactive to a-OT. On the other hand, in immature *E. octoculata*, an additional zone bearing ca. 80% of immunoreactivity to a-OT was detected. A study of the interrelations between the zones detected indicated that in mature leeches, a tight relation exists between the two other zones (reduction with DTT of the two specific zones generated a single immunoreactive peak). This biochemical result suggests, as in *Locusta migratoria*[24], the presence of a monomer and of a dimer in mature leeches. On the other hand, in immature leeches, reduction with DTT of the additional zone characteristic of these leeches gave a single immunoreactive peak distinct of the one found after reduction in mature leeches. This suggests the presence of a possible precursor in immature leeches. Further studies are needed to confirm this hypothesis.

In *E. octoculata*, RIA and ELISA assays indicating that the amount of OT-like substance per sex SG was ca. 5 pmol *vs ca* 0.5 pmol in a non-sex SG. The question arises to know what role could play the supernumerary OT-like immunoreactive cells. A role of these super-numerary neurons in mating has been suggested by Macagno[15]. He found in the Hirudinida *H. medicinalis*, whose copulation is normal, that the number of supernumerary cells in sex SG was much higher than in the Glossiphoniidae *Haementeria ghilianii*, which lack both an evertile penis and a vagina and in which fertilization is accomplished via a spermatophore which can be found attached anywhere on the body wall of the mate. This hypothesis was not contradicted by our observations: there are less supernumerary cells in leeches which exchange spermatophores than in leeches whose copulation is normal (ca. 150 for *E. octoculata* vs. ca. 350 for *H. medicinalis* in Arhynchobdellids, ca. 20 for *H. ghilianii vs ca* 35 for *T. tessulatum* in Rhynchobdellids). However, it must be noted that the number of supernumerary cells varies also according to the group considered. This number is much higher in Arhynchobdellids than in Rhynchobdellids.

This presumed role on reproduction is certainly not the only one which can be attributed to these supernumerary cells. Another hypothesis can be advanced, these cells would exert an anti-diuretic effect, leading to the water retention characteristic of the phase of oocyte growth in the primitive leech *T. tessulatum* and likely indispensable to the important accumulation of precursors of the oocyte yolk in the coelomic fluid[2].

The injection of *E. octoculata* sex SG in mature *T. tessulatum* led to a loss of mass significantly lower than the ones obtained in leeches injected with non-sex SG or with PBS and which were due to the successive drainings required by the weighing of the animals[19]. This result demonstrates the anti-diuretic effect of the *E. octoculata* sex SG in *T. tessulatum*. Moreover, injections to mature *T. tessulatum* of different parts of vertebrate OT (N-terminal part: TA; C-terminal part: MIF), indicate that only MIF has an anti-diuretic effect similar to the one of sex SG. Keeping in mind that the epitope found in the OT-like neurons is close to MIF, this result seems to argue in favour of the statement

that the anti-diuretic effect of the sex SG could be attributed to the OT-like substance.

Nevertheless, experimental results from Malecha[16,17] obtained in *T. tessulatum* have demonstrated a control of water retention only by the supraesophageal ganglia. Two hypotheses can be advanced in order to explain these contradictory observations with the possible anti-diuretic effect of the OT-like localized in the sex SG of *E. octoculata*: the OT-like substance would be either released in the bloodstream under the control of the supraesophageal ganglia, or not released and therefore would not play an hormonal role. Till now, no neurohemal area has been described in leeches at the level of the ventral nerve cord. On the other hand, such a structure exists at the level of the dorsal commissure of the cerebral ganglia[11,37] which could be at the origin of a different role of the OT-like substances in relation to the localization of the neurons producing them. Further investigations are however needed to conclude on an eventual hormonal role on osmoregulation of the OT-like substance contained in sex SG.

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CEREBRAL ANTI-DIURETIC FACTOR RELATED TO THE C-TERMINAL PART OF OXYTOCIN (PROLYL-LEUCYL-GLYCINAMIDE) IN THE RHYNCHOBDELLID LEECH *THEROMYZON TESSULATUM*

(Morphological, biochemical and physiological characterizations)

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Summary : In the brain of the leech *Theromyzon tessulatum*, neurons with morphological characteristics of neurosecretory cells and immunoreactive to an antibody directed against oxytocin (a-OT) were detected.

A radioimmunoassay (RIA) titration of OT-like material in brains at different stages of the life cycle, indicated that in *T. tessulatum* the maximal amount is found at stage 3B, stage correlated both to the water retention phasis and to the vitellogenin synthesis.

With high pressure gel permeation chromatography (HPGPC) followed by reversed-phase high performance liquid chromatography (reversed-phase HPLC) on brain extract, evidence was given of three zones immunoreactive to a-OT. The brain OT-like precursor was identified, it is a protein with a mass of *ca* 17 kDa.

In vivo experiments allowed to demonstrate that the cerebral OT-like substance is an anti-diuretic factor and that Prolyl-Leucyl-Glycinamide (PLGa) has an anti-diuretic effect.

Key Words : Anti-diuretic effect; Immunoassay; Immunocytochemistry; Leech; Oxytocin-like; PLGamide; Reversed-Phase HPLC; Western Blot.

Introduction

The involvement of molecules of the Oxytocin(OT)-Vasopressin(VP) family in the nervous control of salt and water balance has been reported in several families of invertebrates [22].

Indeed, in the Insect *Locusta migratoria*[26,27,28] an arginine-vasopressin-like neuropeptide isolated from the central nervous system (CNS) has a diuretic effect. It acts on Malpighian tubules to enhance the excretion of the primary urine.

In Molluscs, peptides of the OT-VP family have recently been isolated from the CNS of different gastropods [*Lymnaea stagnalis*[37], *Conus geographus* and *Conus striatus*[6]] and of the cephalopod *Octopus vulgaris*[29]. The physiological properties of these peptides are largely unknown. In the opisthobranch *Aplysia californica*, it is known that the osmoregulatory R15 cell of the abdominal ganglion is stimulated by an arginine-vasotocin-like neuropeptide found in the CNS[21].

In Annelids, since the works of Czechowicz[7] and Kulkarni *et al.*[13,14] the CNS of Hirudinae is known to influence water balance and regulate salt and water transport at the level of the integument and nephridia[41].

In the leech *Theromyzon tessulatum*, genital maturity is concomitant with a phasis of water retention. Malecha[16,17] has demonstrated with *in vivo* experiments that the brain is involved in the regulation of this water retention : removal of the supraesophageal ganglion or solely of the posterior paramedial compartments (compartments 4) provokes an important and rapid loss of mass expressing the existence of an anti-diuretic water balance regulator factor (WBRF). In compartment 4, positive immunoreactivities have been detected for three peptides which could be involved in osmoregulation : angiotensin II (AII), oxytocin (OT), vasopressin (VP)[39]. The eventual role of these three peptides in the control of the osmoregulation of *T. tessulatum* has been studied both by injecting mammalian peptides to leeches, and by realizing a passive immunization of leeches with polyclonal antibodies directed against these hormones. Results have shown that if no role can till now be attributed to VP[19], AII would act as a diuretic factor[30]. Concerning the role of OT, Malecha *et al.*[19] have demonstrated that an injection of a-OT provokes a loss of mass equivalent to the one registered after a supraesophageal ganglion removal, which expresses an anti-diuretic role of the *T. tessulatum* OT-like substance. However, the injection of vertebrate OT is ineffective, which allows to think that the leech OT-like substance is different from the mammalian OT.

In fact, results from Salzet *et al.*[33] indicate that the epitope recognized by a-OT is the C-terminal part of OT (Prolyl-Leucyl-Glycinamide : PLGa). Such an epitope has been recently found in great amount at the level of the sex segmental ganglia (sex SG) of three leeches : *T. tessulatum*, *Erpobdella octoculata* and *Hirudo medicinalis*[33]. In *E. octoculata*, this epitope is colocalized with a FMRFamide-like peptide[33]. The biochemical characterization has shown that the OT-like peptide found in these sex SG was present under two forms in mature leeches. By contrast, in immature leeches, three forms were detected : the two forms detected in immature animals and an other one which bore the maximum amount of OT-like material per sex SG. Physiological experiments have shown that the OT-like substance from sex SG may possess an anti-diuretic potential[33].

The aim of this work, is to characterize the cerebral OT-like substance at the morphological, biochemical, and physiological levels in the Rhynchobdellid *T. tessulatum*, which presents during its life cycle a water retention phenomenum correlated to a cœlomic accumulation of yolk proteins[2, 3].

For these reasons, we have undertaken on this species :

- an immunocytochemical study at the level of the brain, in order to know if the OT-like cells possess morphological properties compatible with a neurosecretory role.
- a titration by RIA of the amount of OT-like material present in brains taken at different physiological stages of the life cycle.
- a biochemical investigation of the cerebral OT-like substance and a repartition of the amount borne by each OT-like zone eluted after reversed-phase HPLC during the life cycle.
- a preliminary identification of the cerebral OT-like precursor followed, considering previous results establishing the presence of an OT-like peptide in sex SG of leeches[33], by a comparison of this cerebral precursor with the sex SG OT-like precursor.
- *In vivo* experiments to investigate the anti-diuretic role of the cerebral OT-like substance.

Material and methods

1 - Animals

T. tessulatum (Hirudinae, Rhynchobdellida) reared in our laboratory[17] were used in this study. The life cycle of this animal is subdivided in stages defined by taking as indicators the three blood meals (stage 0 : from hatching till the first blood meal ; stages 1, 2 and 3 : after respectively the first, second and third blood meal). Stage 3 is characterized by a marked increase of mass due to an important water retention and can be subdivided in substages (3A, 3B, 3C, 3D, 3E, 3F) defined according to the degree of sex maturation[20].

2 - Antibody

The polyclonal anti-oxytocin (a-OT) antiserum used in immuno-cytochemistry and in immunoassays (ELISA, DIA and RIA) was raised by immunizing rabbits with synthetic OT (Interchim) coupled to thyro-globulin with gluraraldehyde. The characterization of a-OT was described elsewhere[31].

3 - Immunocytochemical procedures

Both sectioned material and whole-mounts were employed.

a) Whole-mounts

Brains were treated according to the method of Salzet *et al.*[30]. Primary antibody (a-OT) was diluted 1:500, FITC-labeled goat anti-rabbit IgG (Sigma) was used at a dilution of 1:100. Whole-mounts were examined with a Zeiss Axioskop fluorescence microscope.

b) Sections

Anterior parts of leeches including the brain were fixed overnight at 4°C in Bouin Hollande fixative (+ 10 % $HgCl_2$ saturated solution), they were then embedded in paraffin and serially sectioned at 7 µm. After removal of paraffin with toluene and rehydratation, sections were treated by an indirect immunoperoxidase technique [with the primary antibody (a-OT) diluted 1:200 and with goat antirabbit IgG conjugated to horseradish peroxidase as secondary antibody] described elsewhere[38].

The specificity of the immunolabelling obtained with a-OT was tested on consecutive sections by preadsorbing it overnight at 4°C with the homologous peptide [350 µg of synthetic OT (CRB)/ml pure antiserum].

4 - Immunoassays

a) Radioimmunoassay (RIA)

The procedure used has been described elsewhere[33]. In short, for a final dilution of 1:300, the antiserum a-OT bound 28 ± 8.3 % of ^{125}IOT (Amersham). Assay volume was 300 µl (100 µl of a-OT, 100 µl of standard OT (CRB) or sample and 100 µl of ^{125}IOT [10,000 counts per minute (cpm)]. Samples were counted with a γ-counter (LKB). The assay was used to quantify OT-like material in brains of leeches at different stages of the life cycle. Mean values were expressed in pmol of OT per brain. They derived from samples counts reported to a standard curve drawn with the following formula : $B/B_0 = f(\log[OT])$

with B_0 = number of counts given by the solution comprising the 0.1 M carbonate buffer,the tracer ^{125}I (10,000 cpm/100 µl) and the antibody a-OT (1:300).

B = number of counts (cpm) of tracer in competition with different dilutions of samples.

b) Enzyme-linked immunosorbent assay (ELISA)

Immunoassays were conducted according to procedures described elsewhere[32].

c) Dot Immunobinding Assay (DIA)

An aliquot of 1 µl of HPLC fraction or of synthetic OT (CRB) was spotted onto a nitrocellulose membrane (0.45 µm, Schleicher and Schuell BA 85/31) which was then baked 30 min at 110°C. The membrane was blocked, under gentle agitation with PBS [50mM phosphate, 150 mM NaCl, pH 7.4] including 0.05 % Tween 20 and 5 % Skilm milk for 1 hr at room temperature and then incubated overnight at 4°C with a-OT [diluted 1:1000 in PBS/0.05 % Tween 20 (PT)]. After the primary incubation, the membrane was washed for four 5 min periods with PT and incubated for 1 hr with peroxidase-conjugated goat anti-rabbit IgG diluted 1:1000 in PT). Washing in PT was repeated (4 x 5 min). Bound antibody was revealed with a solution comprising 45 mg of 3-3'-diaminobenzidine-tetrahydrochloride (Sigma) and 20 µl hydrogen peroxide (Sigma) in 100 ml of PT.

5 - *In vivo* experiments

In each type of experiment, *T. tessulatum* at stage 3B, which had taken their third meal on the same day, were used. Injections of products were in the subepidermal connective tissue. Injected animals were then kept at room temperature.

a) Injection of fragments of OT (PLGa or TA) or of *T. tessulatum* brain extracts in leeches deprived of their supraesophageal ganglia

Stage 3B animals prealably deprived of their supraesophageal ganglia (according to the technique of Malecha[17]) received, six days after the operation, a single injection of 20 µl of one of the following products : *T. tessulatum* brain extract (5 brain-equivalent), *T. tessulatum* brain extract (5 brain-equivalent) preadsorbed with a-OT, PLGa (1 nmole/animal), TA (1 nmole/animal). Control animals received 20 µl of a solution of extraction buffer [PEP : 0.05 M PBS pH 7.4 supplemented with 2 % Ethylene Diamine Tetra Acetic acid (EDTA) and 1 mM Phenyl methyl Sulfonyl fluoride (PMSF)].

Brain extracts used for injection were prepared as follows : one hundred brains taken on leeches at stage 3B were homogenized with a Dounce at 4°C in 200 µl of PEP. The homogenate was boiled 5 min then centrifuged at 6,000 rpm for 5 min. The supernatant was taken and complemented to 400 µl with 0.05 M PBS. The brain extract preadsorbed with an a-OT recognizing the N- and C-terminal of OT[36] was prepared according to the following procedure : 5 mg Protein A-sepharose (PA) (Pharmacia) were suspended in 0.05 M PBS. The gel was let to swell 1 hr at room temperature and then washed briefly 4-fold in 0.05 M PBS. Ten microliters of pure a-OT were added to the gel and the complex PA + a-OT was diluted in 40 µl of 0.05 M PBS. After 90 min at room temperature, five washings in 0.05 M PBS were carried out. Brain extract was then added for 24 hr under agitation at 4°C. Finally, the complex protein A-a-OT-antigen was centrifuged and the supernatant to be injected dissociated.

b) Injection of Vasotocin and fragment of OT in normal leeches

Non-operated leeches at stage 3B received a single injection of 10 µl of a solution of 1 nmol of either vasotocin or PLGa or TA. Controls were injected with 0.05 M PBS, pH 7.4.

c) Dose/response with PLGa peptide

Normal leeches at stage 3B received a single injection of 10 µl of PLGa peptide at different concentrations (1 pmol, 10 pmol, 100 pmol, 1 nmol, 10 nmol and 100 nmol).

Statistical analysis

At the beginning of the treatments, animals were distributed in lots of 20 leeches having an identical mean body mass. The effect of each treatment was estimated by measuring the change of body mass of the animals between the beginning (injection) and the end of the experiment (2, 4, 8 and 24 hr after injection). Leeches were blotted on tissue paper and weighed to the nearest 0.1 mg. Results are presented as mean (± SD) of the percentage of mass variation, with a minimum of 15 animals for each point. Statistical analysis of results was performed by calculating the mass variation of each leech at a t instant compared to the initial mass (at the time of injection). Statistical comparisons were made with Student's *t*-test.Confidence interval (with $\alpha = 0.5$) of the mean of the relative variations of mass was obtained according to Scherrer[34].

6 - Biochemical procedures

A - Extraction and purification of the OT-like substance

a) Extraction

Brains were dissected in leech Ringer[24] then frozen at -180°C in liquid nitrogen and finally stored at -70°C. Batches of 200 brains were homogenized in 200 µl 1 M acetic acid with a Dounce homogenizer, sonicated (30 sec) twice. Each homogenate was centrifuged 30 min at 4°C and 12,000 rpm. The pellet was dissociated from the supernatant and reextracted as before and then the two supernatants were combined.

b) Peptide characterization

Step I : Sep-Pak Prepurification - Brains of *T. tessulatum* in batches of 200 or 400 were homogenized in 200 or 400 µl of 1 M acetic acid with a Dounce homogenizer and then sonicated (30 sec) twice. Homogenates were centrifuged at 12,000 rpm for 30 min at 4°C. After reextraction of the pellet, the two supernatants were combined. Then, they were applied on C_{18} Sep-pak cartridges (500 µl/cartridge, Waters) and eluted with 5 ml of 50 %

acetonitrile (ACN). Sep-pak eluted fractions were reduced 20-fold by freeze-drying. Total amount of OT-like peptide was determined in direct ELISA.

Step II : Reversed-phase HPLC - The immunoreactive fractions from the Sep-Pak prepurification were subjected to a reversed-phase HPLC on a Vydac C_{18} Protein Peptide (250 x 4.6 mm) column. Elution was performed with a discontinous linear gradient from 0 % A (0.1 % TFA in 100 % deionized water) to 15 % B (0.1 % TFA in 100 % ACN) in 10 min, followed by a linear gradient from 15 to 45 % of B in 30 min at a flow rate of 1 ml/min. Ultraviolet absorbance was monitored at 226 nm. The eluted fractions were reduced to half of their volume by freeze-drying, before being quantified in ELISA.

B- Precursor identification

a) Protein extraction

Brains or sex SG in batches of 200 or 400 were homogenized in 200 or 400 µl of 50 mM Tris/HCl pH 7.4, 150 mM NaCl (TBS) with a Dounce homogenizer and then sonicated (30 sec) twice. Each homogenate was centrifuged at 12,000 rpm for 30 min at 0°C. The pellet was dissociated from the supernatant and reextracted as before. The two supernatants were finally combined.

b) Immunoprecipitation

Five mg of Protein A-Sepharose (PA) (Pharmacia) were suspended in 0.05 M PBS. The gel was led to swell 1 hr at room temperature and then washed briefly 4-fold in 0.05 M PBS by centrifugation. Ten microliters of pure a-OT and then 40 µl of 0.05 M PBS were added to the gel. After 90 min at room temperature, five washings in 0.05 M PBS were carried out by centrifugation. Brain or sex SG extracts were then added to the PA-a-OT for 24 hr under agitation at 4°C Finally, the complex PA-a-OT-antigen was washed 5-fold in 0.05 M PBS, before being treated with the sample buffer in order to dissociate the a-OT-antigen from the PA. This was followed by an electrophoresis and an immunoblot analysis.

c) Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel (SDS-PAGGE) was prepared according to Laemmli[15] except that the separating gel consisted of a 10-25 % polyacrylamide gradient slab gel. Molecular mass standards were :

serum albumin (67 KDa), ovalbumin (45 kDa), carbonic anhydrate (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa).

Proteins reduction was performed by addition in the sample buffer of 5 % ß mercaptoethanol.

d) Immunoblot analysis

After SDS-PAGGE, proteins were electroblotted 5 hr at 250 mA on polyvinylidene difluoride transfer membrane (Immobilon-P, Millipore) in a transfer buffer consisting of 25 mM Tris and 192 mM glycine, 17 % methanol and 0.005 % SDS. After transfer, membranes were blocked with 2 % dried skilm milk in TBS containing 0.05 % Tween 20 (TT). Incubations a-OT (dilutes 1:1000 in TT) [12 hr], and with peroxidase-conjugated goat anti-rabbit IgG (diluted 1/1000 in TT) [2 hr] were performed in TBS/0.05 % Tween 20, on rocking at room temperature. Horseradish peroxidase activity was detected by incubating blots in an oxidative medium [3 ml of a solution of 4-chloro-1-naphtol (10 mg in 5 ml of methanol) + 10 µl of hydrogen peroxide in 50 ml TBS] and then, after washing in TBS, in a solution of 45 mg 3-3'-diaminobenzidine and 15 µl of hydrogen peroxide in 100 ml of TBS. Controls of specificity were realized by preadsorbing a-OT with the OT peptide (100 µg/ml pure serum).

Results

Immunocytochemical investigations

Twenty-five to thirty cells immunoreactive to a-OT were detected in the brain of the leech *T. tessulatum* at stage 3B (Figs. 1a, b,d).

The supraesophageal ganglion contains 6-8 immunoreactive cells (Figs. 1a, b). A medium-sized cell (20-25 µm in diameter) (Fig. 1a) is located in the posterior paramedial compartment of the supraesophageal ganglion, compartment named compartment 4 and known to be involved in the control of water balance. Some cells of the posterior compartments of the supraesophageal ganglion can be seen projecting directly within the dorsal commissure (Fig. 1b) where extensive arborizations are registered (Fig. 1c). In the subesophageal ganglion (Fig. 1d), *ca* 20 cells immunoreacted with a-OT. Two types of preadsorbtions of the a-OT used were realized to check the specificity of the imunostaining observed : with the corresponding peptide [350 µg of OT/ml of pure serum] and with the C-terminal part of OT

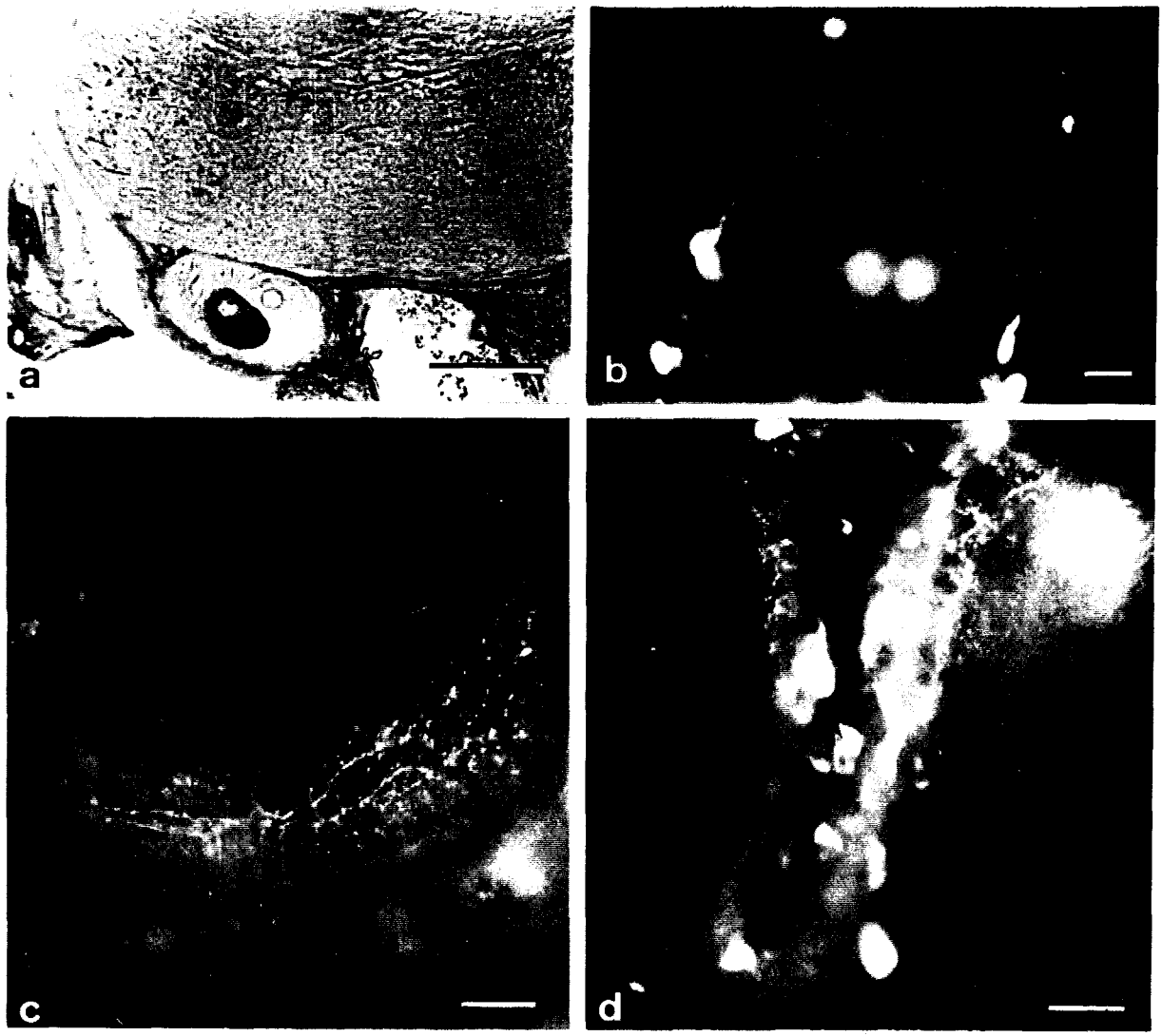

Figure 1a- Frontal section at the level of compartment 4 of the supraesophageal ganglion of *T. tessulatum* showing the cell immunolabelled with a-OT (indirect immuno-peroxidase).

b, c, d- Immunohistochemical fluorescent micrographs of whole-mounts preparations of brains of *T. tessulatum* treated with a-OT. Anterior is to the top of figures.

b- Dorsal view of the supraesophageal ganglion showing an OT-like cell of compartment 5, sending its process directly into the dorsal commissure which is strongly immunolabelled.

c- Dorsal view of the dorsal commissure of the supraesophageal ganglion showing a network constituted of branches which ramify abundantly and form many varicosities.

d- Dorsal view of the subesophageal ganglion focussed at the level of OT-like cells located in the medial compartments.

Bars = 50 µm.

(PLGa : 700 µg/ml of pure serum). Use of these two types of preadsorbed a-OT in immunocytochemistry quenched all immunostaining.

Biochemical investigations

Quantification of the OT-like substance during the different physiological stages of the life cycle.

In order to determine, if a tight relation existed in *T. tessulatum* between the water retention characteristic of the genital maturation and the amount of cerebral OT-like material present in leeches at stages 2 and 3 of their life cycle, an RIA investigation was performed (Fig. 2). Results indicated the presence of a relatively constant amount of OT-like material in the brain (*ca* 0.3 pmol) except during stage 3B, when this amount reached a peak (*ca* 2.0 pmol) (Fig. 2).

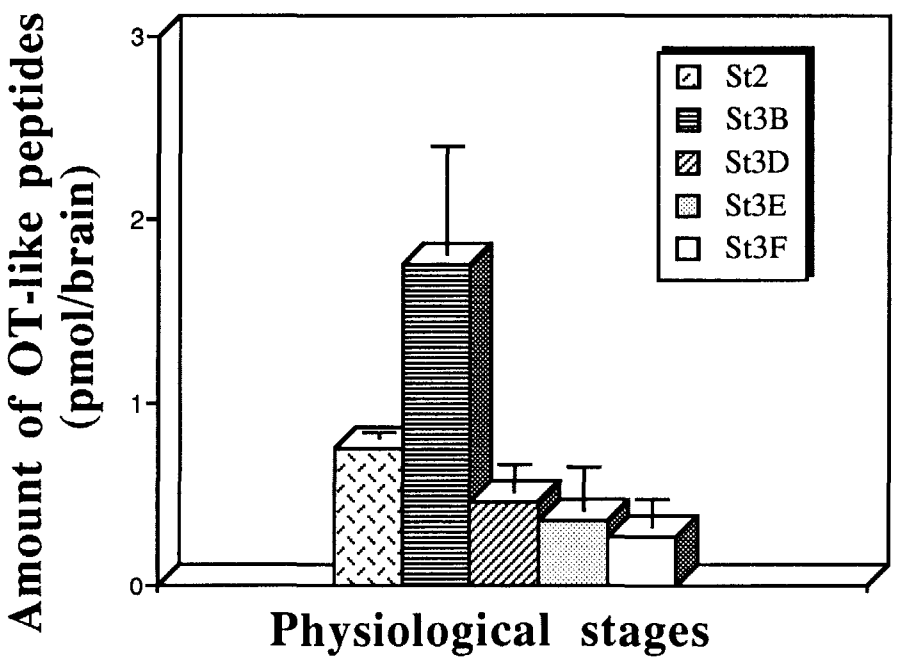

Figure 2- Radioimmunoassays (RIA) titration of the cerebral OT-like peptide at different physiological stages of the life cycle of *T. tessulatum* : Mean amount and SD (pmol/brain) of OT/like substance.
Each mean value is based on 4 determinations
St2: after the second meal
St3: after the third meal
St3B: spermatogenesis and cœlomic accumulation of vitellogenin (stage corresponding to a phasis of water retention).
St3D: oogenesis and egg laying
St3E: hatching
St3F: transport by the parent of the newborn leeches, till their first blood meal.

Biochemical characterization

After purification on Sep-Pak matrix, equivalents of 400 brains of *T.tessulatum* were applied to a high performance gel permeation chromatography (Fig. 3). DIA results indicated that the OT-like substance eluted from the column in a molecular mass range of *ca* 1-5 kDa. Fractions containing OT-like material were pooled and concentrated with Speed-Vac before being applied to a reversed-phase HPLC (Fig. 4). Direct ELISA with a-OT indicated that four zones Z1, Z2, Z3 and Z4 exhibited an OT-like immunoreactivity (Fig. 4). After preadsorption of the a-OT with the synthetic OT peptide, the immunoreactive zones (Z1, Z3 and Z4) disappeared, in contrast to Z2 which subsisted (Fig. 4). So, only three zones immunoreactive to a-OT (Z1, Z3 and Z4) were considered as specific. With a retention time of respectively 15-16 min for Z1, 24-26 min for Z3 and 30-32 min for Z4, none of these three zones corresponded to the ones, found in this system, of either OT (native, reduced or oxidized) or OT fragments (TA, PLGa) or peptides of the OT family (arginine-vasopressin, arginine-vasotocin, lysine-vasopressin, isotocin) (Table I).

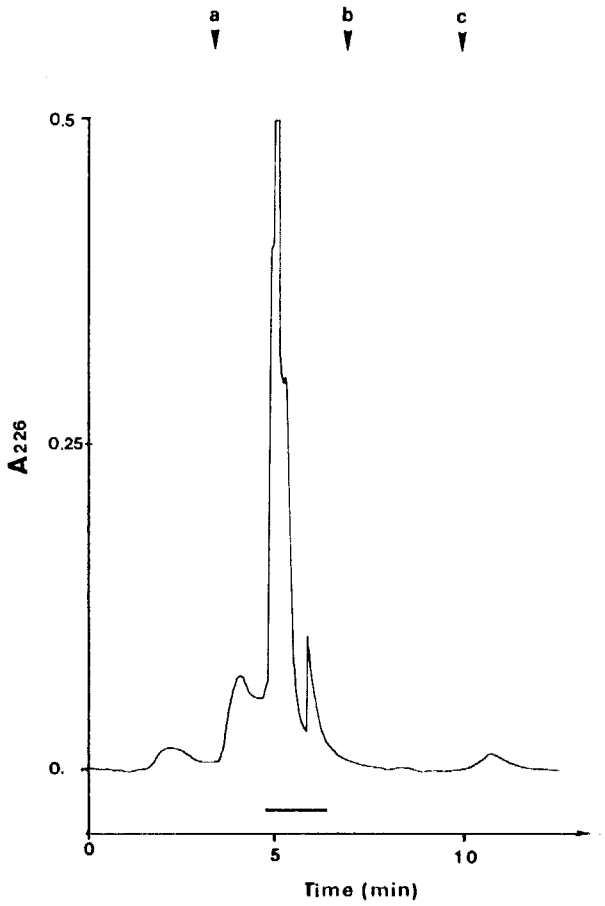

Figure 3- High pressure gel permeation profile of an extract of 400 brains of *T. tessulatum* at stage 3B.
- Elution rate, 1 ml/min ; solvent, ACN 30 %
- Immunoreactive OT-like fractions assayed in DIA are indicated by a bar.
- Arrows indicate the eluted position of standards in identical conditions. (a : hirudin, b : oxytocin, c: L-tryptophane).
- Absorbance was monitored at 226 nm (A 226 nm).

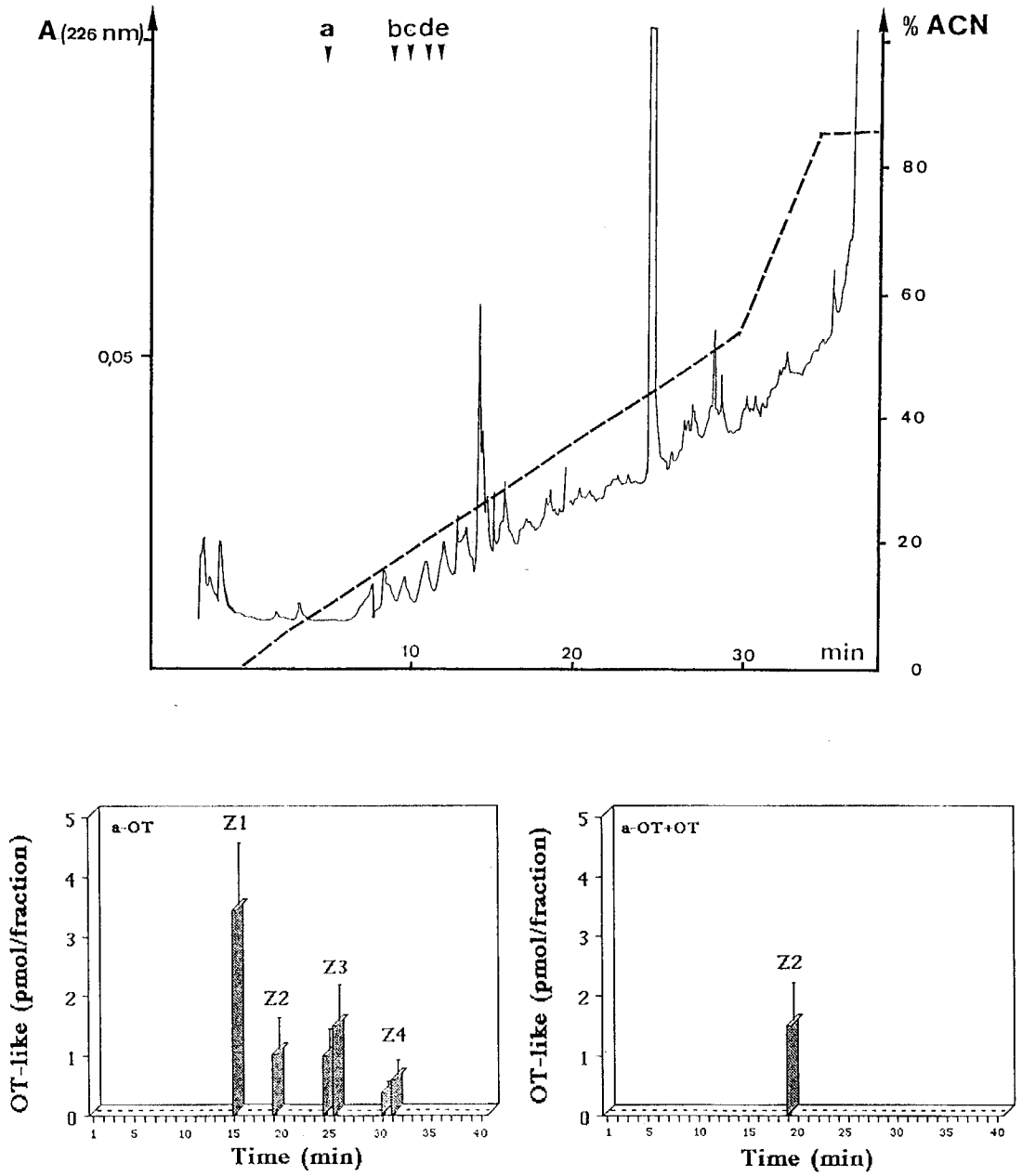

Figure 4- Reversed-phase HPLC elution profile of an extract of 400 brains of *T. tessulatum* at stage 3B.

- Elution rate: 1 ml/min; solvent A : 0.1 % TFA in water; solvent B : 0.1 % TFA in 100 % ACN ; solvent program : gradients of B at 1 %/min from 0 to 50 %.
- The solid line indicates absorbance; the dotted line indicates the gradient.
- Arrows show the respective elution positions of the C-terminal fragment of OT [PLGa] (a), N-terminal fragment of OT [Tocinoic acid](b), oxidized oxytocin (c), native oxytocin (d), reduced oxytocin (e).
- Fractions were assayed for OT-like immunoreactive material (diagrams) with antibody anti-oxytocin preadsorbed (a-OT+OT) or not (a-OT) with the homologous antigen.
- Values given for each assayed fraction correspond to aliquots of fractions of 1 µl. Absorbance was monitored at 226 nm (A 226 nm)

Table I : Retention time after reversed-phase HPLC of the specific immunoreactive eluted zones and of OT, fragments of OT and peptides of the OT family

Peptide	Sequence	Retention time (min)
OT	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH$_2$	14.4
reduced OT	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH$_2$ (SH, SH)	11.3
oxidized OT	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH$_2$ (SO_3-, SO_3-)	14.7
TA	Cys-Tyr-Ile-Gln-Asn-Cys	10.5
PLGa	Pro-Leu-Gly-NH$_2$	5.6
ISO	Cys-Tyr-Ile-Ser-Asn-Cys-Pro-Ile-GlyNH$_2$	13.5
AVP	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH$_2$	14.1
AVT	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-GlyNH$_2$	13.7
LVP	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-GlyNH$_2$	13.1
Z1		15-16
Z3		24-26
Z4		30-32

AVP: Arginine-Vasopressin, AVT: Arginine-Vasotocin, ISO: Isotocin, LVP: Lysine-Vasopressin, OT: oxytocin, PLGa: Prolyl-leucyl-Glycinamide, TA: Tocinoic Acid.

Precursor identification

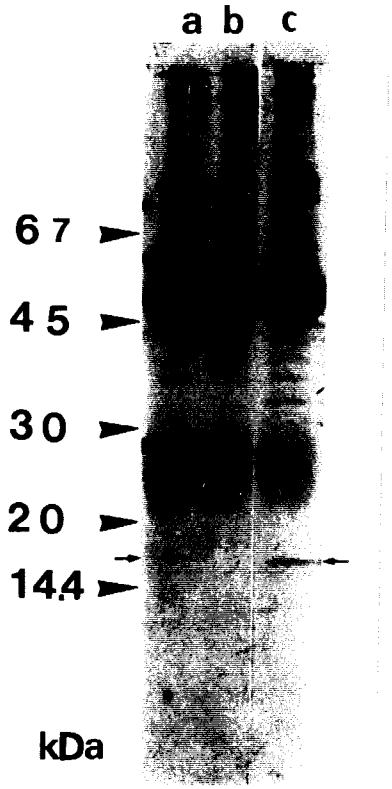

Figure 5- Immunoblot analysis after immunoprecipitation of a brain (lane a) or a sex SG (lane c) extract from *T. tessulatum* at stage 3B.
- Total proteins from brain or sex SG were separated by SDS-PAGGE in reducing conditions, transferred to poly(vinylidene difluoride) membrane and immunoblotted with a polyclonal antibody directed against OT.
- In controls (lane b), only sample buffer was added.
- Small arrows indicate the position of the OT-like precursor.

As an OT-like substance was detected at the level of the sex SG[33], a comparative study was undertaken at the level of the precursor both in the brain and in the sex SG.

After TBS extraction, an extract of brain or of sex SG was subjected to a preliminary immunoprecipitation, then to a western blot analysis. Results are represented in Fig. 5. They indicate that the brain precursor (Fig. 5, lane a) and the sex SG precursor (Fig. 5, lane c) are identical. In both cases, the OT-like precursor is a protein with a mass of *ca* 17 kDa.

Physiological investigations

Evidence of the anti-diuretic effect of the OT-like peptide

Results of the effects on water balance of the injections to leeches deprived of their supraesophageal ganglion (which induced a high and rapid decrease of mass) of either a brain extract (preadsorbed or not with a-OT) or of a fragment of OT (TA : N-terminal part, PLGa : C-terminal part) or of PEP (controls) are shown in Fig. 6. In every case, the injected leeches continued to loose mass. The loss registered in the controls (*ca* 10 %, 8 h post-injection), which is due to the successive drainings required by the weighing of the animals[17], did not significantly differ from the ones registered in leeches injected with either TA or with brain extract preadsorbed with a-OT. On the other hand a significant difference with the controls was obtained 2, 3, 6 and 8 hours post-injection in animals injected with brain extract and 6 and 8 hours after the injection in animals treated with PLGa.

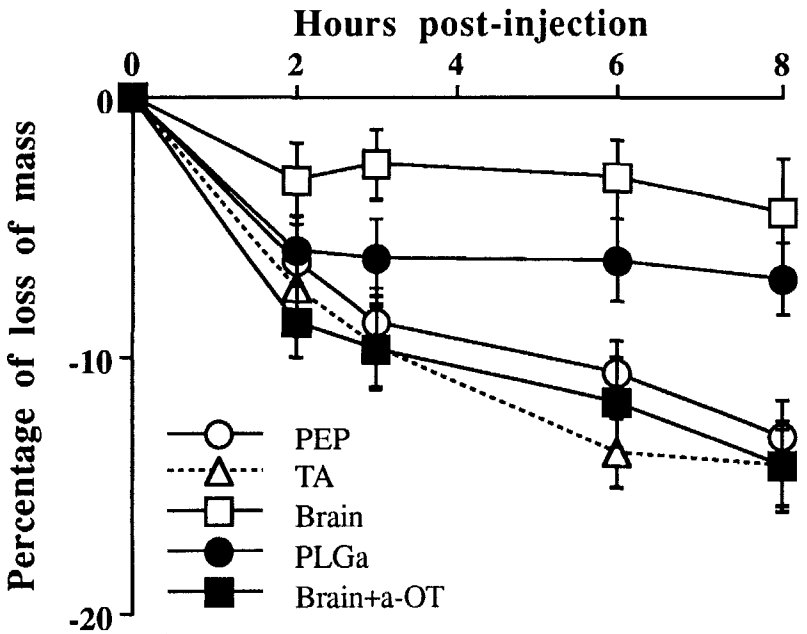

Figure 6- Effect of the injection of stage 3B *T. tessulatum* brain extract preadsorbed (Brain + a-OT) or not (Brain) with anti-oxytocin (a-OT) or of OT fragments (TA or PLGa) on the body mass of stage 3B *T. tessulatum*.

- Each mean value and standard deviation is based on 20 injected animals.
- Dose injected: 5 brain-equivalent for the brain extracts ; 1 nmol for the OT fragments.
- TA : Tocinoic acid ; PLGa : Prolyl-Leucyl-Glycinamide ; PEP : 50 mM PBS supplemented with 2 % ethylene diamine tetra acetic acid and 1 mM phenyl methyl sulfonyl fluoride.

Evidence of the anti-diuretic effect of PLGa peptide

In Fig. 7 are presented results of the effects on osmoregulation of the injections to normal leeches of either vasotocin or TA or PLGa. They demonstrate that compared to controls and in contrast to an injection of vasotocin or TA, only the injection of PLGa has a significant anti-diuretic effect (2, 4, and 6 hours post -injection).

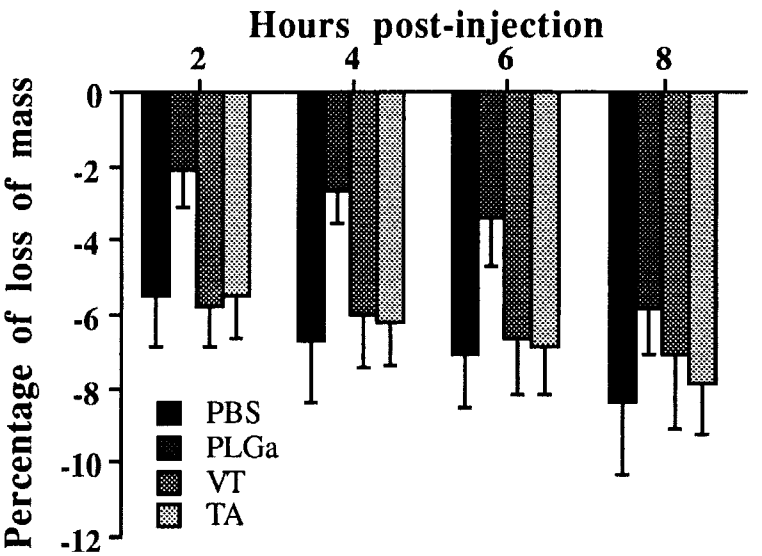

Figure 7- Effect of the injection of 1 nmol of either C-terminal fragment of oxytocin (PLGa) or N-terminal fragment of oxytocin (tocinoic acid: TA) or vasotocin (VT) on the body mass of stage 3B *T. tessulatum* : mean percentual mass variation at different times after the injection.
- Each mean value and standard deviation is based on 20 injected animals.
- PBS: 50mM phosphate buffer saline, pH 7.4.

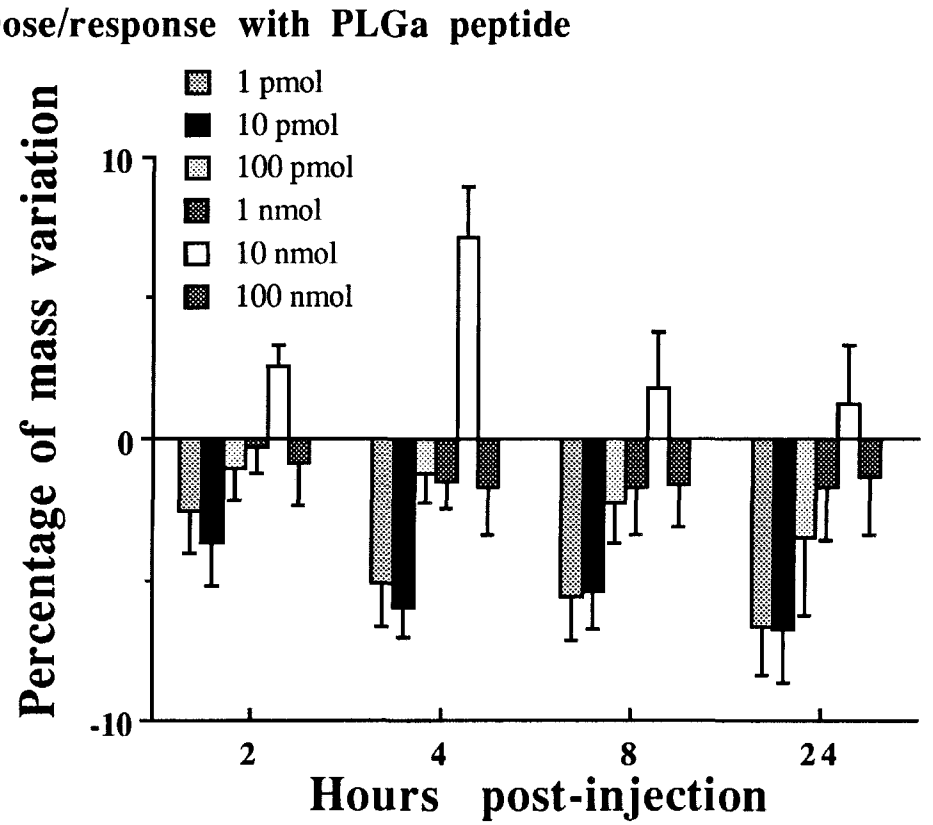

Figure 8- Effect of the injection of different concentrations of PLGa (C-terminal part of OT) on the body mass of stage 3B *T. tessulatum.* : mean percentual mass variation at different times after the start of the experiment.
- Each mean value and standard deviation is based on 20 injected animals.

This investigation was undertaken in order to evaluate the optimal dose of PLGa peptide able to provoke a significant anti-diuretic effect in the injected leeches (Fig. 8). A significant uptake of water was registered 2, 4, and 8 hrs after injection in non-operated leeches injected with 10 nmol. Moreover, 2, 4 and 8 hours post-injection, the effect of doses of 100 pmol, 1 nmol and100 nmol differed significantly from the ones of doses of 1 and 10 pmol.

Discussion

The injection of a brain extract to *T. tessulatum* deprived of their supraesophageal ganglion supresses the effect induced by the decerebration, *i.e* a loss of mass of the animal, which confirms that the water balance regulating factor (WBRF) of *T. tessulatum* discovered in the brain by Malecha[16] is a neurohormone. Besides, results of injections of brain extracts preadsorbed or not by a-OT to leeches deprived of their supraesophageal ganglion suggest that

the OT-like substance detected in the brain is an anti-diuretic factor which could be the WBRF.

With immunocytochemistry, 25-30 cells immunoreactive to a-OT have been detected in the brain of *T. tessulatum* at stage 3B .This number is higher than the one (20 cells) found by Malecha *et al.*[18] in the same species. Three hypotheses can be advanced to explain this difference in the number of cells immunodetected. The first one is that the antibody a-OT used in the present work was mono specific and directed against the C-terminal sequence PXGa (X = non polar amino acid), it thus differed from the one used by Malecha *et al.*, and which was directed against the two parts of OT. The second one is the type of fixation used for whole mounts which would better preserve the antigenic sites. The last one could be the existence of a variation in the number of immunoreactive cells during the physiological stages of the life cycle of *T. tessulatum* as previously demonstrated in the same species for the motiline-like substance[20]. RIA titrations are in line with this last hypothesis, the maximum amount of cerebral OT-like substance being found at stage 3B

With whole mounts it has been possible to detect the axonic extension of certain neurons immunoreactive to a-OT in the brain of *T. tessulatum.* Two of these neurons projected within the dorsal commisssure where is located in Hirudinae a neurohemal area[11, 12, 40]. The varicosities observed at this level can be considered either as synapses[8, 23] or as zones of release of the neurosecretory product[25]. The peptidic secretion could be released into the circulatory system at the neurohemal site and exert an hormonal role. Alternatively, an action as neurotransmitter or neuromodulator at the level of neuron endings located in the dorsal commisure is possible.

The possibility of the involvement of the cerebral OT-like substance in the regulation of water balance in *T. tessulatum* is strengthened by RIA results which showed that the amount of cerebral OT-like substance greatly increases at stage 3B, stage correlated with an important water retention in this leech. These results of RIA titrations are in good agreement with the hypothesis of Baert *et al*[2, 3] who suggested that the important water retention which occurs during the genital maturation of *T. tessulatum*, is essential for the accumulation of vitellogenins in the coelom. Indeed, it is during stage 3B, stage where the amount of OT-like material is maximal, that yolk protein are synthesized. These yolk proteins will be integrated in the oocytes during stages 3C and 3D. The decrease of vitellogenins syntheses during these last two stages[4] is correlated to a decrease in the amount of OT-like material.

Concerning the characterization of the cerebral OT-like substance, immunocytochemical data indicated that in the brain OT-like immunoreactive cells are recognized by an a-OT specific of the C-terminal part of OT (PLGa). Moreover, physiological experiments in *T. tessulatum* have shown that an injection of PLGa to animals deprived of their supraesophageal ganglion has the same physiological effect on water balance that an injection of supraesophageal ganglia extract. The dose/response with PLGa peptide has demonstrated an uptake of water only in normal leeches injected with 10 nmol of PLGa peptide, the other doses assayed having also an anti-diuretic effect. These two results strongly suggest that the OT-like substance is close to vertebrate OT by its C-terminal fragment (PLGa). However, an injection in normal leeches of either OT or VT is ineffective, in contrast to an injection of PLGa. So, it can be postulated that PLGa is either conserved in the leech OT-like peptide and able to mobilize the OT-like receptor or would exist as an endogenous neuropeptide as suggested in vertebrates by Gorzalka *et al.*[10]. PLGa is also considered in Vertebrates as the active metabolite of OT[1] and would result either from a cleavage by a cerebral endopeptidase enzyme[5, 35] or from the TPLGa neuropeptide[10]. This fragment is able to interfere with the OT receptor and to mobilize its proper receptor[10]. So three hypotheses can be advanced for the existence of PLGa in leeches : either it would be released after cleavage by an endopeptidase enzyme and bound on the OT-like receptor or PLGa would be a linear part of the OT-like peptide much longer than the one existing in vertebrate OT or it would exist as an endogenous neuropeptide. This last hypothesis is not sustained by biochemical studies on the cerebral OT-like substance which demonstrated that the three specific zones immunoreactive to a-OT (Z1, Z3, Z4) did not coelute with native, oxidized or reduced OT or with OT fragments (TA or PLGa) or molecules of the OT-VP family. The cerebral OT-like peptide would be biochemically different from vertebrate OT and from molecules of the OT-VP family.

Such a result has also been obtained in the sex SG of the leech *E. octoculata*, where evidence of the presence of an OT-like substance has been given[33]. In mature *E. octoculata* two zones specific to a-OT with a same retention time (RT) and a biochemical behavior identical to Z3 and Z4 were detected. In immature *E. octoculata*, a third form which eluted more quickly than the two others was also described. It presented the same RT and biochemical behavior than Z1. On the other hand, in the brain of *T. tessulatum*, the purification at stages 3A, 3B and 3D of the cerebral OT-like

substance has always given, both in mature and immature animals, three immunoreactive zones as in *E. octoculata* brain.

At this time of the work, only a sequencing of the purified peptides contained in these three zones and injections to *T. tessulatum* of the peptides synthesized from the sequences obtained, will permit to determine which peptides are effective.

The sex SG OT-like peptide possesses the anti-diuretic potential[33]. Moreover, a biochemical study related to the precursor of the OT-like substance indicated that it is identical both in the sex SG and in the brain : it is a single protein with a molecular mass of *ca* 17 kDa. So, the following hypothesis can be advanced : in *T. tessulatum*, the cerebral OT-like substance would be an anti-diuretic factor expressed during stage 3B to facilitate the accumulation of the yolk proteins in the cœlom. By contrast, in sex SG, the OT-like molecule would not act as a neurohormone as in the brain but could act as a neuromodulator or as a neurotransmitter as suggested by Evans *et al.*[9] for the FMRFamide-like peptides of *H. medicinalis*. Thus, in *T. tessulatum*, the OT-like substance could act differently according to its localization.

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Purification de la molécule apparentée à l'ocytocine (OT-like)

Aprés prépurification sur Sep-Pak de ganglions génitaux d'*E.octoculata* à maturité sexuelle et séparation en CLHP en phase réverse, 2 zones (Z1, Z2) contenant des peptides immunoréactifs à l'anti-OT ont été détectées en ELISA (Salzet *et al.*, 1992c). Les étapes chromatographiques successives ont montré que ces 2 peptides sont liés l'un à l'autre : le peptide contenu dans Z1 génére le peptide localisé dans Z2 au cours de la purification.

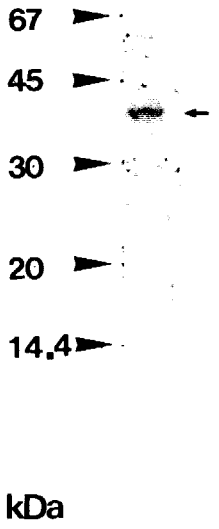
Aprés isolement du matériel immunoréactif contenu dans Z1, le séquençage de celui-ci a donné la séquence suivante : IPEPYVWD. La mesure en spectrométrie de masse du peptide purifié a révélé une masse de 1018,3 ± 0,3 Da, masse en accord avec la masse moyenne calculée (1017.6 Da). La séquence de la molécule apparentée à l'OT est donc différente de celle des peptides de la famille des OT/VP (Acher *et al.*, 1985).

Etude du précurseur de la molécule apparentée à l'ocytocine

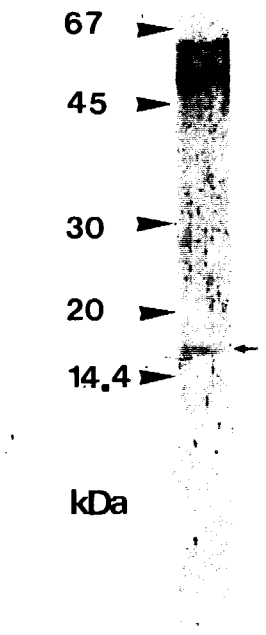
Aprés séparation d'un extrait protéique de ganglions génitaux d'*E. octoculata* en HPGPC, l'analyse en ELISA a révélé une zone immunoréactive à l'anti-OT correspondant à des protéines s'éluant de la colonne entre 30 et 45 kDa [Z1]. L'étude en Western blot des protéines contenues dans Z1 a mis en évidence une bande immunoréactive à l'anti-OT correspondant à une protéine d'une masse moléculaire de *ca* 34 kDa [après SDS-PAGGE en milieu non réducteur (Fig. 1a)] et de *ca* 17 kDa [après SDS-PAGGE en présence de ß-mercaptoéthanol (Fig. 1b)] qui pourrait être la Pro-OT-like.

La même démarche expérimentale a été utilisée pour l'analyse des produits de traductions issus d'ARN totaux extraits à partir de ganglions génitaux d' *E. octoculata*. Une zone immunoréactive correspondant à des protéines s'éluant de la colonne entre 15 et 30 kDa a été détectée aprés HPGPC. Cette zone contient une protéine de *ca* 19 kDa, qui pourrait être la PréPro-OT-like (Fig. 1c) immunoréactive à l'anti-OT mise en évidence en western blot aprés SDS/PAGGE en milieu réducteur.

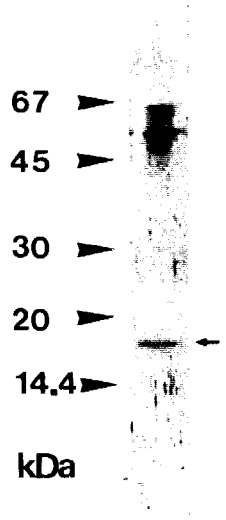
A l'inverse de la Pro-OT-like qui est un homodimére de *ca* 34 kDa, la PréPro-OT-like est un monomère de *ca* 17 kDa. La faible différence de masse (*ca* 2 kDa) observée en SDS/PAGGE en milieu réducteur, entre la Pro-OT-like et la PréPro-OT-like permet de suspecter l'addition d'un peptide signal au niveau de la PréPro-OT-like. Celle-ci a pu être confirmée par la différence de temps de rétention observée entre ces 2 protéines en C3 RP-HPLC(15,5 min *vs* 16,7 min).



A



B



C

Figures 1A, B, C
Identification aprés western blot du précurseur de la molécule apparentée à l'ocytocine (OT-like).

Après séparation en HPGPC d'un extrait de ganglions génitaux d'*E. octoculata*, la fraction immunoréactive à l'anti-OT, déterminée en ELISA et contenant le précurseur de l'OT-like est analysée en SDS-PAGGE en milieu non réducteur (A) ou en présence de ß mercapto-éthanol (B) et transférée sur une feuille d'Immobilon P. L'immunorévélation a été effectuée par l'anti-OT.

Après séparation en HPGPC de produits de traduction d'ARN totaux (C) extraits de ganglions génitaux d'*E. octoculata*, la fraction immunoréactive à l'anti-OT, déterminée en ELISA et contenant le précurseur de l'OT-like est analysée en SDS-PAGGE en présence de ß mercaptoéthanol et transférée sur une feuille d'Immobilon P. L'immunorévélation a été effectuée par l'anti-OT.

Les flèches situées à droite indiquent la position des précurseurs de la molécule apparentée à l'ocytocine.

La recherche de nouveaux épitopes pouvant être portés par la Pro-OT-like, s'est révélée infructueuse. En effet, l'analyse en ELISA et en Western-blot d'un extrait protéique de ganglions génitaux d'*E. octoculata* a donné des résultats négatifs tant avec l'anti-FMRFa, alors que les épitopes FMRFa-like et OT-like sont colocalisés dans les mêmes cellules des ganglions génitaux d'*E. octoculata* (Salzet *et al.*, 1992 c), qu'avec un anticorps anti-neurophysine (pool de MSEL- et VLDV-neurophysine).

Comme le peptide "apparenté à l'ocytocine "(peptide IPEP), le précurseur de l'OT-like est différent de celui des peptides de la famille des OT/VP (Acher *et al.*, 1985).

Biobliographie

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Molécule immunoréactive à l'anti-lysine-vasopressine

Article présenté

(6) Salzet, M., Bulet, P., Vandorsselaer, A. and Malecha, J., Isolation and structural characterization of a Lysine-conopressin in the central nervous system of the Pharyngobdellid leech *Erpobdella octoculata*, en préparation 2.

Etant donné que la molécule OT-like isolée chez *E. octoculata* est différente des peptides de la famille des OT/VP, nous avons recherché chez cette espèce une autre molécule de cette famille : la LVP-like, ceci d'autant plus que des peptides de cette famille OT/VP avaient été retrouvés chez l'Insecte *Locusta migratoria* (Proux *et al.*, 1987) et chez des Mollusques Gastéropodes [*Conus geographus* et *Conus striatus* : Cruz *et al.* (1987) ; *Aplysia kurodai* : McMaster *et al.* (1992) ; *Lymnaea stagnalis* :Van Kesteren *et al.* (1992)] et Céphalopodes [*Octopus vulgaris* : Reich (1992)]. Après avoir quantifié par dosages ELISA cette LVP-like, nous l'avons purifiée à partir de CNS. Les résultats (Salzet *et al.*, en préparation 2) ont montré que cette molécule possède la même séquence que la Lysine-conopressine isolée chez les Mollusques Gastéropodes *C. geographus*, *A. kurodai* et *L. stagnalis*.

Isolation and structural characterization of a Lysine-conopressin in the central nervous system of the Pharyngobdellid leech *Erpobdella octoculata.*

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ABSTRACT

Several neuropeptides are susceptible to act on the control of hydric balance in leeches. Two of these peptides were previously characterized from the central nervous system (CNS) of the leech *Erpobdella octoculata* : an angiotensin II amide (Salzet et al., in preparation) and an oxytocin-like peptide (Salzet *et al.*, in press).

This paper reports the isolation from the CNS of this same leech of another of these neuropeptides : a lysine-vasopressin-like molecule (LVP-like). Its purification was performed by reversed-phase HPLC combined with both DIA and ELISA for LVP. Characterization of the LVP-like by reduction followed by an alkylation, automated Edman degradation and mass spectrometry resulted in the structure of a nonapeptide : CFIRNCPKG, with a molecular mass of 1034.2 ± 0.6 Da. This LVP-like exactly corresponded both in sequence and molecular mass to the lysine-conopressin peptide previously isolated by Cruz *et al.* (1987) in *Conus geographus.*

Abbreviations : AVP, arginine-vasopressin ; DIA, dot immunobinding assay ; ELISA, enzyme linked immunosorbent assay ; HPLC, high performance liquid chromatography; LC, lysine-conopressin ; LVP, lysine-vasopressin ; OT, oxytocin ; SG, segmental ganglia.

INTRODUCTION

The advent of immunocytochemical techniques has strongly influenced thinking in invertebrate endocrinology. With antisera directed against various vertebrate neuropeptides, a large number of immuno-reactivities has been demonstrated at the level of fibres and neurons of invertebrate nervous systems (De Loof and Schoofs, 1990). The improvement of chromatographical, immunological and molecular biology techniques has greatly facilitated the purification and the determination of the amino acid sequences of neuropeptides in invertebrates . A comparison of sequences of neuropeptides isolated in vertebrates and invertebrates has shown the existence of neuropeptides families e.g. RFamides, proopio-melanocortin, tachykinins, opioids, gastrin /cholecystokinin peptides, oxytocin/ vasopressin peptides.

In this paper, we focused our interest on peptides of the oxytocin (OT) / vasopressin (VP) family. These molecules control hydric balance in vertebrates (Laevitt *et al.*, 1987) and in some invertebrates (Moore *et al.*, 1981; Proux *et al.*, 1987). Their biological function was conserved during evolution (Mühlethaler *et al.*, 1984). However, the physiological actions of these peptides depend on the amino acid residue in position 8, which is polar in VP-related (VP-like) molecules and non-polar in OT-related (OT-like) peptides (Acher *et al.*, 1985).

In vertebrates, six OT-like and four VP-like hormones have been identified (Acher *et al.*, 1985). In invertebrates, although peptides related to the OT/VP family have been detected immunocytochemically in all phylla (Mizuno and Takeda, 1988a, b), only six peptides related to the OT/VP family have been isolated till now, five are related to vasotocin (VT-like) and one to isotocin (IT-like). Among the VT-like molecules, the ones isolated in the Molluscs *Lymnaea stagnalis* (Van Kesteren *et al.*, 1992) and *Aplysia kurodai* (McMaster *et al.*, 1992) are identical and correspond to the lysine-conopressin peptide, firstly isolated in another Mollusc, *Conus geographus* (Cruz *et al.*, 1987). Lysine-conopressin differs from the VT molecule by a substitution of amino acid residues in positions 2, 4 and 8. In contrast, the two other VT-like peptides isolated respectively in the Mollusc *Conus striatus* (Cruz *et al.*, 1987) and in the Insect *Locusta migratoria* (Proux *et al.*,1987) present, compared to the VT molecule, substitutions in amino acid residues in positions 2 and 4. The sixth peptide of the OT/VP family detected in invertebrates, the cephalotocin peptide isolated in the Cephalopod *Octopus vulgaris* (Reich,

1992), presents 78% of homology with IT, which positions it into the OT lineage.

Based on these results, the hypothesis of a molecular filiation of these peptides has been advanced by Acher *et al.* (1985) and Van Kesteren *et al.* (1992). An ancestral molecule would have given, 400 million years ago, two lineages of macromolecular precursors (OT and VP). Each lineage would have then been subjected to independent substitutions. The precursors of present day mammals would be processed on the one hand into OT or mesotocin and likely a VLDV-neurophysin, and on the other hand into VP and likely a MSEL-neurophysin (Acher *et al.*, 1985).

In the light of the evolutionary conservancy of neuropeptides, the identification of a peptide of OT/VP family in the central nervous system of the common leech *Erpobdella octoculata,* animal belonging to the oldest group of cœlomate Metazoa (the Annelida), was undertaken. This investigation was realized by a combination of reversed-phase HPLC, automated Edman degradation and mass spectrometry.

MATERIALS and METHODS

Animals

E.octoculata , collected at Harchies (Belgium) and then kept in the dark at 15°C in pond water, were used in this study.

Dissections

After anaesthetization in 0.01 % chloretone, animals were pinned out, dorsal side up, in leech Ringer (Muller *et al.*, 1981). Central nervous systems (CNS), consisting of the brain and of a fragment of nerve cord including segmental ganglia 1-8, were dissected, frozen at -180°C in nitrogen and finally stored at -20°C until use for biochemical purification.

Chemicals

3-3'-diaminobenzidine-tetra-hydrochloride (DAB), hydrogen peroxide and synthetic lysine-vasopressin (LVP) were obtained from Sigma, the trifluoroacetic acid (TFA), sequencer grade, from Pierce and peroxidase-conjugated goat anti-rabbit IgG from Pasteur Diagnostics. All organic solvents were HPLC grade and purchased from Merck. Deionized water was obtained from a Milli-Q system (Millipore).

Antibody

The polyclonal antibody anti-lysine-vasopressin (a-LVP), raised in rabbit and kindly provided by Dr G. Tramu (Laboratoire de Neurocytochimie fonctionnelle, Université de Bordeaux I, Talence, France) was used in DIA and ELISA procedures. Its specificity was described elsewhere (Tramu *et al.*, 1983).

Immunoassays

a - ELISA procedures

- Immunoassays were conducted according to the procedures of Salzet *et al.* (1992) with a-LVP used at a dilution of 1:1000.

- The quantification of the LVP-like peptide in CNS extracts was done in direct ELISA according to Salzet *et al.* (1992).

b - Dot immunoassay (DIA)

Aliquot of 1 µl of each collected fraction from the reversed-phase HPLC was spotted onto a nitrocellulose membrane (0.45 µm pore size, Schleicher and Schuell) which was then baked 30 min at 110°C. The membrane was blocked, under gentle agitation 1 hr at room temperature with PBS (50 mM Phosphate buffer, 150 mM Sodium chloride, pH 7.4) including 0.05 % Tween 20 and 5 % Skilm milk and then incubated overnight at 4°C with a-LVP [diluted 1:1000 in PBS/0.05 % Tween 20 (PT)]. After the primary incubation, the membrane was washed for four 5 min periods with PT and incubated for 1 hr with peroxidase-conjugated goat anti-rabbit IgG diluted 1:1000 in PT. Washing in PT was repeated (4 x 5 min). Bound antibody was revealed with a solution comprising 45 mg of DAB and 20 µl hydrogen peroxide in 100 ml of PT.

Carboxymethylation reactions

Two microliters of purified LVP-like peptide (50 pmol) were added to 100µl of a freshly prepared solution of 0.1 M dithiothreitol (DTT) in 0.1 M Tris/HCl (pH 8.5). After a 2h incubation, 6 µl of a freshly prepared 0.2 M iodoacetic acid (IAA) in Tris/HCl buffer were added. After 1-2 h in the dark, the mixture was transferred to an injection vial, acidified with 95 µl of 1 M acetic acid and finally injected in a column of reversed-phase HPLC.

Purification of the LVP-like peptide

Step I : Sep-Pak prepurification - CNS in batches of 200 or 400 were homogenized in 200 or 400 µl of 1 M acetic acid with a Dounce homogenizer and then sonicated (30 sec) twice. Homogenates were centrifuged at 12,000 rpm for 30 min at 4°C. After reextraction of the pellet, the two supernatants were combined. Then, they were applied on C_{18} Sep-pak cartridges (500 µl/cartridge, Waters) and eluted with 5 ml of 50 % acetonitrile (ACN). Sep-pak eluted fractions were reduced 20-fold by freeze-drying. Total amount of LVP-like material was quantified in ELISA.

Step II : Reversed-phase HPLC - Immunoreactive fractions from the Sep-Pak prepurification were subjected to a reversed-phase HPLC on a Vydac C_{18} protein peptide (250 x 4.6 mm) column. Elution was performed with a discontinous linear gradient from 0% A (0.1% TFA in 100% deionized water) to 15% B (0.1% TFA in 100% ACN) in 10 min, followed by a linear gradient from 15 to 45% of B in 30 min at a flow rate of 1 ml/min. Ultraviolet absorbance was monitored at 226 nm or 215 nm. Eluted fractions were reduced to a half of their volume by freeze-drying, before being assayed for immunoreactivity in DIA.

The immunoreactive fraction containing the LVP-like material was pooled to 10.000 CNS-equivalent and chromatographed on the same column with a shallower gradient. The solvent program consisted in a linear gradient 0 to 15 % B in 10 min followed by 15 to 45 % B in 40 min at a flow rate of 1ml/min. After reduction by freeze-drying, fraction aliquots of 0.5 µl were tested in DIA and the immunoreactive material separated on a more resolutive column, a narrowbore.

Step III. Final purification - The immunoreactive peptide was purified by two successive steps on a narrowbore (250 x 2 mm) column (Beckman) with a linear gradient from 0 to 60% B in 60 min at a flow rate of 300 µl/min. Amounts of LVP-like material were determined in direct ELISA, before being subjected to an automated Edman degradation.

All HPLC purifications were performed with a Beckman Gold HPLC system equiped with a photodiode array detector Beckman 168.

Amino acid Sequence analysis

Automated Edman degradation of 100 pmol of peptide and detection of phenylthiohydantoin (PTH) derivates were performed on a pulse liquid automatic sequentator (Applied Biosystems, model 473).

Mass Spectrometry

The purified peptides were dissolved in water/ methanol (50/50, v/v) containing 1% acetic acid and analyzed on a VG BioTech Bioq mass spectrometer (Manchester). This instrument consists of an electrostatic ion spray source operating at atmospheric pressure, followed by a quadrupole mass analyser.

RESULTS and DISCUSSION

Isolation of the LVP-like peptide.

CNS of *E. octoculata* were subjected to a peptide extraction in 1 M acetic acid (pH 2). The crude extract was prepurified using C_{18} Sep-Pak cartridges, reduced 20-fold by freeze drying and applied to a reversed-phase HPLC column. The total amount of LVP-like material determined at this step of purification was *ca* 1 pmol/ CNS. Eluted fractions tested in direct ELISA, revealed an immunoreactive zone (Z1) at a retention time (RT) ranging from 19 to 20 min (Fig. 1). Results obtained after preadsorption of a-LVP by LVP synthetic peptide established the specificity of the immunodetection. A comparison with synthetic peptides of the OT/VP family eluted in same conditions, indicated that the LVP-like peptide was different from arginine-VP, VT, LVP, IT, OT peptides (Table I).

The immunoreactive zone containing the LVP-like material (Z1) from 10.000 CNS was chromatographed on the same column with a shallower gradient (Fig. 2). A large peak with a RT ranging from 21 to 24 min was resolved. Four fractions (F1, F2, F3 and F4) were collected. DIA indicated that these four fractions were immunoreactive to a-LVP. However, ELISA indicated that the major amount of LVP-like peptide (*ca* 0.85 pmol/CNS) was contained in F2. F2 was separated on a narrowbore column and gave an immunoreactive peak at a RT of 24.4 min (Fig. 3). This peak was finally purified (Fig. 4) on a narrowbore column in same conditions as precedently. Quantification in ELISA indicated an amount of 0.80 pmol of LVP-like/CNS.

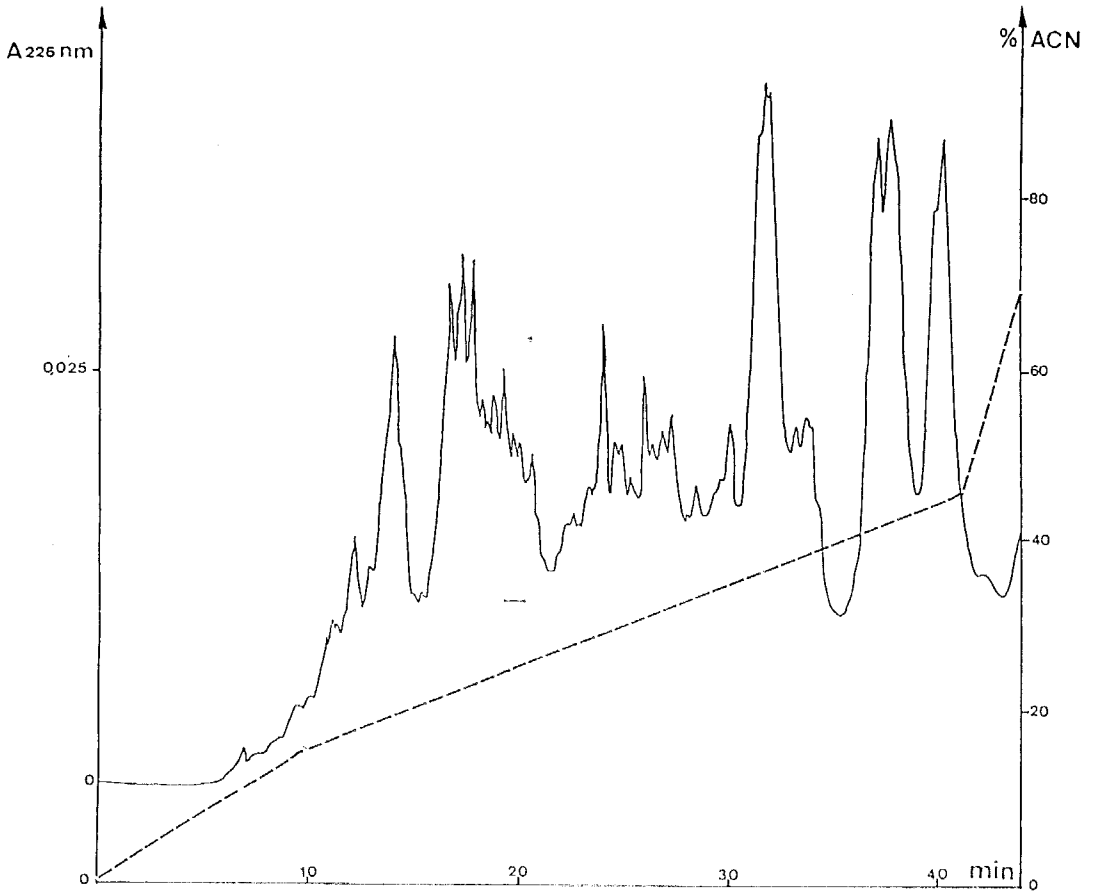

Figure 1 : *C18 reversed-phase HPLC elution profile of an extract of 400 CNS of E. octoculata.*
- Elution rate: 1 ml/min; solvent A: 0.1 % TFA in water; solvent B: 0.1 % TFA in 100 % ACN; solvent program : gradients of B at 1.5 %/min from 0 to 15 % followed by 1 %/min from 15 to 45 %.
- The solid line indicates absorbance; the dotted line indicates the gradient.
- The immunoreactive zone to a-LVP was indicated by a solid bar.
- Absorbance was monitored at 226 nm (A226 nm)

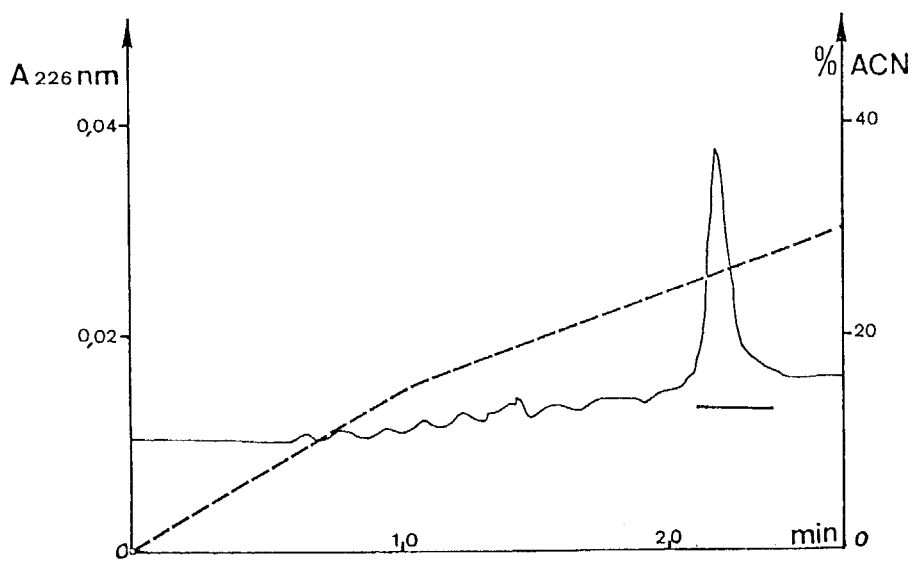

Figure 2 : *C18 reversed-phase HPLC on a Vydac column of the LVP-like immunoreactive material previously separated on a C_{18} reversed-phase HPLC column.*
- Elution rate: 1 ml/min; solvent A: 0.1 % TFA in water; solvent B: 0.1 % TFA in 100 % ACN; solvent program : gradients of B at 1.5 %/min from 0 to 15 % followed by 0.8 %/min from 15 to 45 %.
- The solid line indicates absorbance; the dotted line indicates the gradient.
- The immunoreactive zone to a-LVP was indicated by a solid bar.
- Absorbance was monitored at 226 nm (A226 nm)

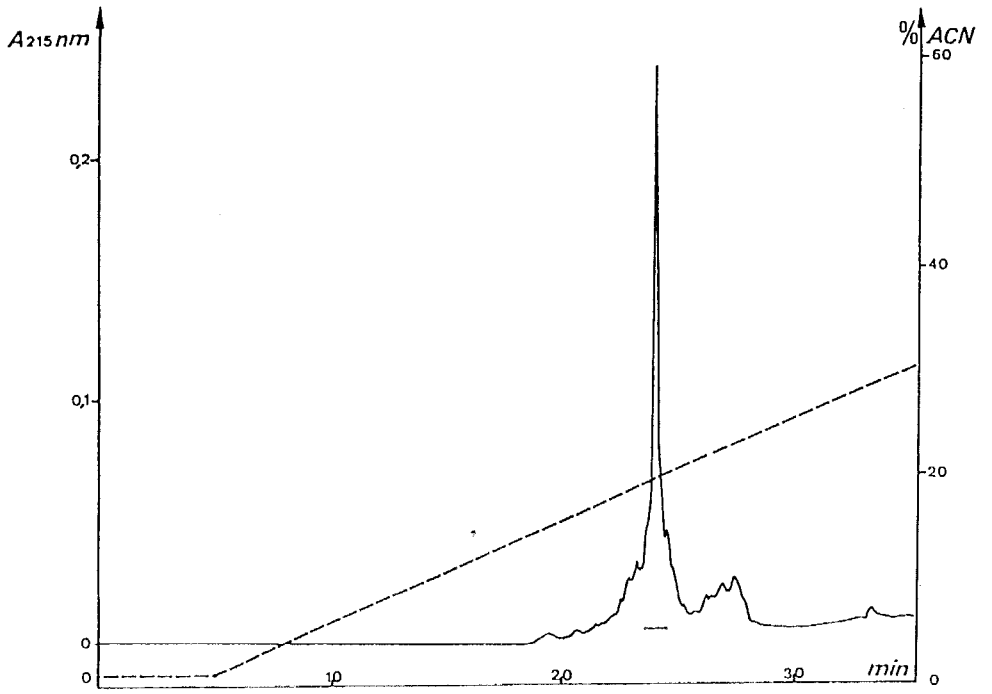

Figure 3 : *C_{18} reversed-phase HPLC on a narobore column of the LVP-like immunoreactive material previously separated on a C_{18} reversed- phase HPLC column.*
- Elution rate: 0.3 ml/min; solvent A: 0.1 % TFA in water; solvent B: 0.1 % TFA in 100 % ACN; solvent program: gradients of B at 1 %/min from 0 to 60 %.
- The solid line indicates absorbance; the dotted line indicates the gradient.
- Absorbance was monitored at 215 nm (A215 nm)

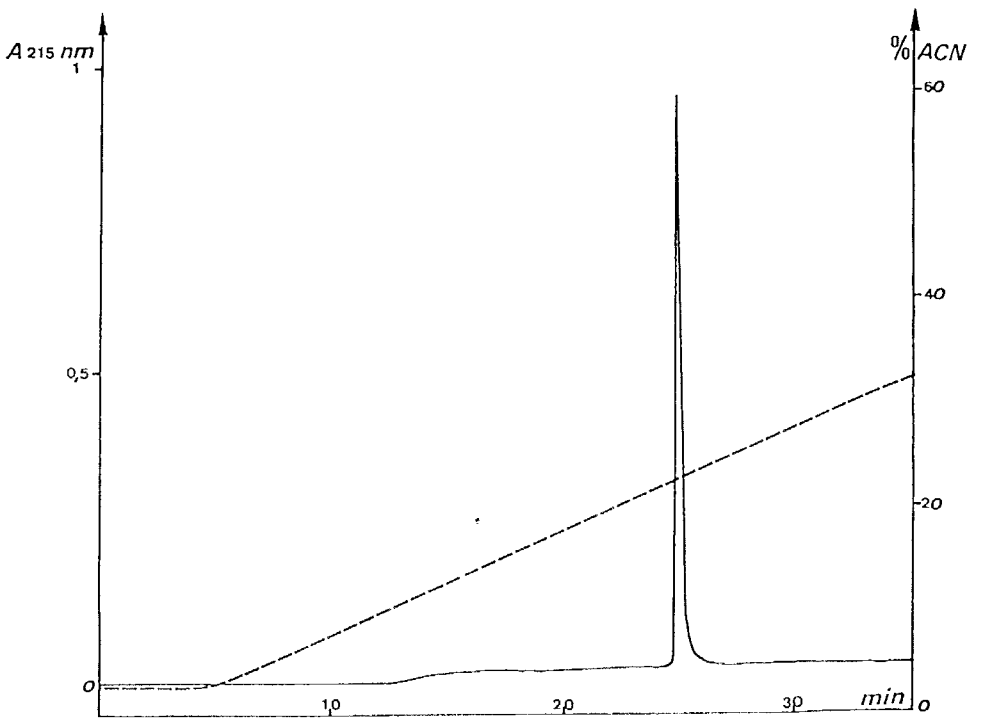

Figure 4 : *Final step of purification on a narobore column of the LVP-like peptide.*
- Elution rate: 0.3 ml/min; solvent A: 0.1 % TFA in water; solvent B: 0.1 % TFA in 100 % ACN; solvent program : gradients of B at 1 %/min from 0 to 60 %.
- The solid line indicates absorbance; the dotted line indicates the gradient.
- Absorbance was monitored at 215 nm (A215 nm)

Table I : Retention times after RP-HPLC of the LVP-like peptide and of different peptides of the OT/VP family

Peptide	Sequence	Retention time (min)
OT	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH$_2$	21.5
ISO	Cys-Tyr-Ile-Ser-Asn-Cys-Pro-Ile-GlyNH$_2$	21
AVP	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH$_2$	19
AVT	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-GlyNH2	17.8
LVP	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-GlyNH2	18
LVP-like		19.4

AVP: Arginine-Vasopressin, AVT: Arginine-Vasotocin, ISO: Isotocin, LVP: Lysine-Vasopressin, OT: oxytocin.

Characterization of the LVP-like peptide

The amino acid sequence determination of the LVP-like peptide gave successively the following nine amino acids residues : X, F, I, R, N, X, P, K, G. (X: non-identified PTH residue). No PTH amino acids were further detected in cycles 10 and 11. In cycles 1 and 6, no PTH amino acids appeared. This could be expected since reduction and alkylation of cysteine (Cys) residues was omited before sequencing. However, presence of a disulfide bridge in the LVP-like peptide was demonstrated by the shift of the LVP-like peptide (from 19.32 min to 16.3 min) after reduction with DTT and alkylation with IAA.

Postulating that we had to deal with an OT/VP related peptide with Cys at positions 1 and 6 involved in a disulfide bridge, the sequence of this peptide may be therefore: CFIRNCPKG(NH_2), which corresponds to the lysine-conopressin (LC). Its calculated mass (1034.5 Da) is in a good

agreement with the *m/z* obtained (1034.2 ± 0.6 Da) which is close to the one (1034 Da) reported by Cruz *et al.* (1987) for the LC of *C. geographus*.

We can thus conclude on the presence in the CNS of *E. octoculata*, of a LC peptide also detected in the Molluscs *A. kurodai*, *L. stagnalis* and *C. geographus*. This presence of LC in leeches strenghtens the hypothesis of Van Kesteren *et al.* (1992) that typical OT/VP molecule must have been yet present in Archaemetazoa, from which vertebrates and invertebrates diverged 600 million years ago. Such an hypothesis was also reinforced by data related to the cDNA sequencing of the LC precursor in *L. stagnalis* (Van Kesteren *et al.*, 1992). The deduced amino acid sequence of the *L. stagnalis* preproconopressin indicates that it is organized as the OT/VP-like precursors of vertebrates *i.e.* with a signal sequence followed by conopressin and a remarkably conserved neurophysin-domain having a divergent copeptin-homologous C-terminal sequence. These last results demonstrate the existence of a typical architecture of the precursors of the OT/VP pro-hormone in an invertebrate.

The *L. stagnalis* neurophysin presenting 49% of homology with MSEL-neurophysin and 45% of homology with the VLDV-neurophysin , it can be proposed that the preproconopressin would be at the origin of the OT and VP lineages. Moreover, the presence of a LC in leeches and the fact that the leech OT-like molecule possesses a structure different from peptides of the OT/VP family (Salzet *et al.*, unpublished data) allow to suggest that LC is the ancestral molecule of the OT/VP family in cœlomates.

Aknowledgements

We are indebted to Dr. J. Hoffmann (Laboratoire des bases cellulaires et moléculaires de de la réponse immunitaire des Insectes, URA 1490 CNRS, Strasbourg, France), for its scientific collaboration for the peptide sequencing.

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Molécules immunoréactives à l'anti-FMRFamide

Article présenté

(7) Salzet, M., Bulet, P. and Malecha, J., GDPFLRFamide : a novel leech RFamide peptide extracted from the sex segmental ganglia of the Pharyngobdellid *Erpobdella octoculata*, en préparation 3.

Macagno *et al.*, (1986) avaient montré chez *Hirudo medicinalis* l'existence au niveau des cellules surnuméraires des ganglions génitaux d'une immuno-réactivité à l'anti-FMRFa (a-FMRFa). Le même résultat a été obtenu chez *E. octoculata* où la substance FMRFa-like est colocalisée avec celle immunoréactive à l'a-OT. Les résultats expérimentaux d'injections d'extraits de ganglions génitaux d'*E. octoculata* chez *T. tessulatum* montrent qu'ils exercent un effet anti-diurétique (Salzet *et al.*, 1992c). Par contre les extraits des autres ganglions segmentaires sont sans effet (Salzet *et al.*, 1992c). La différence observée dans les effets physiologiques exercés par les deux types de ganglions (génitaux et non génitaux) est liée à la présence dans les ganglions génitaux de cellules surnuméraires contenant à la fois une substance OT- et FMRFa-like. L'un ou l'autre de ces deux types de molécules des ganglions génitaux pouvait donc être responsable de l'effet observé. C'est pourquoi, dans la phase initiale de nos recherches, nous avons décidé d'isoler en même temps que la substance OT-like, celles apparentées aux FMRFa (RFa).

Le FMRFa et trois peptides qui lui sont apparentés [FLRFa, FM(O)RFa et GDPFLRFa] ont été purifiés à partir des ganglions génitaux d'*E. octoculata* (Salzet *et al.*, en préparation 3).

GDPFLRFamide : A novel leech RFamide peptide extracted from the sex segmental ganglia of the Pharyngobdellid *Erpobdella octoculata.*

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ABSTRACT

In Hirudinea, Evans *et al.*, (1991) isolated from the nerve cord of *Hirudo medicinalis* five RFamide peptides [YMRFa, YLRFa, FMRFa, FLRFa and YGGKYMRFa]. In this leech (Macagno *et al.*, 1986) and in the Pharyngobdellid leech *Erpobdella octoculata* (Salzet *et al.*, 1992b) evidence was given of the presence in the two sex segmental ganglia (sex SG) of the nerve cord of supernumerary cells immunoreactive to an antibody directed against FMRFa. In *E. octoculata*, a colocalization of RFamides and of an OT-like epitope was demonstrated in these supernumerary cells of the sex SG (Salzet *et al.*, 1992b). This paper reports the isolation of four RFamide peptides [FM(O)RFa, FMRFa, FLRFa and GDPFLRFa] from the sex SG of *E. octoculata* by a combination of antiserum specificity, Edman degradation and mass spectrometry. Two of these peptides [GDPFLRF and FM(O)RFa] are novel leech RFa peptides.

Abbreviations : DIA, dot immunobinding assay ; ELISA, enzyme linked immunosorbent assay ; HPLC, high performance liquid chromatography ; M(O), methionine sulfoxide; RFa; Arg-Phe-amide ;sex SG, sex segmental ganglia.

Introduction

Since their discovery in the central nervous system of the clam, *Macrocallista nimbosa* by Price and Greenberg (1977), RFamides (RFa), a family of peptides having in common a C-terminal sequence (Arg-Phe-amide) have been identified and isolated throughout the animal kingdom (for a review, see Walker,1992).

In Annelids, authentic FMRFamide (FMRFa) is present in the Polychaete *Nereis virens* (Krajniak and Price, 1990). In a related species, *Nereis diversicolor*, Baratte *et al.* (1991) have isolated three RFa peptides [FM(O)RFa, FMRFa and FTRFa]. Immunohistochemical and pharmacological data suggest that RFa are involved in the control of heartbeat and body wall tone, in the Polychaete *Sabellastarte magnifica* (Diaz-Miranta *et al.*, 1989) and in the Oligochaete *Eisenia foetida* (Fujii *et al.*, 1989).

In *Hirudo medicinalis*, a FMRFa-like immunoreactivity has also been reported in the central nervous system (CNS) (Evans and Calabrese, 1989 ; Kuhlman *et al.*, 1985a). Biochemical and immunocytochemical studies have localized RFa peptides in the heart excitatory motor neurons (HE) and in the heart accessory modulating neurons (HA) that innervate the leech heart and also in terminals in the heart (Evans and Calabrese, 1989 ; Kuhlman *et al.*, 1985a ; Li and Calabrese, 1987 ; Maranto and Calabrese, 1984). In *H. medicinalis*, Evans *et al.* (1991) have isolated from the nerve cord, five RFa peptides [YMRFa, YLRFa, FMRFa, FLRFa and GGKYMRFa].

The physiological roles of RFa peptides are well known in *Hirudo*, where they act in the control of heartbeat (Kuhlman *et al.*, 1985a, b) and in the contraction of longitudinal muscles (Norris and Calabrese, 1987). FMRFa applications increase the strength and accelerate the rate of myogenic contractions. They can also induce myogenic contractions in quiescent hearts (Calabrese and Maranto, 1984 ; Kuhlman *et al.*, 1985b ; Li and Calabrese, 1987). Furthermore, bath application of FMRFa mimics many of the effects of electrical stimulation of HE and HA cells (Calabrese and Maranto, 1984 ; Kuhlman *et al.*, 1985b ; Li and Calabrese, 1987). In addition, bath application with FMRFa and several related peptides (YMRFa, FLRFa, pQDPFLRFa, YGGFMRFa and LPLRFa)elicit contractions in longitudinal muscles (Norris and Calabrese, 1990). RFa peptides would also act as neurotransmitters. Indeed, in *H. medicinalis*, the

action on heartbeat of the swim initiating interneurons 204 (Weeks and Kristian, 1978) could be due to the central release of RFa peptides which may act in conjunction with serotonin, which is released centrally from Retzius neurons during swimming (Willard, 1981).

An immunostaining of the supernumerary cells of the sex SG with anti-FMRFamide was reported by Macagno *et al.* (1986) in *H. medicinalis.* Recently, we demonstrated (Salzet *et al.*, 1992b) immunocytochemically and biochemically the presence of an RFa epitope colocalized with an oxytocin-like epitope in the sex SG of the leech *Erpobdella octoculata* . The aim of this work was to isolate and characterize the RFa peptides contained in the sex SG of *E. octoculata.*

Material and Methods

Animals

Mature *E. octoculata* collected at Harchies (Belgium), were used in this study. They were kept in the dark at 15°C in freshwater (Salzet *et al.*, 1992a) .

Dissections

After anesthetization in 0.01% chloretone, leeches were pinned out, dorsal side up, in leech Ringer (Muller *et al.*, 1981). Dissected sex SG (SG5 and SG6) were frozen at -180°C in nitrogen and finally stored at -20°C until biochemical purification.

Chemicals

3-3'-diaminobenzidine-tetra-hydrochloride (DAB), synthetic peptides (FMRFa and FLRFa) and hydrogen peroxide were obtained from Sigma, the trifluoroacetic acid (TFA), sequencer grade, from Pierce and peroxidase-conjugated goat anti-rabbit IgG from Pasteur Diagnostics. All organic solvents were HPLC grade and were purchased from Merck. Deionized water was obtained from a Milli-Q system (Millipore).

Antibody

The polyclonal antibody anti-FMRFa (646) (a-FMRFa), raised in rabbit and kindly provided by Dr J. Van Minnen (Free University,

Amsterdam, Netherlands) was used in DIA and ELISA procedures. It is specific of the C-terminal part (RFa) of FMRFa (Schot *et al.*, 1984).

Immunoassays

a - ELISA procedures

- Immunoassays were conducted according to procedures described elsewhere (Salzet *et al.*, 1992a). Polyclonal antibody (a-FMRFa) was used at a dilution of 1:1000.

- The quantification of RFa-like peptides in sex SG extracts was done in direct ELISA according to Salzet *et al.* (1992a).

b - Dot immunoassay (DIA)

An aliquot of 1 µl of HPLC fractions was spotted onto a nitrocellulose membrane (0.45 µm pore size, Schleicher and Schuell) which was then baked 30 min at 110°C. The membrane was blocked, under gentle agitation at room temperature, 1 hr with PBS (50 mM Phosphate buffer, 150 mM Sodium chloride, pH 7.4) including 0.05 % Tween 20/5 % Skilm milk, then incubated overnight at 4°C with a-FMRFa [diluted 1:1000 in PBS/0.05 % Tween 20 (PT)]. After the primary incubation, the membrane was washed for four 5 min periods with PT and incubated for 1 hr with peroxidase-conjugated goat anti-rabbit IgG diluted 1:1000 in PT. Washing in PT was repeated (4 x 5 min). Bound antibody was revealed with a solution comprising 45 mg of DAB and 20 µl hydrogen peroxide in 100 ml of PT.

Purification of RFa peptides

Step I : Sep-Pak prepurification - Sex SG in batches of 200 or 400 were homogenized in 200 or 400 µl of 1 M acetic acid with a Dounce homogenizer and then sonicated (30 sec) twice. Homogenates were centrifuged at 12,000 rpm for 30 min at 4°C. After reextraction of the pellet, the two supernatants were combined. Then, they were applied on C18 Sep-pak cartridges (500 µl/cartridge, Waters) and eluted with 5 ml of 50 % acetonitrile (ACN). Sep-pak eluted fractions were reduced 20-fold by freeze-drying. Total amount of RFa peptides was obtained in ELISA on a fraction aliquot of the reduced extracts.

Step II : Reversed-phase HPLC - The immunoreactive fractions from the Sep-Pak prepurification were subjected to a reversed-phase HPLC on a Vydac C_{18} Protein Peptide (250 x 4.6 mm) column. Elution was performed with a discontinous linear gradient from 0 % A (0.1 % TFA in 100 % deionized water) to 15 % B (0.1 % TFA in 100 % ACN) in 10 min, followed by a linear gradient from 15 to 45% of B in 30 min at a flow rate of 1 ml/min. Ultraviolet absorbance was monitored at 226 nm. The eluted fractions were reduced to a half of their volume by freeze-drying, before being assayed for immunoreactivity in DIA.

The immunoreactive compounds present in different zones were separately subjected to a reversed-phase HPLC on a Vydac column with a shallower gradient. The solvent program consisted in a linear gradient 0 to 15 % B in 10 min followed by 15 to 45 % B in 40 min at a flow rate of 1 ml/min. After, reduction by freeze-drying, fraction aliquots of 0.5 µl were tested in DIA.

Step III. Final purification - The different immunoreactive substances were then separated on a Vydac column with the same conditions as precedently.

Fractions collected manually, were quantified in ELISA, before being subjected to an automated Edman degradation.

All HPLC purifications were performed with a Beckman Gold HPLC system equiped with a photodiode array detector Beckman 168.

Peptide oxidation

To demonstrate the presence or the absence of methionine, fraction aliquots of purified peptides (50 pmol) were evaporated almost to dryness, diluted in water and subjected to an oxidation prior to be rechromatographed on a Vydac column with a linear gradient of 0 to 60 % B in 60 min at a flow rate of 1 ml/min. The immunoreactive fractions which possessed a methionine, were converted in methionine sulfoxide. Aliquots of purified fractions were treated with 2 % hydroxygen peroxide (1 hr at room temperature). Peptides which possessed a methionine eluted earlier in the gradient.

Amino acid Sequence analysis

Automated Edman degradation of 100 pmol of peptide and detection of phenylthiohydantoin derivates were performed on a pulse liquid automatic sequentator (Applied Biosystems, model 473).

Mass Spectrometry

The purified peptides were dissolved in water/ methanol (50/50, v/v) containing 1% acetic acid and analyzed on a VG BioTech Bioq mass spectrometer (Manchester). This instrument consists of an electrostatic ion spray source operating at atmospheric pressure, followed by a quadrupole mass analyser.

RESULTS

Isolation of RFa peptides.

Sex SG (SG5 + SG6) of mature *E. octoculata* were subjected to a peptide extraction in 1 M acetic acid (pH 2). The crude extract was purified using C_{18} Sep-Pak cartridges, reduced 20-fold by freeze drying and applied to a reversed-phase HPLC column. The total amount of RFa determined at this step of purification was *ca* 18 pmol/mature sex SG (Table I). Eluted fractions tested in direct ELISA revealed (Fig. 1) five immunoreactive zones (Z1, Z2, Z3, Z4 and Z5). After preadsorption of a-FMRFa by FMRFa peptide, four of these zones disappeared, in contrast to zone Z5, eluting between 43-44 min, which persisted (Fig. 1). Only, Z1, Z2, Z3 and Z4, which corresponded respectively to eluted zones with a retention time of respectively 17-18 min (Z1), 21-22 min(Z2), 23-24 min (Z3) and 29-30 min(Z4), were thus considered as specific. A comparison with synthetic peptides of the RFa family indicated that FM(O)RFa, FMRFa, and FLRFa eluted with the same retention time than respectively Z1, Z2 and Z3.

Compounds contained in respectively Z1, Z2, Z3 and Z4 were further purified separately on the same column with a shallower gradient. In these conditions Z1, Z2 and Z3 gave the RFa immunoreactive material P1, P2 and P3 which presented the same retention time than respectively FM(O)RFa [19.4 min], FMRFa [22.3 min] and FLRFa [23.4 min] whereas Z4 gave P4 at 26.2 min.

In a final step on a Vydac column (Figs. 2a, b, c, d), P1, P2, P3 and P4 possessed a retention time of respectively 19.4 min, 22.3 min, 23.4 min

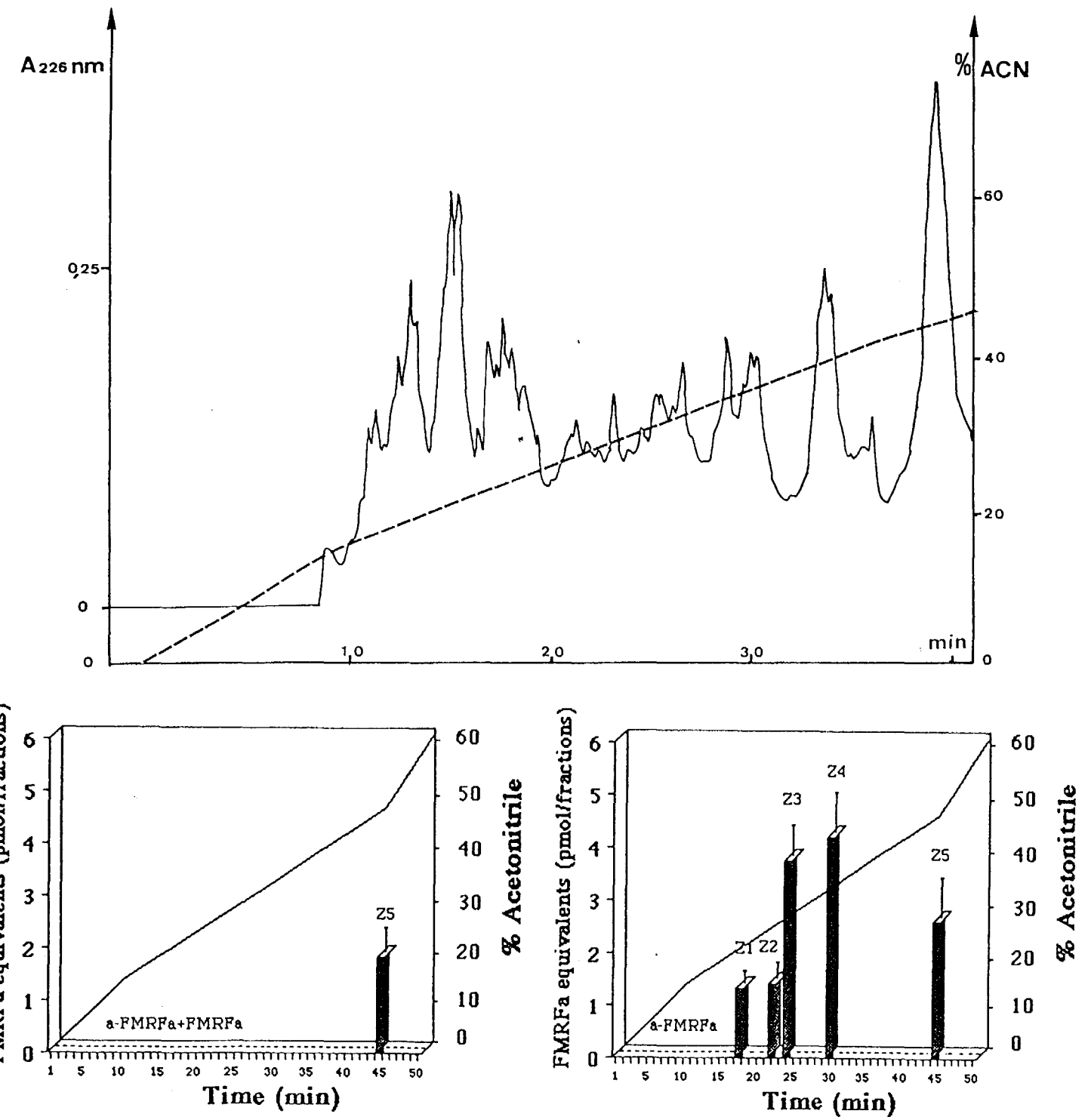

Figure 1 : *C_{18} reversed-phase HPLC elution profile of an extract of 400 sex SG from mature E. octoculata .*

- Elution rate : 1 ml/min; solvent : 0.1 % TFA in water ; solvent B : 0.1 % TFA in 100 % ACN ; solvent program : gradients of B at 1.5 %/min from 0 to 15 % followed by 1 %/min from 15 to 45 %.
- The solid line indicates absorbance ; the dotted line indicates the gradient.
- Fractions were assayed for FMRFa-like immunoreactive material (diagrams) with antibody anti-FMRFamide preadsorbed (a-FMRFa + FMRFa) or not (a-FMRFa) with the homologous antigen.
- Values given for each assayed fraction correspond to aliquots of fractions of 1 µl.
- Absorbance was monitored at 226 nm (A 226 nm).

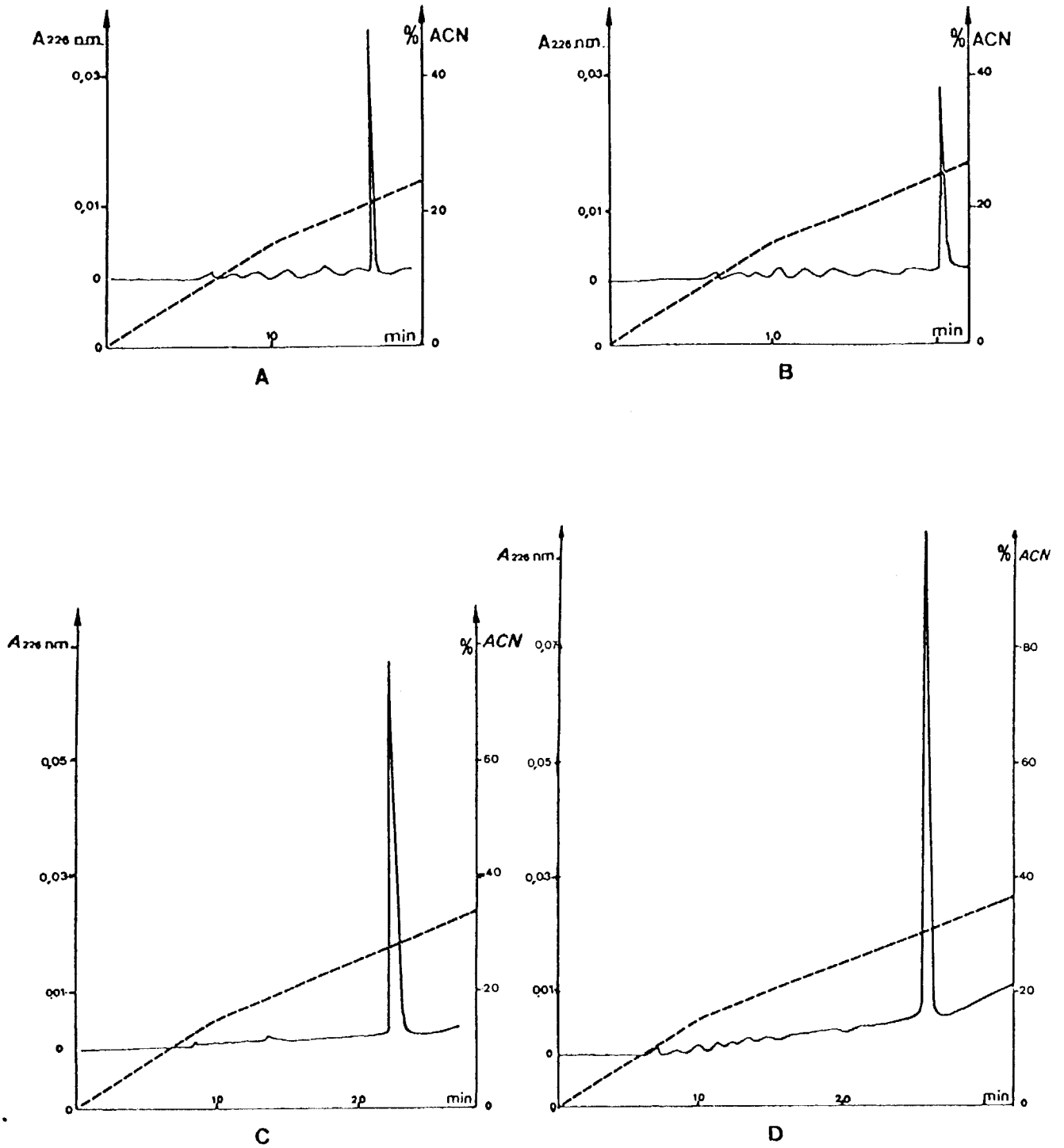

Figure 2 : *Final step purification on C_{18} reversed-phase HPLC of peptides P1* (A), *P2* (B), *P3* (C) *and P4* (D) *previously separated on C_{18} reversed-phase HPLC column.*

- Elution rate : 1 ml/min ; solvent A : 0.1 % TFA in water ; solvent B : 0.1 % TFA in 100 % ACN ; solvent program : gradients of B at 1.5 %/min from 0 to 15 % followed by 0.8 %/min from 15 to 45 %.
- The solid line indicates absorbance ; the dotted line indicates the gradient.
- Absorbance was monitored at 226 nm (A 226 nm).

and 26.2 min. In these conditions P1, P2 and P3 had the same retention time than FM(O)RFa, FMRFa and FLRFa peptides.

The comparison of the total amount of RFa determined just after Sep-Pak prepurification with the sum of each RFa amount, showed 60 % of recovery after the three biochemical purification steps.

Characterization of the immunoreactive peptides P1 and P2

It is known that the oxidized analog of FMRFa peptide [FM(O)RFa] bears a methionine sulfoxide instead of a methionine and that being more hydrophilic, it elutes earlier than the unoxidized peptide (Krajniak and Price, 1990). In order to establish that P1 corresponded to FM(O)RFa and P2 to FMRFa, P1 and P2 were oxidized. The separation of these peptides was then undertaken on a Vydac column. It showed a shift of P2 in P1. These results indicated the possibility of the existence of a methionine sulfoxide in P1 and of an unoxidized methionine in P2.

Analysis of P1 - Since this peptide was immunoreactive to the a-FMRFa (antibody specific of the C-terminal part of the FMRFa) and coeluted with methionine sulfoxife FMRFa, it can be proposed that P1 possesses the same structure as the FM(O)RFamide peptide. The molecular mass of *m/z* 614.6 ± 0.14 Da obtained by mass spectrometry (Table II) was in good agreement with the mean of the calculated monoisotopic mass of FM(O)RFa (615.3 Da) which confirmed the presence of FM(O)RFa in the sex SG of mature *E. octoculata*. The amount of FM(O)RFa was estimated at *ca* 1.8 pmol/sex SG (Table I).

Table I : Total amount of RFamide peptides after sep-Pak prepurification and amounts of each identified RFamide peptide isolated at the final step of purification present in sex segmental ganglia (sex SG) of *E. octoculata*.

		Amount (pmol/sex SG)
Total RFamide		17.69 ± 4.86
Peptide (P #)		
FM(O)RFa	(P1)	1.82 ± 0.87
FMRFa	(P2)	1.45 ± 0.33
FLRFa	(P3)	3.75 ± 1.45
GDPFLRFa	(P4)	3.58 ± 1.75

Values are expressed as a mean ± SD (from 4 determinations)

Analysis of P2 - Based on the chromatographic behavior of P2 which coeluted with synthetic FMRFa peptide, the hypothesis that P2 is the FMRFa peptide, was confirmed by mass spectrometry measurement (Table II). The molecular mass obtained in mass spectrometry (*m/z* 598.6 ± 0.5 Da) was extremely close to the calculated monoisotopic mass of FMRFa (599.27 Da). These results allowed to conclude that authentic FMRFa is present in the sex SG of mature *E. octoculata*. Its amount was estimated at *ca* 1.5 pmol/ sex SG (Table I).

Table II : Mass spectrometry measurements and deduced sequences of the purified peptides P1, P2, P3 and P4 present in the two sex segmental ganglia of mature *E. octoculata*.

Peptide	Sequence	Calculated monoisotopic mass	*m/z* (mean ± SD)
P1	FM(O)RFa	615.3	614.6 ± 0.14
P2	FMRFa	599.3	598.6 ± 0.5
P3	FLRFa	581.3	580.6 ± 0.6
P4	GDPFLRFa	850.42	849.9 ± 0.35

m/z : mass spectrometry measurement. Each molecular species produced a series of multiply-charged protonated molecular ions from which the molecular mass was determined by simple calculation. Calculation was performed using the multiply-charged ions from a separate introduction of horse heart myoglobin (16,951.4 Da). Molecular masses are given as average values based on the atomic weights of the elements (C = 12.011, H = 1.00794, N = 14.0067, O = 15.9994 and S = 32.06) ; only average masses were measured.

Characterization of the immunoreactive peptide P3

P3 was further purified on a Vydac column. In the two reversed-phase HPLC systems used, this peptide coeluted with synthetic FLRFa, as confirmed by coinjection of P3 with FLRFa on the Vydac column. Since P3 was recognized by an antiserum a-FMRFa specific of RFa, we proposed that P3 is FLRFa. This hypothesis was confirmed by the mass spectrometry measurement (Table II) which indicated a molecular mass of 580.6 ± 0.6 Da for P3, which was in good agreement with the mean of the calculated monoisotopic mass of FLRFa (581.3). The amount of FLRFa determined was *ca* 3.75 pmol/sex SG (Table I).

Characterization of the immunoreactive peptide P4

The amino sequence of P4 obtained by automated Edman degradation was GDPFLRF (Table II). Since P4 was recognized by a-FMRFa, the primary sequence of P4 can be completed to GDPFLRFa. This sequence is in accordance with the mass spectrometry measurement of P4 (Table II) which gave a molecular mass of *m/z* 849.87 ± 0.35, molecular mass very close to the calculated monoisotopic mass of GDPFLRFa (850.42). We can thus conclude on the presence of GDPFLRFa in the sex SG of mature *E. octoculata*. The amount of GDPFLRFa was found to be *ca* 3.5 pmol/sex SG (Table I).

DISCUSSION

Three steps of reversed-phase HPLC separation were needed to isolate four RFa [FM(O)RFa, FMRFa, FLRFa and GDPFLRFa] in the sex SG of mature *E. octoculata*. The carboxyl terminal amidation was confirmed by obtention of the structural proof with mass spectrometry and antibody recognition.

If we compare our results, obtained on the sex SG of *E. octoculata*, with the ones of Evans *et al.* (1991) obtained on the nerve cord of *H. medicinalis*, we can register that two of the four peptides identified in *E. octoculata* [FMRFa and FLRFa] were also identified in *H. medicinalis*. However, in *E. octoculata*, two novel peptides were demonstrated : FM(O)RFa and GDPFLRFa. Four hypotheses may explain this difference of results between *E. octoculata* and *H. medicinalis* : a difference of species, the degree of maturity of the animals, the fact that sex SG were used for *E. octoculata* and whole nerve cords for *H medicinalis* and the different procedures of extraction used.

Concerning FM(O)RFa, data are in favour of the existence of an endogenous peptide : This peptide is also found in other Annelids [*N. diversicolor* (Baratte *et al,.* 1991) and *N. virens* (Krajniak and Price, 1990)] and oxidation of FMRFa peptide could be a process of inactivation of the peptide (Price, 1986). Nevertheless, another possibility is that FM(O)RFa would be generated by an artefactual oxidation in the course of the different purification steps.

Concerning the heptapeptide GDPFLRFa found in the sex SG of *E. octoculata*, it is the first time that such a peptide has been extracted in

leeches. Nevertheless, in Hirudinae, it was known since the works of Norris and Calabrese (1990) that this peptide, although not yet purified in leeches at this time, elicit contractions equal or greater than those induced by FMRFa. With regards to its localization in a specific type of cells, the supernumerary cells of the sex SG, immunoreactive to a-FMRFa in this leech (Salzet *et al.*, 1992b), a role on reproduction can be suggested. Indeed it has to be noted that in the Mollusc *Lymnaea stagnalis*, a role of RFa in egg-laying was found : XDPFLRFa (with X = S or G) acts on the release, by the cerebral caudo dorsal cells, of CDCH, neurohormone that induces egg-laying (ter Maat *et al.*, 1986).

The presence of GDPFLRFa in leeches seems to demonstrate, as suggested by Evans *et al.* (1991), the existence of an ancestral RFa peptide gene common to Annelids and Molluscs. In *L. stagnalis*, the FMRFa precursor contains 9 copies of FMRFa and 2 FLRFa peptides (Linacre *et al.*, 1990). Recently, it has been demonstrated by Saunders *et al.* (1991) that the heptapeptides [GDPFLRFa/SDPFLRFa] isolated by Ebberink *et al.* (1987) in *L. stagnalis* are encoded contiguously by an exon 3' to the FMRFa coding region. In this gene, 7 copies of GDPFLRFa, 6 copies of SDPFLRFa and 3 putative peptides [SDPYLRFa, SDPFFRFa and EFFPLa] were found. A comparison of the amounts of tetra- and heptapeptides detected in *H. medicinalis* by Evans *et al.* (1991) with the amounts of tetra- and heptapeptides detected in *E. octoculata* (Table I), shows that a given peptide was found in equivalent amount in the two species. Moreover, for a given species (either *H.medicinalis* or *E. octoculata*), amounts of tetrapeptides and heptapeptides were equivalent. From that, we can postulate that leeches RFa peptides are located on a same gene, as in *L. stagnalis*. Nevertheless, the genic organization of the leech RFa gene may differ from the one of *L. stagnalis*, the number of copies for each *L. stagnalis* RFa being different. Only a genic study, in leeches, will permit to check this hypothesis.

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Molécules immunoréactives à l'anti-angiotensine II

Articles présentés

(8) Salzet, M., Verger-Bocquet, M., Wattez, C. and Malecha, J., Evidence for angiotensin-like molecules in the central nervous system of the leech *Theromyzon tessulatum* (O. F. M). A possible diuretic effect. *Comp. Biochem. Physiol.*, 101 A, (1992d) 83-90.

(9) Salzet, M., Bulet, P., Wattez, C., Verger-Bocquet, M. and Malecha, J., Angiotensin II-like peptide in the central nervous system of the leech *Erpobdella octoculata.* Localization, isolation, sequence analysis, biological properties and precursors characterization, en préparation 4.

(10) Salzet, M., Wattez, C., Baert, J.L. and Malecha, J., Biochemical evidence of angiotensin II-like peptides and precursors in the brain of the Rhynchobdellid leech *Theromyzon tessulatum.*, en préparation 5.

Compte tenu des résultats relatifs à la cartographie du follicule 4 du ganglion supra-œsophagien chez *T. tessulatum*, mettant en évidence dans ce follicule des cellules immunoréactives à l'AII, une étude précise de ces cellules a été entreprise. Elles constituent un groupe de 4 à 5 neurones caractérisés par leur sécrétion colorable par l'orangé G et classés par Hagadorn *et al.* (1963) dans la catégorie des cellules de type ß. Leur taille, comprise entre 35 et 40 µm, et leur sécrétion, présente sous l'aspect de flaques, nous a conduit à les appeler "cellules à flaques" ou"ß giant cells" (Salzet *et al.*, 1992d).

L'emploi des techniques immunohistochimiques, à la fois sur coupes et *in toto*, a montré que ces cellules unipolaires possèdent un prolongement axonique se dirigeant vers la commissure dorsale où il se dichotomise. Une branche se dirige vers le ganglion sous-œsophagien, l'autre se dirige vers la commissure. Au niveau de l'aire neurohémale, de nombreuses varicosités sont détectées. Celles-ci constituent, soit des synapses (Muller, 1979, Deriemer et Macagno, 1981), soit des zones de rejet des produits de neurosécrétion (Orchard et Webb, 1980). Les observations permettent de supposer, soit un rôle hormonal, soit un rôle de neurotransmetteurs ou neuromodulateurs de la substance AII-like de ces cellules (Salzet *et al.*, 1992d).

Dans le but de mieux appréhender la fonction de ces neurones renfermant une substance apparentée à l'AII (AII-like), des dosages ELISA de l'AII-like, au cours du cycle vital de *T. tessulatum*, ont été réalisés. Ils ont montré une augmentation notable du taux d'AII-like juste après le troisième repas de sang (Salzet *et al.*, 1992d). Cette relation entre le repas de sang et l'augmentation du taux d'AII-like est à rapprocher des travaux de Zerbst-Boroffka (1973), chez la sangsue *Hirudo medicinalis* qui ont mis en évidence une augmentation (x par 8) du volume d'urine excrétée 15 minutes après un repas de sang. Cette excrétion urinaire est due au compactage du sang ingéré au moment du repas par élimination de l'eau et des ions en excès. L'existence d'une substance diurétique avait été suspectée depuis longtemps chez la sangsue mais on ne possédait jusqu'à présent aucune donnée concernant sa nature chimique. La substance AII-like pouvait être une bonne candidate pour ce rôle.

Dans ce contexte, avant d'isoler la molécule apparentée à l'AII reconnue au sein du follicule 4, nous avons d'abord cherché à savoir si l'AII ou ses produits de dégradation pouvaient avoir un effet diurétique chez des *T. tessulatum* en phase de rétention d'eau. Les résultats des injections d'AII, AIII, ou des fragments (1-4) et (5-8) de l'AII, à des*T. tessulatum.* de stade 3B, ont clairement établi que l'AII, l'AIII et le fragment (5-8) de l'AII ont un effet diurétique, alors que le fragment (1-

4) de l'AII est sans effet (Salzet *et al.*, 1992d). Ceci a permis de conforter l'hypothèse qu'une des molécules diurétiques de la sangsue pourrait être apparentée à l'AII des Vertébrés. Compte tenu des résultats immunohistochimiques obtenus après préadsorption de l'a-AII par les fragments de l'AII et de l'effet diurétique de l'AII, et surtout de son fragment 5-8, nous pouvions supposer que l'AII-like et l'AII pouvaient être très proches au niveau structural. Néanmoins, seul l'isolement de cette AII-like chez *T. tessulatum*, pouvait nous permettre de déterminer son pourcentage d'homologie avec l'AII des Vertébrés.

Les dosages ELISA effectués ont montré qu' *E. octoculata* contenait par système nerveux central (SNC) une quantité de peptide AII-like 10 fois supérieure à celle detectée chez *T. tessulatum*. Pour cette raison, nous avons effectué l'isolement de l'AII-like chez *E. octoculata* après avoir vérifié que le comportement chromatographique de cette molécule était semblable chez les 2 sangsues. Quatre étapes chromatographiques ont été nécessaires pour purifier cette substance et son séquençage a abouti à une structure primaire identique à celle de l'AII de Vertébrés : DRVYIHPF. Par contre les résultats de spectrométrie de masse ont révélé un défaut de masse de 1 kDa par rapport à sa masse isotopique moyenne calculée, ce qui établit la présence d'une amidation C-terminale de la molécule. L'effet diurétique de ce peptide purifié a pu être démontré chez des*T. tessulatum* en phase de rétention d'eau. L'ensemble de ces résultats a permis de conclure que la molécule apparentée à l'AII chez *E. octoculata* est une AII amide (AIIa) de structure primaire DRVYIHPFamide à activité diurétique (Salzet *et al.*, en préparation 4).

Bien que l'effet diurétique de la molécule isolée d'*E. octoculata* ait été vérifié chez *T. tessulatum* , il fallait confirmer nos résultats préliminaires montrant que la molécule d'AII-like était la même chez *T. tessulatum* et *E. octoculata.*

Chez *T. tessulatum*, après séparation chromatographique, 3 peptides apparentés à l'AII ont été isolés au niveau du système nerveux central (Salzet *et al.*, en préparation 5). L'un co-migre avec l'AIIa précédemment isolée, les 2 autres présentent un temps de rétention plus faible, traduisant un caractère biochimique différent. Ces 2 derniers pourraient être des sous-produits issus de la dégradation du composé le plus hydrophobe. Cette hypothèse est étayée par la spécificité de l'anticorps , celui-ci étant dirigé contre la partie C-terminale de l'AII, par la co-migration de ces 2 peptides avec respectivement l'AIV et l'AVII de Vertébrés (produits de dégradation de l'AII), par les résultats des dosages ELISA et par l'obtention récente de la séquence du pic le moins hydrophobe (séquence : HPF) qui correspond bien à l'AVII (Salzet *et al.*, inédit). Ces résultats ont permis de

conclure à l'existence chez *T. tessulatum* d'une molécule proche de l'AIIa, ainsi qu'à la présence de produits de dégradation de cette molécule. Ces derniers résultats suggèrent la possibilité de l'existence chez *T. tessulatum* d'un système enzymatique comparable à celui des Vertébrés.

A l'inverse, l'étude du précurseur de l'AII-like chez *T. tessulatum* et *E. octoculata*, a révélé des différences significatives entre ces 2 espèces et également entre ces 2 espèces et les Vertébrés. En effet, chez *E. octoculata*, la pro-AII amide est présente sous 2 formes *i.e.* une protéine de *ca* 18 kDa et une autre de *ca* 14 kDa (Salzet *et al.*, en préparation 4). Par contre, chez *T. tessulatum*, une seule protéine de *ca* 18 kDa a été détectée (Salzet *et al.*, en préparation 5). La caractérisation des Pré-Pro-AII amide a permis de mettre en évidence 2 protéines de respectivement *ca* 19 kDa et *ca* 15 kDa chez *E. octoculata* et une seule protéine de *ca* 19 kDa chez *T. tessulatum*. La présence de 2 protéines chez *E. octoculata*, laisse supposer l'existence soit de 2 ARN messagers codant pour l'AII amide, soit d'une dégradation de la protéine de *ca* 19 kDa en une protéine de *ca* 15 kDa. Seules des études en présence d'inhibiteurs de protéases et en Northern blot pourront lever cette ambiguïté.

L'étude des précurseurs de l'AIIa a montré une différence de masse entre la Pro-AIIa et la Pré-Pro-AIIa. Ce résultat est confirmé par ceux obtenus en C3 CLHP. L'existence d'un peptide signal additionné à la pro-hormone est probable.

Trois épitopes, reconnus respectivement par l'a-AII, l'a-γ-MSH et le Tt159, sont colocalisés au sein des "ß giant cells" (Verger-Bocquet *et al.*, 1992). Nous avons cherché à savoir si ces épitopes sont portés par le même précurseur. La réponse est positive. Les 3 anticorps reconnaissent la Pro-AIIa chez *T. tessulatum* (Salzet *et al.*, en préparation 5). Nous sommes donc en présence, comme dans le cas du Pro-FMRFamide des Mollusques *Aplysia californica* (Taussing and Scheller, 1986) ET *Lymnaea stagnalis* (Linacre *et al.*, 1990), d'un précurseur multipeptidique.

La purification des épitopes reconnus par l'a-γ MSH et par le Tt 159, a été entreprise. Deux substances immunoréactives à la γ MSH ont été purifiées. La moins hydrophobe a pour séquence primaire XFRW (X = H, V, ou A) (Salzet *et al.*, inédit), séquence commune à toutes les MSH; l'autre est en cours de séquençage. L'existence d'αMSH a été signalée chez le Mollusque *A. californica* (Taussing and Scheller, 1986) et chez l'Insecte *Locusta migratoria* (Schoof *et al.*, 1988). Chez *A. californica,* l'α MSH serait portée par le précurseur du FMRFamide. L'α-MSH interagit sur l'ocytocine pour le contrôle de l'équilibre hydrominéral chez les Vertébrés (Howe et Ray, 1985 ; Wesley et Gilmore, 1985).

Nous n'avons par contre aucune donnée sur le rôle de cette molécule chez les sangsues.

L'étude du précurseur de l'AII-like des Hirudinées fournit des résultats différents de ceux connus chez les Vertébrés. En effet chez ces derniers, l'angiotensinogène (Ao) est une protéine globulaire de 60 kDa non multi-peptidique. Deux hypothèses peuvent être émises pour expliquer cette différence :

- l'identité de structure primaire entre l'AII des sangsues et celle des Vertébrés résulte d'une simple coïncidence. Dans ce cas ces molécules proviendraient de précurseurs codés par des gènes n'ayant aucune parenté entre eux.

- l'AII des sangsues et celle des Vertébrés sont issues d'une même protéine ancestrale codée par un même gène. Dans ce cas, ce gène aurait évolué d'une façon très divergente chez les Invertébrés et les Vertébrés au cours de l'évolution.

Compte tenu de la longueur de la séquence de l'AIIa et du maintien probable de son rôle physiologique, la seconde hypothèse semble la plus vraisemblable.

La connaissance du gène codant ce neuropeptide chez les Hirudinées reste indispensable pour trancher en faveur de l'une de ces deux hypothèses.

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EVIDENCE FOR ANGIOTENSIN-LIKE MOLECULES IN THE CENTRAL NERVOUS SYSTEM OF THE LEECH *THEROMYZON TESSULATUM* (O.F.M.). A POSSIBLE DIURETIC EFFECT

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Abstract—1. Cells in the central nervous system of the leech *Theromyzon tessulatum* were revealed with an antiserum against angiotensin II. Among these cells, a group of 4–5 pairs of neurons, called β giant cells, and located in the posterior compartments of the supraesophageal ganglion was particularly investigated.

2. The amount of angiotensin II-like substance(s) in the brain increased notably in the days immediately following the third meal.

3. Injections of angiotensin II, fragments 1–4 or 5–8 of angiotensin II and of angiotensin III into stage 3 leeches showed that fragment 5–8 of angiotensin II was the most effective: it provokes a loss of mass of the leeches, which could express a diuretic effect.

INTRODUCTION

In the semelparous leech *Theromyzon tessulatum*, genital maturity coincides with a period of water retention (Malecha, 1979, 1983). This retention is controlled by an antidiuretic factor secreted by neurons of the posterior compartments of the supraesophageal ganglion and more likely by those of the paramedial ones (compartment 4) (Malecha, 1983). The biochemically homologous cells constituting compartment 4 were detected immunocytochemically with antibodies against vertebrate peptide hormones (Malecha *et al.*, 1986; Verger-Bocquet *et al.*, 1986, 1988). These molecules generally have a very ancient origin (Scharrer, 1978) and their physiological activity is sometimes maintained during evolution (Mühlethaler *et al.*, 1984; Vincent and Simmonet, 1986; Proux *et al.*, 1987; Veenstra, 1988). Among the substances demonstrated in the supraesophageal ganglion of *T. tessulatum*, some are immunologically related to vasopressin, oxytocin and angiotensin, i.e. substances involved in vertebrate hydromineral homeostasis. An experimental study concerning the physiological role of oxytocin and vasopressin was undertaken in *T. tessulatum* by injecting the animals either with the antibody directed against them or with synthetic mammalian hormones. Lysin- and arginin-vasopressin are ineffective (Malecha *et al.*, 1989a), which is in line with results obtained in *Hirudo medicinalis* (Zerbst-Boroffka and Wenning, 1986), whereas the injection of anti-oxytocin provoked a loss of mass equivalent to the one registered after supraesophageal ganglion removal. The antidiuretic hormone of *T. tessulatum* thus could be structurally related to mammalian oxytocin without nevertheless being identical; the injection of this hormone to leeches being ineffective (Malecha *et al.*, 1989a). On the other hand, apart from some immunocytochemical data, little is hitherto known about the angiotensin-like substance in *T. tessulatum*. The main objectives of the present investigation were to collect data on the cells containing angiotensin-like material and to investigate the eventual role of the angiotensin-like substance(s) in osmoregulation.

MATERIALS AND METHODS

Animals

T. tessulatum used in this study were bred in the laboratory. The different stages of the life of this animal were defined by taking as indicators the three blood meals (Malecha *et al.*, 1989b). In short, the food intakes define the following stages:

—Stage 0: from hatching to the first meal;
—Stage 1: after the first meal;
—Stage 2: after the second meal;
—Stage 3: after the third meal.

Generally, sexual maturity occurs after the third meal.

Electron microscopy

Supraesophageal ganglia were fixed for 3 hr at 4°C in phosphate-buffered glutaraldehyde (3%). After washing overnight with buffer, they were post-fixed in 1% osmium-tetroxide in the same buffer at room temperature for 1 hr and embedded in araldite. Ultra-thin sections contrasted with uranyl acetate and lead citrate (Reynolds, 1963) were examined with a JEOL JEM 100 CX electron microscope.

Antiserum

The antiserum against angiotensin II (AII) used in this study was kindly provided by Dr G. Tramu (University of Bordeaux, France). It was obtained by immunizing rabbits with human synthetic AII coupled to human serum albumin via glutaraldehyde (Aguirre *et al.*, 1989).

Immunohistochemical procedures

Both sectioned material and whole-mounts were employed. Immunohistochemical controls for the specificity of staining were performed by incubation of the primary antiserum with synthetic AII (Sigma Chemical Co., St Louis, MO, U.S.A.) prior to application.

Sections. Anterior parts of leeches at stage 2 or 3 were fixed overnight at 4°C in Bouin Hollande fixative supplemented with 10% mercuric chloride. Paraffin sections (7 μm) were subjected to an indirect immunoperoxidase method described elsewhere (Verger-Bocquet *et al.*, 1987).

Whole-mounts. Immunocytology of whole-mounts was carried out using a method derived from that followed by Pearson and Lloyd (1989) and Shankland and Martindale (1989). All steps were performed at 4°C with gentle agitation. Central nervous systems of leeches at stage 3 were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.2 for 1 hr. Specimens to be processed for immunofluorescence were then rinsed in three changes of 0.1 M PBS pH 7.2 containing 1% Triton X 100 (PB-T) over 6 hr, dehydrated to 80% ethanol (50%, 60%, 70%, 5 min/step) and washed briefly with PB-T (3 × 5 min). After incubation with primary antibody solution [anti-AII, diluted 1:500 with PBS containing 1% Triton X 100 and 1% normal goat serum (PB + T + NGS)] for 36 hr, the material was then washed in three changes of PB + T for 5–8 hr and incubated in the secondary solution (FITC-labelled goat anti-rabbit IgG (Sigma), diluted 1:100 with (PB + T + NGS) overnight. Material was then washed for 18 hr in PB + T, washed for an additional 5 hr in PBS and mounted in buffered glycerol (pH 9.0) containing 4% *n*-pyrogallate. Whole-mounts were examined and photographed under UV fluorescence with a Zeiss Axioskop microscope.

ELISA assay procedure

ELISA was performed on 96 well maxisorp microtiter plates (Nunc, Sweden).

Preparation of brains. One hundred brains were dissected, homogenized in 200 μl 1 N acetic acid at 4°C, sonicated and then centrifuged 15 min at 4°C at 15,000 rpm. The pellet was re-extracted as above, the supernatants combined and immediately used.

Antigen coating. Antigens (brains taken at different stages of the animal's life cycle or synthetic AII) were diluted in carbonate buffer (0.1 M, pH 9.6) to obtain the equivalent of 30 brains or of 10 pg–100 ng of synthetic AII (Sigma) per well. Aliquots of each (100 μl) were added to wells. After incubation (3 hr) at 37°C, the non-specific sites were blocked by the addition of PBS (0.01 M, pH 7.4) containing 2% BSA (1 hr at 37°C, 200 μl/well). After being treated four times with washing solution (PBS containing 0.1% BSA + 0.05% Tween 20), plates were stored at 4°C until use.

First antibody. The antiserum against AII diluted 1:1000 in PBS, preadsorbed (100 μg/ml undiluted serum) or not with AII, was distributed in duplicates (100 μl/well) and incubated at 37°C for 2 hr. Wells were then washed four times with washing solution.

Secondary antibody. Peroxidase-conjugated goat anti-rabbit IgG (Pasteur Diagnostics) was diluted 1:10,000 with PBS. One hundred microliter were added to each well and incubated at 37°C for 2 hr. Wells were then washed four times with washing solution.

Enzyme reaction. Plates were treated for 10 min with *O*-phenylenediamine (0.5 mg/ml) in citrate buffer pH 5.5 with 0.05% H_2O_2 (100 μl/well). Colour development was stopped by adding 1 N HCl (100 μl/well). Plates were read on an Immunoreader MR 700 (DYNATECH) at 490 nm.

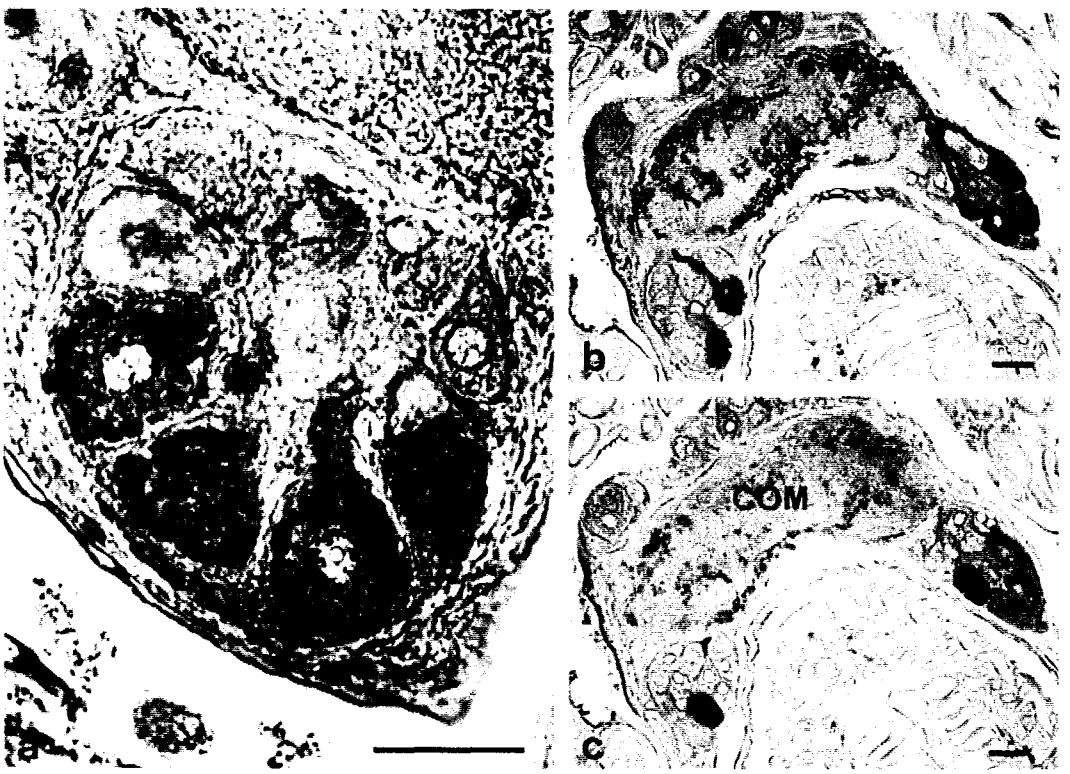

Fig. 1. Immunoperoxidase preparations of frontal sections through compartment 4 of the supraesophageal ganglion of *T. tessulatum*. (a) Anti-angiotensin II (a-AII) immunoreactive β giant cells. (b) (c) Adjacent sections treated either with a-AII (b) or with a-AII preadsorbed with AII (c). β giant cells and numerous fibres in the dorsal commissure stained positively for AII (b). The a-AII staining capacity is abolished after adsorption with AII (c). COM: dorsal commissure. Bar equals 30 μm.

In vivo experiments. Leeches (stage 3), which had taken their third meal on the same day, were used. Injections were in the subepidermal connective tissue. Products (AII, AII(1–4), AII(5–8), AIII) were dissolved in a leech Ringer (Muller *et al.*, 1981). Each animal received 10 μl of a solution of 1 nmol ml. Control animals received Ringer. The effect of the treatment was estimated by measuring the change of body mass of the animals between the beginning and the end of the experiment. Animals were blotted on tissue paper and weighed to the nearest 0.1 mg.

Statistical analysis of results was performed by calculating the mass variation of each leech at t instant compared to the initial mass. Confidence interval (with $\alpha = 0.05$) of the mean of the relative variations of mass was obtained according to Scherrer(1984).

RESULTS

Immunohistochemical study

This investigation done both on sectioned material (Fig. 1) and on whole-mounts (Fig. 2) shows that the supraesophageal ganglion contains about 18 cells which could be stained with AII antiserum. The

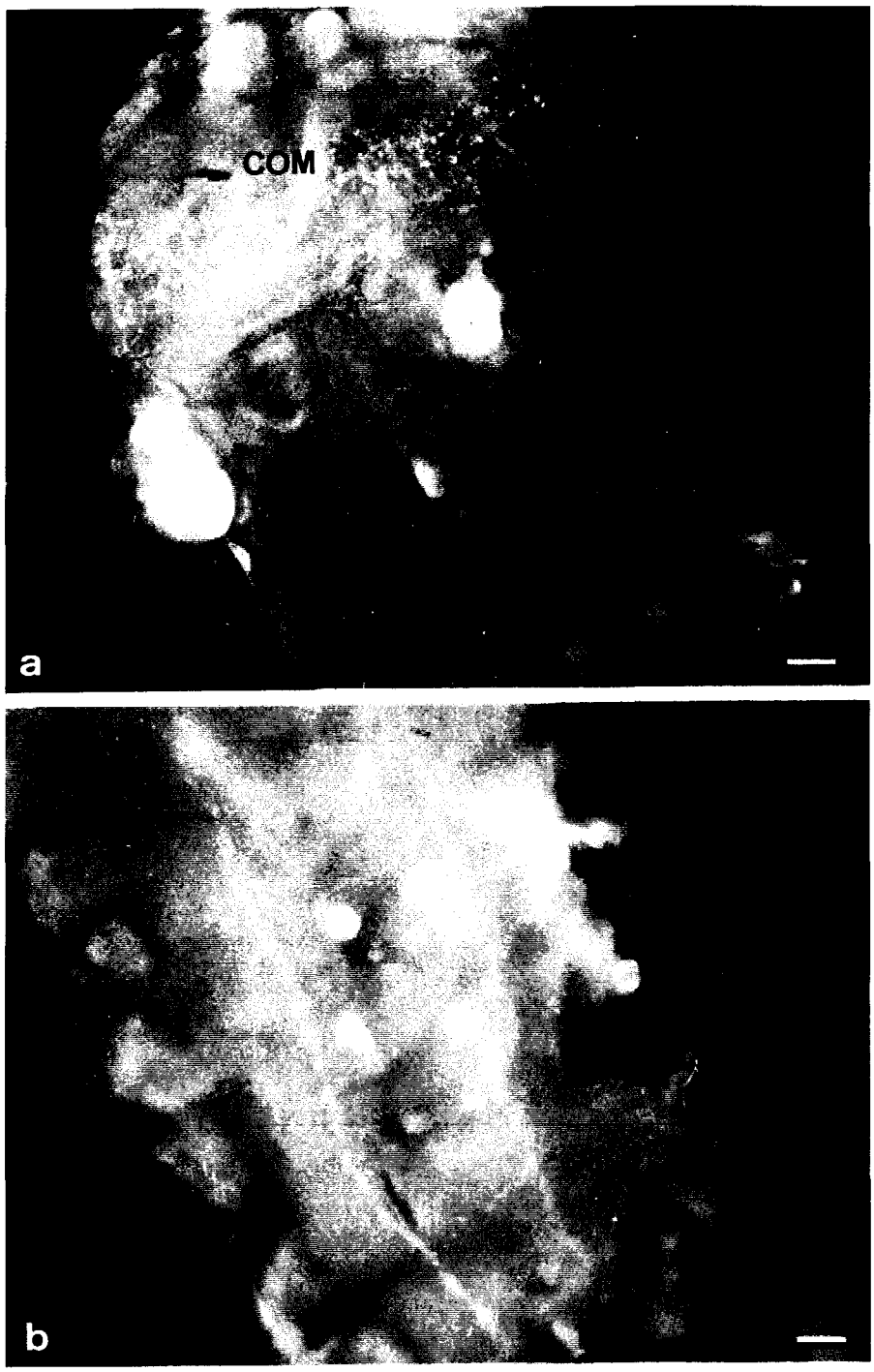

Fig. 2(a–b)

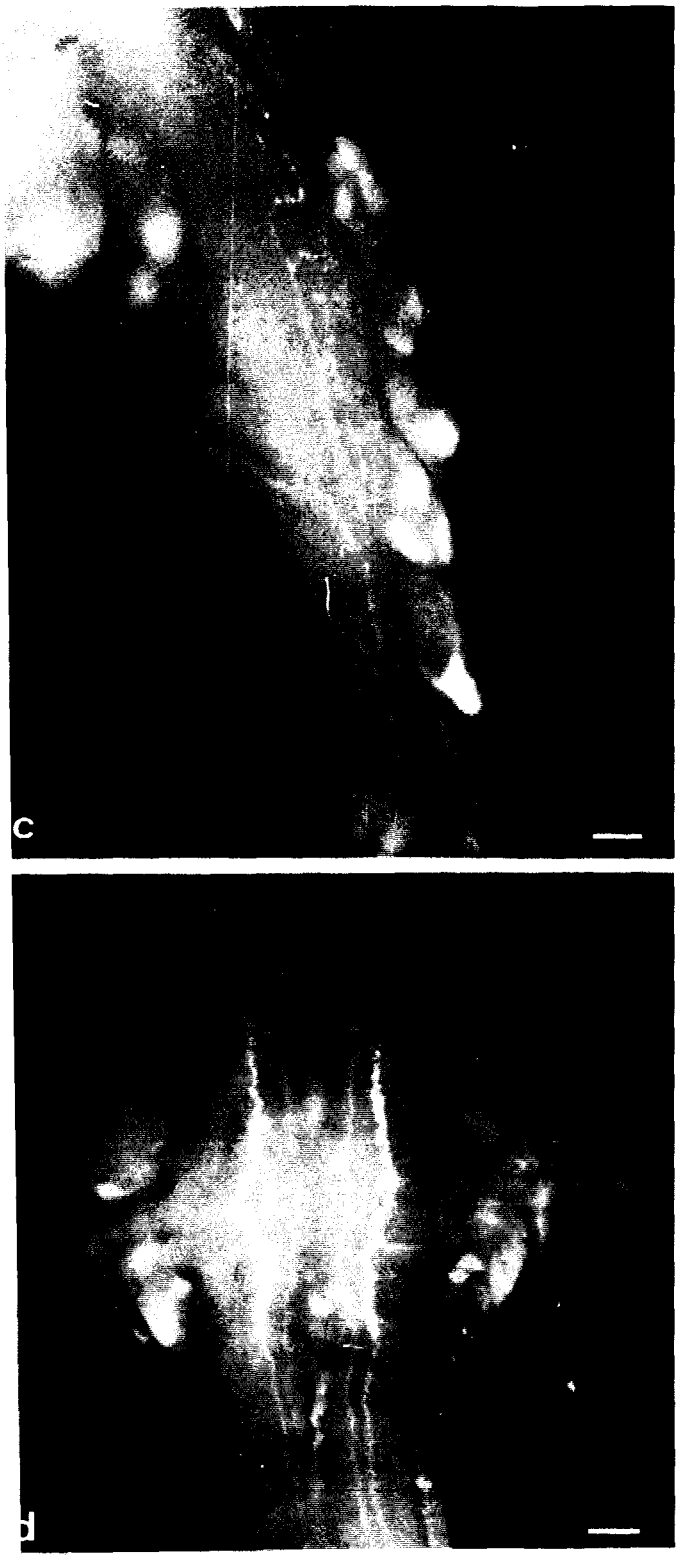

Fig. 2(c–d)

Fig. 2. Immunohistochemical fluorescent micrographs of whole-mount preparations of central nervous systems of *T. tessulatum* treated with anti-angiotensin II. In all preparations, anterior is to the top. (a) Dorsal view of the supraesophageal ganglion. Immunoreactivity is localized in the posterior compartments at the level of the β giant cells and in the dorsal commissure. COM: dorsal commissure. (b) Dorsal view of the subesophageal ganglion. Four cells were strongly labelled in the medial compartments. (c) Lateral view of the subesophageal ganglion (ventral is to the right). Immunoreactive cells are located in the medial compartments. Note also the presence of immunoreactive axonal tracts. (d) Dorsal view of a segmental ganglion. Immunoreactive processes are visible within the neuropile. Bar equals 50 μm.

Table 1. Immunohistochemical specificity tests of the antiserum to angiotensin II (a-AII) used to stain the β giant cells of compartment 4 of the supraesophageal ganglion

Staining reaction with a-AII (1:300)	
Blocked by	Unaffected by
AII: 350 nmol/ml	AII(1–4): 1 μmol/ml
AIII: 350 nmol/ml	AII(5–8): 1 μmol/ml
	AII(1–4) + AII(5–8): 1 μmol/ml

AII: angiotensin II, AIII: angiotensin III, AII(1–4) and AII(5–8): fragments 1–4 and 5–8 of AII. Incubation with the different peptides (at a concentration indicated in nmol or in μmol/ml of undiluted serum) are at 4°C for 6 hr.

presence of 2–4 cells in the anterior compartments (1, 2 and 3) was inconsistent. Four or five pairs of immunoreactive cells were always observed in compartment 4 (Fig. 1a, b). Other immunoreactive cells were sometimes detected in the posterior part of the supraesophageal ganglion. The immunoreactive cells figured in compartment four were remarkable with their great size (30–40 μm) and their secretion typically dispersed in masses at the periphery of the cell body (Fig. 1a). They correspond to the β giant cells described in *Theromyzon rude* by Hagadorn (1958). The process which arose from each of these neurons divided into two branches as it reached the dorsal commissure. One branch ramified there abundantly and formed many varicosities (Fig. 2a). The other ran to the periesophageal connectives and extended in the ventral commissure. The processes coming from the β giant cells constituted two parallel cords which extended into the interganglionic connectives of the ventral nerve cord where they received fibres coming from neurons of the subesophageal (Fig. 2b) and segmental ganglia.

The subesophageal ganglion contained about 15 small cells containing AII-like material. Among the immunoreactive cells situated in the medial compartments (Fig. 2c) two pairs (Fig. 2b) showed strong immunolabelling.

Six to eight angiotensin-like immunoreactive cells were detected in each segmental ganglion of the ventral nerve cord. Their commissure was an area of neuritic arborization (Fig. 2d).

Preadsorption of the antiserum against AII with AII (Fig. 1c) or AIII abolished the β giant cells labelling. On the other hand, the positive staining was not quenched when the antiserum against AII was preadsorbed with AII(1–4) or AII(5–8) or with their association (Table 1).

Ultrastructural characteristics of the β *giant cells of compartment 4 of the supraesophageal ganglion*

Their secretion consisted of moderately electron-dense ovoid granules of a size *ca* 220 nm × 140 nm (Fig. 3a, b).

Injections of synthetic mammalian peptides

Results are presented in Figs 4 and 5. A loss of mass in the controls injected with the physiological saline used to dissolve the peptide was regularly registered (Malecha *et al.*, 1989a). It is due to successive blotting required during the weighing of the animals.

The weight changes of the leeches injected with AII(1–4) was closed to the one of the controls. The series injected with AII, AIII and AII(5–8) showed a significant decrease of mass. The maximum response was found with AII(5–8) (Fig. 4). In order to further

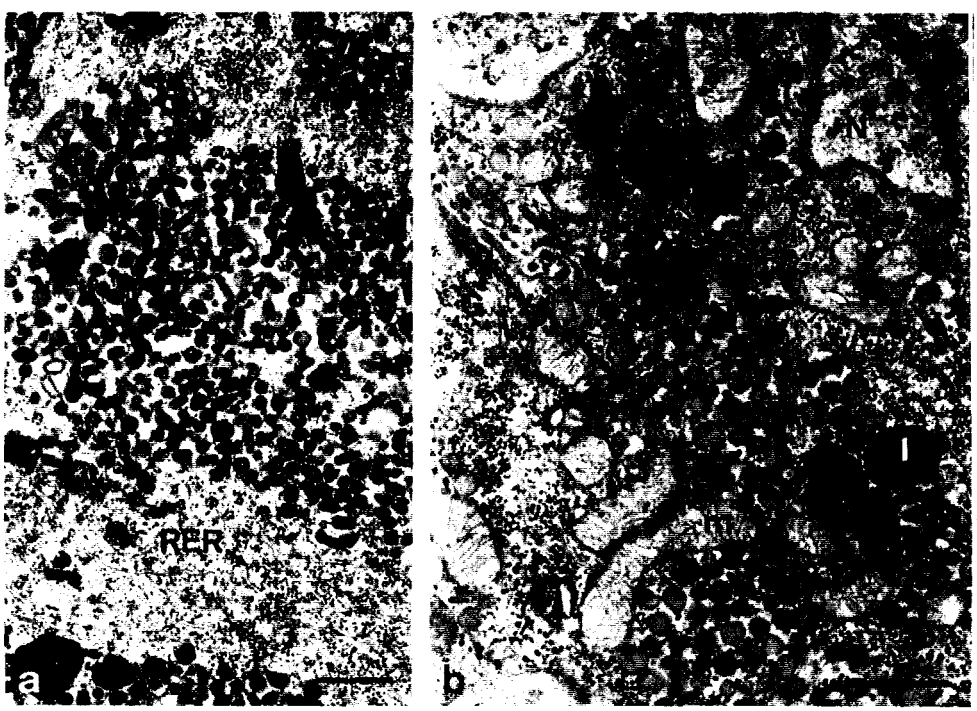

Fig. 3. Electron micrographs of the electron-dense ovoid granules in the perikaryon of β giant cells of compartment 4 of the supraesophageal ganglion (a and b). Bar equals 0.5 μm. G: Golgi apparatus; l: lysosome; m: mitochondrion; N: nucleus; RER: rough endoplasmic reticulum.

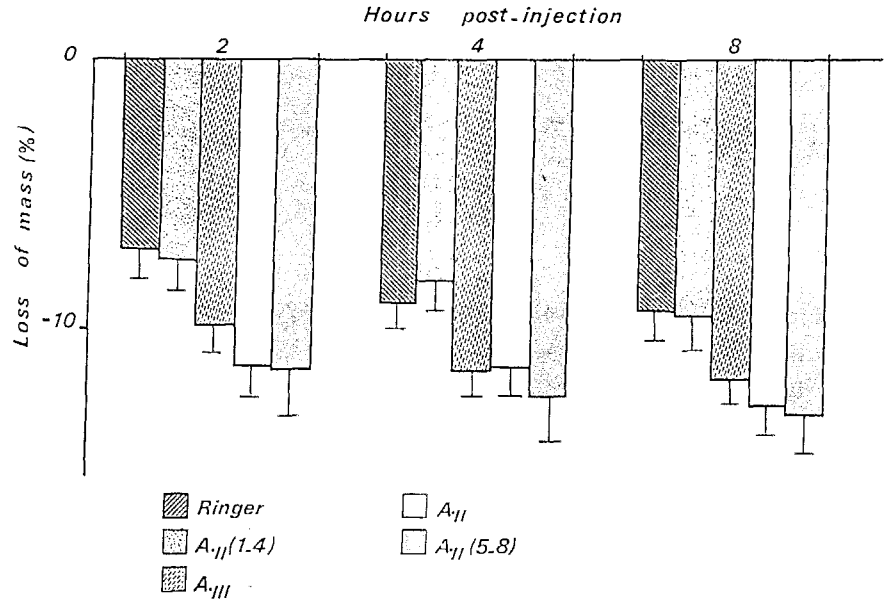

Fig. 4. Effect of the injection of angiotensins (1 nmol ml) upon osmoregulation of *T. tessulatum*: mean percentual loss of mass at different times after the start of the experiment. Each mean value and SD is based on 20 injected animals.

investigate its activity, leeches were injected with varying amounts of AII(5–8) (Fig. 5). The most efficient concentration was of 0.8 nmol/ml. A lower one (0.16 nmol/ml) was ineffective. The highest concentrations assayed (4, 20 and 100 nmol/ml) did not provoke significant differences compared to the controls.

Amounts of angiotensin-like substances with enzyme-linked immunosorbent assay (ELISA) (Fig. 6)

Assays done at stage 2, prior to the third and last meal of blood showed the presence of *ca* 10 pg of AII per brain. The amount of peptide increased noticeably in the days immediately following this meal (15 pg per brain) and remained unchanged at *ca* 12 pg per brain until the end of stage 3.

DISCUSSION

In *T. tessulatum*, the immunoreactivity to an antiserum against AII of a population of neurons distributed in the whole central nervous system seemed to be linked to the presence of a substance chemically very close to AII or AIII. The reactivity of the

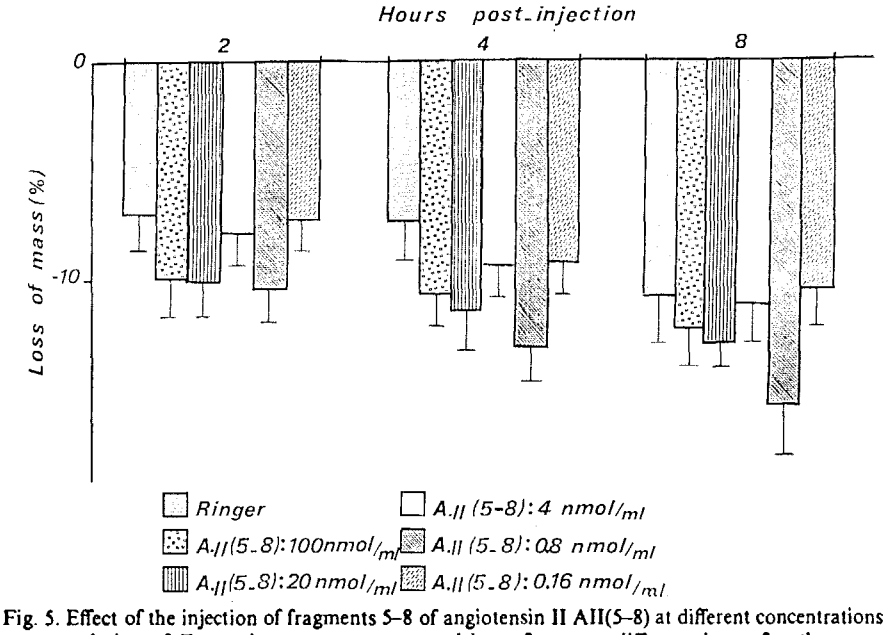

Fig. 5. Effect of the injection of fragments 5–8 of angiotensin II AII(5–8) at different concentrations upon osmoregulation of *T. tessulatum*: mean percentual loss of mass at different times after the start of the experiment. Each mean value and SD is based on 40 injected animals.

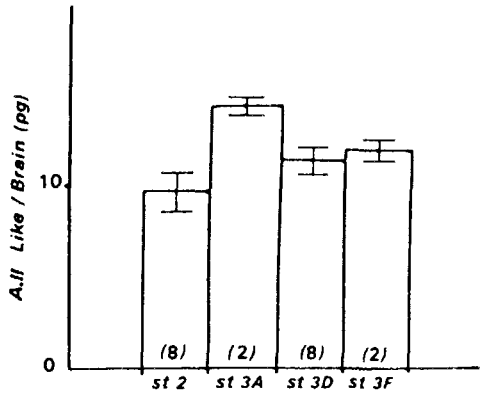

Fig. 6. Mean amounts and SD (pg/brain) of AII-like substance(s) in *T. tessulatum* at different stages (st) of the life cycle. The number of determinations is given between brackets. Values are the result of the difference between the value obtained with the antiserum against AII and that obtained with the antiserum against AII preadsorbed with AII. St 2: after the second meal. St 3A: immediately after the third meal. St 3D: yolk accumulation in eggs. St 3F: before death.

antiserum after saturation by fragments 1–4 + 5–8 of AII could be due to the existence of a binding site depending upon the conformation of the molecule. In the supraesophageal ganglion, 4–5 pairs of neurons located in the posterior compartments were remarkable for their great size (30–40 μm), hence their name of giant cells,and the special aspect of their secretion in light microscopy. This histological appearance has not been found in any other cell of the central nervous system and could be related to the different embryological origin of the brain and of the segmental ganglia (Weisblat *et al.*, 1980, 1984). These giant cells are unipolar neurons whose cellular process divides in the dorsal commissure into two branches. One branch abundantly ramifies at the level of the neurohaemal area located between compartments 4, close to a large blood vessel (Hagadorn, 1958; Webb, 1980). The numerous varicosities have been considered either as synapses (Muller, 1979; Deriemer and Macagno, 1981) or as zones of release of the neuroscecretory product (Orchard and Webb, 1980). The other branch runs to the subesophageal ganglion and its course has to be investigated. The axonal process of the giant cells is very similar to that of neurosecretory cells in another leech, *Macrobdella decora* (Orchard and Webb, 1980).

The peptidic secretion could be released into the circulatory system at the neurohaemal site and exert an hormonal role. Alternatively, an action as neurotransmitter or neuromodulator at the level of neuron endings located in the dorsal commissure is possible.

The secretion of the β giant cells of compartment 4 of the supraesophageal ganglion was of a single type of moderately electron-dense granules, more or less ovoid (140 × 220 nm). A comparison of our findings with those from Hagadorn *et al.* (1963), in a related species (*Theromyzon rude*) is difficult. Indeed, an improved preservation of the structures due to the evolution of techniques has allowed us to demonstrate more cell types in compartment 4 than the four cell types described by Hagadorn. No homology was found with the neurosecretory granules observed by Webb and Orchard (1979) and Webb (1980) in *M. decora*.

The small neurons of the segmental ganglia arborize extensively throughout the neuropile. With the technique used, cellular processes were never detected in the roots which leads us to suppose that they would consist essentially of interneurons rather than sensory or effectory neurons.

The injection of AII, of AII(5–8) and of AIII resulted in a decrease of mass expressing a diuretic effect of these molecules. The fragment 5–8 of AII at a very low concentration (0.8 nmol/ml) seems to be the most effective. The existence of a diuretic hormone in Hirudinea has been suspected for a long time. In *Hirudo medicinalis* an 8-fold increase of the urine volume excreted was registered 15 min after a blood meal and persisted for many hours (Zerbst-Boroffka, 1973). No proof for the existence of a diuretic hormone has been brought till now (Zerbst-Boroffka and Wenning, 1986). The angiotensin-like substance(s) could fulfil this role since the amounts found in the brain immediately after the third meal (stage 3A) were clearly higher than those found during the rest of stage 3.

The possible diuretic role of angiotensin must be examined carefully. The results of the injections of synthetic angiotensins were indeed in contradiction to the finding that the removal of the compartments of the brain in which the β giant cells reside provoked a decrease of mass (Malecha, 1983) and that a passive immunization of leeches by injection of anti-AII was ineffective (Malecha *et al.*, 1989a). These contradictory results could be explained either by the existence in *T. tessulatum* of several different substances with a diuretic function as demonstrated for example in the locust (Phillips, 1983) or by an indirect action of the injected peptides on diuresis. In vertebrates, it is well known that the injection of AII causes the secretion of catecholamine (Carroll and Opdyke, 1982), vasopressin (Keil *et al.*, 1975) or vasotocin (Reboreda and Segura, 1989). The diuretic effect observed in *T. tessulatum* could be due either to the stimulation or to the inhibition of the secretion of a biologically active factor. The diuretic effect of angiotensin could also result from the competition for degradative enzymes exerted by the injected peptides towards the biologically active endogenous peptides (La Bella *et al.*, 1985).

The exact role of the angiotensin-like substance(s) of *T. tessulatum* can only be examined when the endogenous molecule(s) will be chemically known.

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Angiotensin II-like peptide in the central nervous system of the leech *Erpobdella octoculata.* Localization, isolation, sequence analysis, biological properties and precursors characterization

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Abstract

Cells immunoreactive to an antibody directed against angiotensin II (a-AII) were detected in the central nervous system (CNS) of the pharyngobdellid leech, *Erpobdella octoculata*. The isolation of the angiotensin II-like (AII-like) peptide from this CNS, which is the first one reported in an Invertebrate, was performed by reversed-phase HPLC. The automated gas-phase sequencing revealed a primary sequence identical to the one of Vertebrate AII. However, mass spectrometry established a C-terminal amidation in the peptide. The isolated peptide, so called AIIamide, exerted a diuretic effect when injected in the leech used for the bioassay, *Theromyzon tessulatum*. The AIIamide precursors, proteins of *ca* 15 and 19 kDa, were detected both in CNS extracts and in CNS translated products.

Key Words : Angiotensin II amide/ Angiotensin II-like precursors/ Diuretic effect/ Leeches

Introduction

Angiotensin II (AII), discovered in Vertebrates by Page and Helmer[1], is composed of 8 amino acid residues and is produced by a chain of reactions from a precursor, the angiotensinogen (Ao)[for a review, see Saavedra[2]]. Besides its presence in the kidney, evidence for its existence has also been reported in the brain[3]. Moreover, recent works have demonstrated in Vertebrate brain the presence and the expression of both Ao mRNA[3] and renin[4], enzyme which cleaves Ao at a single site located between amino acids 10 and 11, to produce the inactive decapeptide, the angiotensin I (AI) which will be then cleaved in AII by the angiotensin-converting enzyme[5]. The roles of Vertebrate cerebral AII are multiple. It stimulates the secretion of catecholamine[6], vasopressin[7] or vasotocin[8]. Its involvement in the control of osmoregulation is well established in mammalians[9, 10] and non-mammalian Vertebrates[8, 11, 12]. This latest action of AII was also observed in an Invertebrate, the freshwater leech *Theromyzon tessulatum* [13]. In *T. tessulatum* as in other leeches, the internal medium is hypertonic in comparison to the environment, which leads to a constant osmotic inflow of water compensated by the excretory activity of nephridia. In this animal, hydric balance is controlled by two categories of hormones : anti-diuretic ones and diuretic ones. Some periods of the life cycle of *T. tessulatum* are characterized either by an increased diuresis or by a water retention. Indeed, an increased diuresis occurs within the hours following a blood meal, this in order to eliminate water and ions in excess of the ingested blood and thus to concentrate red blood corpuscles [14]. One of the substances involved in this physiological process could be AII [13]. On the other hand, a water retention is observed during the phasis of sexual maturation[15]. This phenomenon seems necessary to the accumulation of vitellogenins in the cœlomic fluid[16, 17] and is controlled by an antidiuretic hormone[15].

In *T. tessulatum*, immunoreactivities to an antibody raised against AII (a-AII) were detected in the posterior paramedial compartments of the supraesophageal ganglion. The immunoreactive cells presented characteristics of neurosecretory cells, and an epitope close to AII or angiotensin III (AIII) was demonstrated[13]. The ELISA dosages done at different physiological stages of the life cycle of *T. tessulatum*, revealed an increase in the cerebral AII-like substance amount just after a blood meal[13]. Moreover, injections of AII, AIII, fragment (5-8) of AII in *T. tessulatum*, in water retention phasis, indicated a diuretic effect of these synthetic peptides[13]. These results allowed to speculate that the AII-like peptide is close to Vertebrate AII. Amounts of AII-like detected

in *T. tessulatum* are low (*ca* 10 fmol/brain)[13]. In order to find a species possessing higher amounts of AII-like material, we assayed a very common leech, *Erpobdella octoculata*, which was found suitable to isolate the AII-like substance.

Leeches constitute a very homogenous group in which the characteristics of the segmental ganglia are specially well conserved from one species to another[18]. From this, we could emit the hypothesis that we will not find fundamental differences between the neuropeptides isolated in *E. octoculata* and *T. tessulatum*. Moreover, a preliminary investigation in reversed-phase HPLC indicated that chromatographic behaviors of the *T. tessulatum* AII-like peptide and *E. octoculata* AII-like peptide are identical (Salzet *et al*,. in preparation).

This paper, which investigates the AII-like substance of the Pharyngobdellid leech *Erpobdella octoculata*, deals with :

- the localization in the CNS of cells immunoreactive to a-AII.
- the purification and sequencing of the AII-like peptide.
- a preliminary characterization of the AII-like precursors
- a demonstration of the diuretic effect of the purified AII-like peptide.

Material and Methods

Animals

E. octoculata collected at Harchies (Belgium), were used for the biochemical purification.

Dissections

After anaesthetization in 0.01 % chloretone, animals were pinned out, dorsal side up, in leech Ringer[18]. Central nervous systems (CNS), consisting of the brain and of a fragment of nerve cord including segmental ganglia 1-8, were dissected, frozen at -180°C in nitrogen and finally stored at -20°C until use for biochemical purification.

Chemicals

3-3'-diaminobenzidine-tetra-hydrochloride, (DAB), 4-chloro-1-naphtol, hydrogen peroxide, synthetic peptide (AII) and FITC-labeled goat anti rabbit IgG were obtained from Sigma, the trifluoroacetic acid, sequencer grade from Pierce, the peroxidase-conjugated goat anti-rabbit IgG from Pasteur Diagnostics and protein A-sepharose from Pharmacia. All organic solvents were HPLC

grade and purchased from Merck. Deionized water was obtained from a Milli-Q system (Millipore).

Antibody

The polyclonal antiserum against angiotensin II (a-AII) used in this study and previously characterized by Aguirre *et al.*[19], was kindly provided by Dr G. Tramu (Laboratoire de Neurocytochimie fonctionnelle, Université de Bordeaux I, Talence, France).

Immunocytochemistry

Whole-mounts of CNS were treated according to Salzet *et al.*[13]. Primary antibody (a-AII) was diluted at 1:500 ; FITC-labeled goat anti-rabbit IgG was used at a dilution of 1:200. Whole-mounts were examined with a Zeiss Axioskop fluorescence microscope.

Immunoassays

a - ELISA procedures

Immunoassays were conducted according to procedures described elsewhere[20] with a-AII used at a dilution of 1:1000.

b - Dot immunoassay (DIA) procedure

An aliquot of 1 µl of HPLC fractions was spotted onto a nitrocellulose membrane (0.45 µm pore size, Schleicher and Schuell) which was then baked 30 min at 110°C. The membrane was blocked, under gentle agitation at room temperature, 1 hr with PBS (50 mM Phosphate buffer, 150 mM Sodium chloride, pH 7.4) including 0.05 % Tween 20 and 5 % Skilm milk and then incubated overnight at 4°C with a-AII [diluted 1:1000 in PBS/0.05 % Tween 20 (PT)]. After the primary incubation, the membrane was washed for four 5 min periods with PT and incubated for 1 hr with peroxidase-conjugated goat anti-rabbit IgG diluted 1:1000 in PT. Washing in PT was repeated (4 x 5 min). Bound antibody was revealed with a solution comprising 45 mg of DAB and 20 µl hydrogen peroxide in 100 ml of PT.

Purification of angiotensin II-like peptide

Step I : Sep-Pak Prepurification - CNS in batches of 200 or 400 were homogenized in 200 or 400 µl of 1 M acetic acid with a Dounce homogenizer and then sonicated (30 sec) twice. Homogenates were centrifuged at 12,000 rpm

for 30 min at 4°C. After reextraction of the pellet, the two supernatants were combined. Then, they were applied on C_{18} Sep-pak cartridges (500 µl/cartridge, Waters) and eluted with 5 ml of 50 % acetonitrile (ACN). Sep-pak eluted fractions were reduced 20-fold by freeze-drying. Total amount of AII-like peptide was obtained in direct ELISA.

Step II : Reversed-phase HPLC - The immunoreactive fractions from the Sep-Pak prepurification were subjected to a reversed-phase HPLC on a Vydac C_{18} Protein Peptide (250 x 4.6 mm) column. Elution was performed with a discontinous linear gradient from 0% A (0.1% TFA in 100 % deionized water) to 15 % B (0.1 % TFA in 100 % ACN) in 10 min, followed by a linear gradient from 15 to 45 % of B in 30 min at a flow rate of 1 ml/min. Ultraviolet absorbance was monitored at 215 nm. The eluted fractions were reduced to half of their volume by freeze-drying, before being assayed for immunoreactivity in DIA.

The immunoreactive compounds were separately subjected to a reversed-phase HPLC on a Vydac column with a shallower gradient. The solvent program consisted in a linear gradient from 0 to 15 % B in 10 min followed by 15 to 45 % B in 40 min. After reduction by freeze-drying, fraction aliquots of 0.5 µl were tested in DIA. The immunoreactive materials were then separated on a narobore column.

Step III. Final purification - Immunoreactive peptides were purified on a narobore (250 x 2 mm) column (Beckman) with a linear gradient from 0 to 60 % B in 60 min. Fractions, collected manually, were assayed in DIA. The immunoreactive compound was finally subjected to a reversed-phase HPLC with the same column as above in non-acidified conditions. The purified peptide was subjected to an automated Edman degradation.

All HPLC purifications were performed with a Beckman Gold HPLC system equiped with a photodiode array detector Beckman 168.

Amino acid Sequence analysis

Automated Edman degradation of 20 pmol of peptide and detection of phenylthiohydantoin derivates were performed on a pulse liquid automatic sequencer (Applied Biosystems, model 473).

Mass Spectrometry

The purified peptides were dissolved in water/ methanol (50/50, V/V) containing 1 % acetic acid and analyzed on a VG BioTech Bioq mass spectrometer (Manchester). This instrument consists of an electrostatic ion spray

source operating at atmospheric pressure, followed by a quadrupole mass analyser.

Biological assay

Rhynchobdellid leeches of the species *Theromyzon tessulatum*, bred in the laboratory and fed on ducks were used. The physiological stage of the animals was determined according to Malecha *et al* [21]. Leeches at stage 3B, stage which corresponds to the water retention phasis, received a subepidermal injection of 10 µl of an aqueous solution of 1 pmol of purified AII-like peptide. Controls were injected with a same volume of deionized water. In all cases, injected animals were kept at room temperature.

Before injections, animals were distributed in lots of 20 having an identical mean body mass. The effect of each injection was estimated by measuring the change of body mass of the animals between the beginning (injection) and the end of the experiment (1, 2, 4 and 5 hr after injection). Leeches were blotted on tissue paper and weighed to the nearest 0.1 mg. Results are presented as a mean ± SD (in per cent) representing the difference between the mass variations registered in stage 3B *T. tessulatum* injected either with the purified peptide or with deionized water. Statistical analysis of results was performed by calculating the mass variation of each leech at a t instant compared to the initial mass (at the time of injection). Statistical comparisons were made with Student's *t*-test. Confidence interval (with $\alpha = 0.5$) of the mean of the relative variations of mass was obtained according to Scherrer[22].

Protein identification

a - CNS protein extracts

CNS in batches of 200 or 400 were homogenized in 200 or 400 µl of TBS (50 mM Tris/HCl pH 7.4, 150 mM NaCl) with a Dounce homogenizer and then sonicated (30 sec) twice. Each homogenate was centrifuged at 12,000 rpm for 30 min at 0°C. The pellet was dissociated from the supernatant and reextracted as before. The two supernatants were finally combined and subjected either to an immunoprecipitation or to a high pressure gel permeation chromatography (HPGPC).

The CNS extracts were applied to a high pressure gel permeation column (SEC2000, ultraspherogel, 7.5 x 300 mm, Beckman) associated to a precolumn (ultraspherogel, 7.5 x 40 mm, Beckman), eluted with 30 % ACN at a flow rate of 0.3 ml/min and monitored with diode array at both 215 nm and

280 nm. The eluted fractions were concentrated 5-fold by freeze-drying and tested in ELISA. The positive fractions were then subjected to an electrophoresis and to an mmunoblot analysis.

Five milligrams of Protein A-sepharose (PA) were suspended in PBS (50 mM Phosphate buffer, pH 7.4, 150 mM sodium chloride). The gel was let to swell 1 hr at room temperature and was then washed briefly 4-fold in PBS by centrifugation. Ten microliters of pure a-AII and 40 µl of PBS were then successively added to the gel. After a 90 min incubation under gentle agitation at room temperature, five washings in PBS were carried out by centrifugation. CNS extracts (500 µl) were then added to the complex PA-a-AII. Incubation was conducted 24 hr under agitation at 4°C. Finally, the new complex PA-a-AII-antigen was washed 5-fold in PBS, before being treated with the sample buffer in order to dissociate the a-AII-antigen from PA and subjecting them to an electrophoresis and to an immunoblot analysis.

b- *In Vitro* translated products of CNS RNA extracts

CNS in batches of 400 were subjected to a total RNA extraction by the guanidium isothiocyanate method[23]. Total RNA were then subjected to a translation in a mixture containing 30 µl of rabbit reticulocyte lysate and 20 µl of a solution containing 30 µg of total RNA for 1 hr at 30°C. Translation was stopped on ice. The translated products underwent the same procedures that the protein extracts.

Polyacrylamide gel electrophoresis and Immunoblot analysis

Sodium dodecyl sulphate/polyacrylamide gels (SDS-PAGGE) were prepared according to Laemmli[24] except that the separating gel consisted of a 10-25 % polyacrylamide gradient slab gel. Molecular mass standards were : serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrate (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa). After SDS-PAGGE, proteins were electroblotted 5 hr at 250 mA on poly(vinylidene difluoride) transfer membrane (Immobilon-P, Millipore) in a transfer buffer consisting of 25 mM Tris and 192 mM glycine, 17 % methanol and 0.005 % SDS. After transfer, membranes were blocked with 2 % dried skilm milk in TBS containing 0.05 % tween 20 (TT). Incubations with a-AII (diluted 1:1000 in TT) [12 hr] and with peroxidase-conjugated goat anti-rabbit IgG (diluted 1:1000 in TT) [2 hr] were performed in TT, on rocking at room temperature. Peroxidase activity was detected by incubating blots successively in an oxidative medium [3 ml of a solution of 4-chloro-1-naphtol (10 mg in 5 ml of methanol) + 10 µl of

H_2O_2 in 50 ml TBS] and then, after washing in TBS, in a solution of 45 mg DAB and 15 µl of H_2O_2 in 100 ml of TBS]. Control of specificity was realized by preadsorbing a-AII with the AII peptide (100µg/ml of pure a-AII).

Results

Immunocytochemical investigations

About twenty cells immunoreactive to a-AII were detected in the brain (supraesophageal ganglion + subesophageal ganglion). Among these cells, two were particulary remarkable. Located in neuromere II (according to the numbering of Sawyer[25]) of the subesophageal ganglion, they present axons which contact each other in the neuropile (Fig. 1).

Figure 1

Immunohistochemical fluorescent micrograph of a whole-mount preparation of a subesophageal ganglion of *E. octoculata* treated with anti-AII (dorsal view). Anterior is to the top of figure.Two cells located in neuromere II are strongly labelled.

Bar = 50 µm

At the level of the nerve cord, four cells immunoreacted with a-AII in each segmental ganglion.

Biochemical investigations

Peptide isolation

Four hundreds CNS of *E. octoculata* were subjected to a peptide extraction in 1 M acetic acid (pH 2). The crude extract was purified using C_{18} Sep-Pak cartridges, reduced 20-fold by freeze drying and applied to a C_{18}

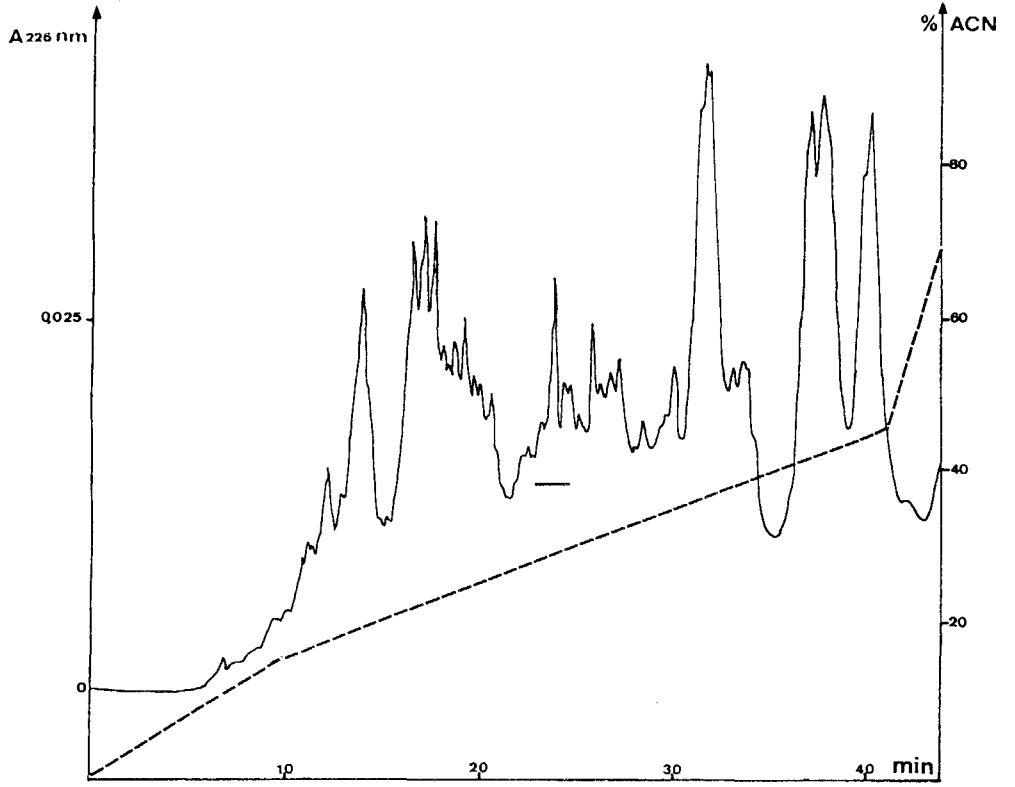

Figure 2 : *C_{18} reversed-phase HPLC separation of C_{18} Sep-Pak prepurified extracts of 400 CNS of E. octoculata.*
- Elution rate : 1 ml/min; solvent A : 0.1 % TFA in water ; solvent B : 0.1 % TFA in 100 % ACN ; solvent program : gradients of B at 1.5 %/min from 0 to 15 % followed by 1 %/min from 15 to 45 %.
- The solid line indicates absorbance and the dotted line the gradient.
- The solid bar indicates the elution position of the zone immunoreactive to a-AII.
- Absorbance was monitored at 226 nm (A226 nm).

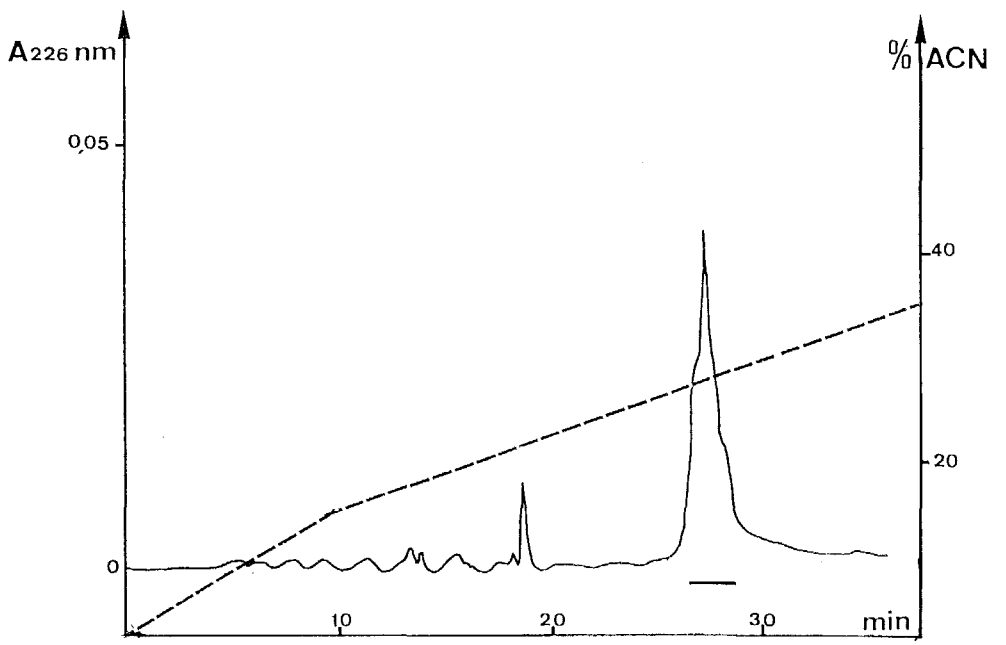

Figure 3 : *C_{18} reversed-phase HPLC of the AII-like immunoreactive fraction previously separated on a C_{18} reversed-phase HPLC column.*
- Elution rate : 1 ml/min ; solvent A : 0.1 % TFA in water ; solvent B : 0.1 % TFA in 100 % ACN ; solvent program : gradients of B at 1.5 %/min from 0 to 15 % followed by 0.75 %/min from 15 to 45 %.
- The solid line indicates absorbance and the dotted line the gradient.
- The solid bar indicates the elution position of the zone immunoreactive to a-AII.
- Absorbance was monitored at 226 nm (A226 nm).

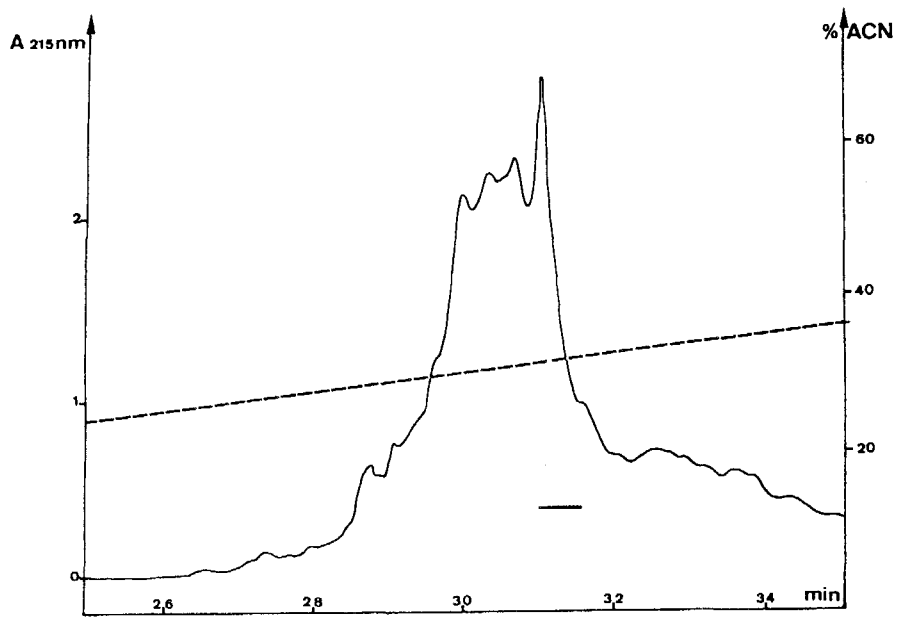

Figure 4 : *C_{18} reversed-phase HPLC on a narobore column of the AII-like immunoreactive material previously separated on a C_{18} reversed-phase HPLC column.*
- Elution rate : 0.3 ml/min ; solvent A : 0.1 % TFA in water ; solvent B : 0.1 % TFA in 100 % ACN ; solvent program : gradients of B at 1 %/min from 0 to 60 %.
- The solid line indicates absorbance and the dotted line the gradient.
- The solid bar indicates the elution position of the zone immunoreactive to a-AII.
- Absorbance was monitored at 215 nm (A215 nm).

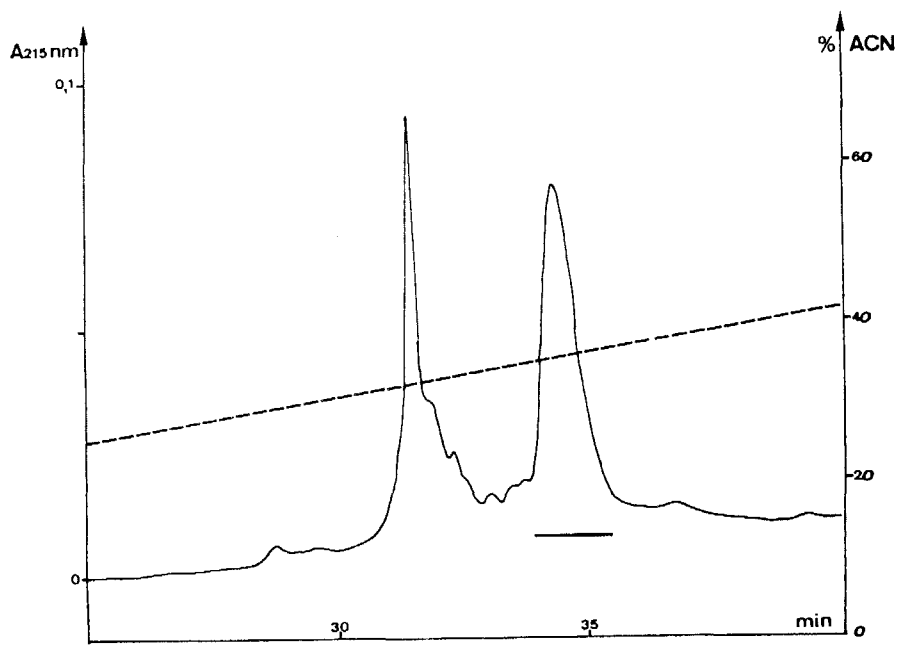

Figure 5 : *C18 reversed-phase HPLC on a narobore column, in non-acid conditions, of the AII-like peptide*
- Elution rate : 0.3 ml/min ; solvent A : 100% water ; solvent B :100 % ACN ; solvent program : gradients of B at 1 %/min from 0 to 60 %.
- The solid line indicates absorbance and the dotted line the gradient.
- The solid bar indicates the zone immunoreactive to a-AII.
- Absorbance was monitored at 215 nm (A215 nm).

reversed-phase HPLC column (Fig. 2). Fractions aliquots (1 µl) were assayed in DIA. An immunoreactive zone to a-AII was detected at a retention time (RT) ranging from 23 to 25 min. In the same conditions, synthetic AII eluted from the column with a RT of 24.8 min.

In a second step, these fractions containing the AII-like peptide were pooled to 4000 CNS-equivalent and chromatographed on the same column with a shallower gradient (Fig. 3). After DIA, a zone immunoreactive to a-AII with a RT ranging from 26.25 to 28.25 min was detected. In the same conditions, synthetic AII eluted from the column with a RT of 27.5 min.

In a third step, this immunoreactive zone was chromatographed on a narrowbore column with a linear gradient of ACN, in acid conditions. As seen in Fig. 4, several peaks were resolved, but ELISAs on the collected fractions revealed a major zone immunoreactive to a-AII with a RT ranging from 31 to 31.5 min, whereas Vertebrate AII eluted at 31.1 min in these conditions.

This major immunoreactive zone was then purified in non-acid conditions. Results indicated (Fig. 5) the presence of two peaks(P1 and P2) with respective RT of 31.4 min for P1 and 34.3 min for P2. However, DIA indicated that only the peptide contained in P2 was immunoreactive. Synthetic AII also gave a shifted peak at a RT of 34.3 min, in the same non-acid conditions. This shift in non-acid conditions could be explained by the fact that Vertebrate AII possesses an isoelectric point of 7.2, which would provoke an aggregation of the AII peptide. The same explanation could be advanced for the fact that when the AII-like peptide was replaced in acid conditions and rechromatographed on a narrowbore column, it gave a shifted peak at 31 min.

Characterization and primary stucture determination of the AII-like peptide.

The automated Edman degradation on 20 pmol of the purified AII-like peptide gave the following amino acid residues with a repetitive yield of 95 % : D, R, V, Y, I, H, P, F. The primary structure of the AII-like peptide is thus identical to Vertebrate AII. A mass spectrometry measurement of the intact peptide yielded a molecule of *m/z* 1044.36 ± 1.43 *vs* 1046.56 for the calculated average isotopic mass.

Biological assay

Intracoelomic injections to *T. tessulatum* in water retention phasis of 1 pmol of the purified AII-like of *E. octoculata* were effective. They provoked,

compared to controls injected with deionized water, a significant loss of mass 1, 2, 4 and 5 hrs post injection (Table I).

Table I : Difference of mass variation (in per cent) registered between Stage 3B *T. tessulatum* injected either with leech purified AIIamide or with deionized water.

Hours post-injection			
1	2	4	5
-20.36 ± 3.1	-19.33 ± 2.9	-16.37 ± 3.5	-13.61 ± 4.3

Each value is a mean ± standard deviation based on 20 animals.

Precursors characterization

After TBS extraction, supernatants of CNS extracts of *E. octoculata* were fractionated on HPGPC. The collected fractions were assayed in direct and inhibiting ELISA. Results revealed a specific immunoreactive zone (Z1) which corresponded to proteins eluting from the column with a molecular mass ranging from 10 to 25 kDa (Fig. 6). The immunopositive fractions showed, after SDS-PAGGE in presence of ß-mercaptoethanol and immunoblot analysis, the presence of two proteins with molecular masses of *ca* 14 and *ca* 18 kDa which could correspond to the Pro-AII-like : PA II (Fig. 7, lane a).

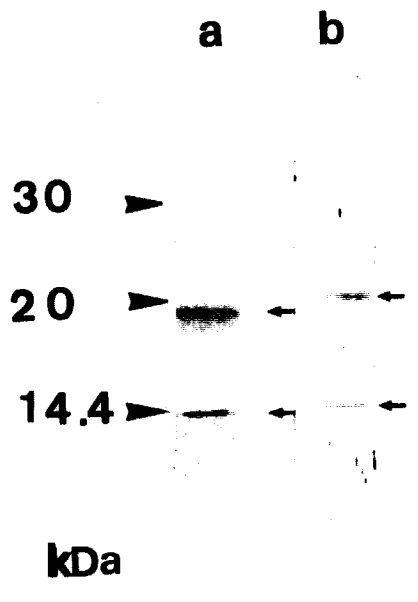

Figure7

Immunoblot analysis of the a-AII-like immunoreactive zones after HPGPC of a CNS extract (7a) or of translated total RNA extracted from CNS of E. octoculata (7b).

- Total proteins from CNS or translated products were separated by SDS-PAGGE in reducing conditions, transferred to poly (vinylidene difluoride) membrane and immunoblotted with a-AII.
- Small arrows indicate the position of the AII-like precursors.

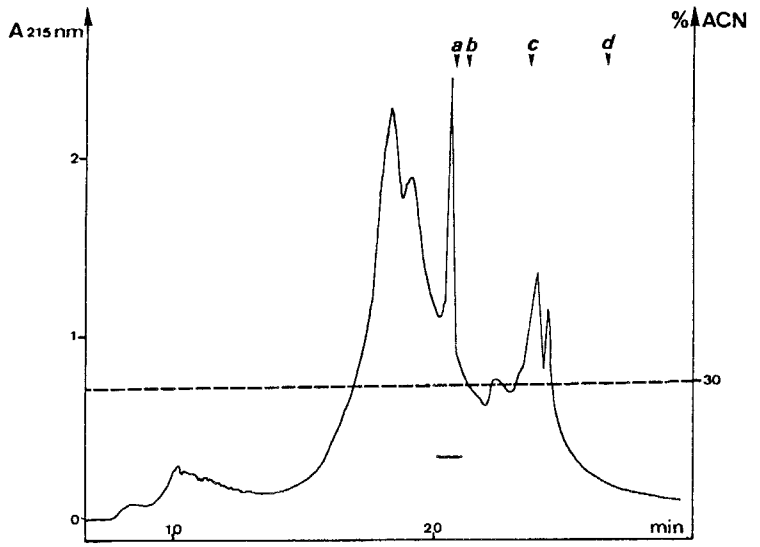

Figure 6 : *HPGPC conducted on an extract of 400 CNS from E. octoculata.*
- Elution rate : 0.3 ml/min, solvent : ACN 30%
- Arrows indicate the eluted position of standards in identical conditions (a : ovohemerythrin, b : cytochrom C, c : hirudin, d : angiotensin II).
- The solid line indicates absorbance and the dotted line the gradient.
- The solid bar indicates the zone immunoreactive to a-AII.
- Absorbance was monitored at 215 nm (A215 nm).

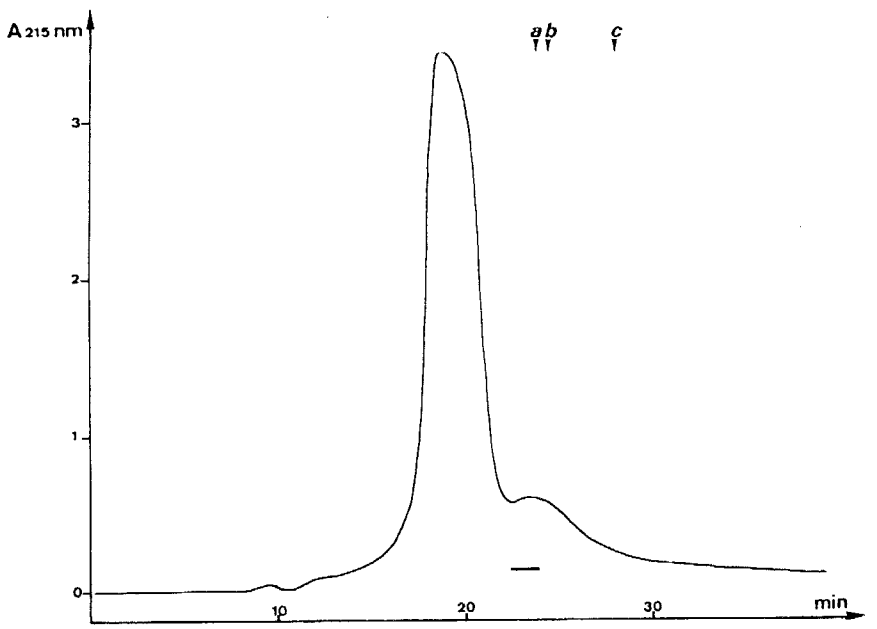

Figure 9 : *HPGPC conducted on translated total RNA extracted from CNS of E. octoculata*
- Elution rate : 0.3 ml/min, solvent : ACN 30%
- The solid line indicates absorbance and the dotted line the gradient.
- Arrows indicate the eluted position of standards in identical conditions (a : ovohemerythrin, b : cytochrom C, c : hirudin).
- Absorbance was monitored at 215 nm (A 215 nm).

In order to know if the protein of *ca* 14 kDa was generated during the preparation, an immunoprecipitation was conducted on a CNS crude extract. Two proteins with a molecular mass of respectively *ca* 14 kDa and *ca* 18 kDa were again found (Fig. 8, lane b).

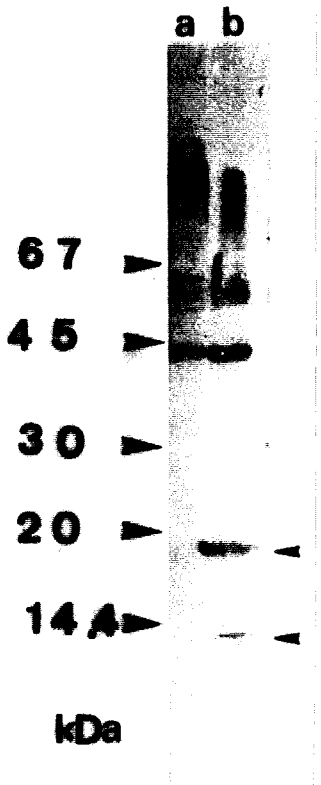

Figure 8
Immunoblot analysis after immunoprecipitation of a CNS extract of 400 CNS from E. octoculata (lane b).

- In controls (lane a), only the sample buffer was added.
- The immunoprecipitated proteins were separated by SDS-PAGGE, transferred to poly(vinylidene difluoride) membrane and immuno-blotted with a-AII.
- Arrows indicate the position of the immunostained proteins.

An identical investigation was performed on *in vitro* translated products. An immunoreactive zone was detected. It corresponded to proteins eluting from the HPGPC column with a molecular mass ranging from 10 to 25 kDa (Fig. 9). The immunoblot analysis revealed (Fig. 7, lane b) the presence of two proteins with a molecular mass of *ca* 19 and *ca* 15 kDa which could correspond to the PrePro-AII-like : PPAII.

In order to know if the protein of *ca* 15 kDa was generated during the preparation, an immunoprecipitation was conducted on CNS translated products. Two proteins with molecular masses of respectively *ca* 15 kDa and *ca* 19 kDa were found (Fig. 10, lane 1). However, when the immunoprecipitation was conducted on translated products stored at 4°C for 72 hr or at room temperature for 24 hr, only the protein of *ca* 15 kDa was found (Fig. 10, lane 2).

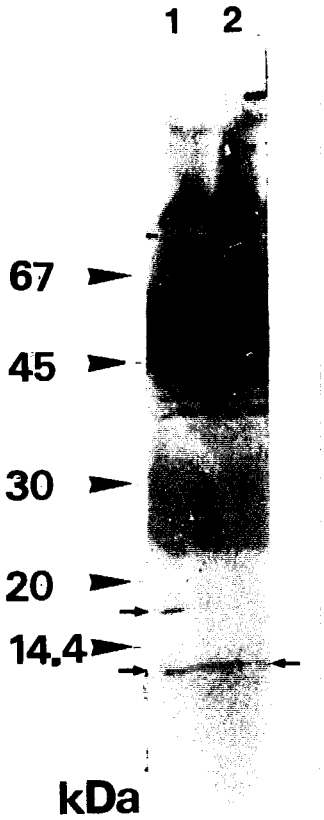

Figure 10
Immunoblot analysis after immunoprecipitation of translated total RNA extracted from CNS of E. octoculata with (lane 2) or without (lane 1) degradation.

- The immunoprecipitated proteins were separated by SDS-PAGGE, transferred to poly(vinylidene difluoride) membrane and immunoblotted with a-AII.
- Small arrows indicate the position of the specific immunostained proteins.

Discussion

Cells immunoreactive to an antiserum raised against AII are present in the central nervous system of *E. octoculata*. AII-like immunoreactive cells have also been described in the CNS of another leech, *T. tessulatum*[13], but in a greater number. Among the AII-like cells immunoreactive in *E. octoculata*, we did not find the equivalent of the ß giant cells, characteristic by their typical secretion, detected in the supra-esophageal ganglion of *T. tessulatum* [13]. In contrast, at the level of the sub-esophageal ganglion, a type of cell immunoreactive to a-AII not described in *T. tessulatum* was found ; these cells were remarkable by their axons contacting each other at the level of the ventral commissure.

In spite of a number of AII-like cells lower in the CNS of *E. octoculata* than in the CNS of *T. tessulatum*, amounts of AII-like material were found to be higher (*ca* 100 fmol/CNS *vs ca* 10 fmol/CNS) in *E. octoculata*. In this species, four reversed-phase HPLC steps were needed to isolate the AII-like peptide. During all steps of purification, Vertebrate AII coeluted with AII-like and a total overlapping between AII-like and AII scans or first derivate of the scans was obtained (data not shown). These results suggested that AII-like and AII possess a similar amino acid composition. The primary sequence

obtained with the automated Edman sequencer gave a primary sequence of AII-like peptide identical to the one of Vertebrate AII. However, the mass spectrometry realized on purified AII-like showed a *m/z* of 1044.36 ±1.43 Da *vs* 1046.56 Da for the calculated mass. This measured mass is in good agreement with the calculated isotopic mass of an amidated AII peptide (1045.56) which suggests that AII-like is an amidated AII. It is the first time that an AII is reported in an Invertebrate. Moreover, this molecule differs from the one of Vertebrates by its C-terminal amidation. The presence of this molecule in an animal belonging to the oldest Cœlomates Metazoa (Annelids), leads to suppose that AII is a very old molecule. The fact that its primary structure is identical to the one of Vertebrate AII shows that it is a well conserved molecule whose role must be fundamental for the animal physiology[26].

In leeches, the existence of a diuretic hormone is suspected since a long time. In *Hirudo medicinalis*, an 8-fold increase of the urine volume excreted was registered 15 min after a blood meal[14]. Moreover, in *T. tessulatum*, Salzet *et al*[13] demonstrated that the AII-like peptide amount increases just after a blood meal and that an injection of the Vertebrate metabolites of AII *i.e.* AII, AIII and fragment (5-8) of AII in this leech, during its water retention phasis, has a diuretic effect. These results suggested that the leech AIIamide is a diuretic hormone, which was strengthened by the diuretic effect of the purified leech AIIamide when injected in *T. tessulatum*.

These results obtained in leeches contrast with the ones obtained in Vertebrates, where injection of AII triggers drinking behavior in fishes[27], reptiles[28], birds[29] and mammals[30].

This existence of a role of AII which could differ between Vertebrates and Invertebrates could be compared to what is found for another peptide : the vasopressin-like of the Insect *Locusta migratoria*[31]. However, recent investigations in Vertebrates[32] have established that AII would exert both a diuretic and an anti-diuretic effect. Mühlethaler *et al.*[33] and Vincent and Simonnet[34], proposed that molecules which possess a very old phylogenic origin, keep their physiological function during evolution. So, we suggest that in leeches, as in Vertebrates, AII would exert an action on hydric balance. It has to be noted that investigations on the AII receptor indicated that the analog of AII, the AIIamide, is 100-fold more active than AII on the permeability of the frog skin[35].

After HPGPC fractionation and immunoblot analysis evidence was given of the existence in CNS extracts or in CNS RNA extracts of two proteins : a major one [*ca* 18 kDa in CNS extracts (PAII)*vs ca* 19 kDa in translated products

of CNS RNA extracts (PPAII)] and a minor one [*ca* 14 kDa (PAII) in CNS extracts *vs* 15 kDa (PPAII) in translated products of CNS RNA extracts]. The slight difference of mass between the PAII and the PPAII and the observation of a slight increase in retention time between PPAII and PAII after C3 reversed-phase HPLC (data not shown), led to think that a signal peptide might be associated in PPAII.

The immunoprecipitation on CNS crude extracts and CNS translated products has established that the lower protein was not generated during the purification. So, at this level of the work, two hypotheses can be advanced : either it exists two mRNA encoding the AIIamide or the protein of *ca* 15 kDa is a product of degradation of the protein of *ca* 19 kDa. When transcripted products were quickly subjected to an immunoprecipitation, two proteins were detected. On the other hand, when the analysis was not done quickly and thus subjected to conditions of degradation, only the protein of *ca* 15 kDa subsisted, which allowed to speculate that the lower protein was a product of degradation of the larger one. However, further investigations with protease inhibitors and a northern blot analysis have to be undertaken to definitely elucidate this problem.

This study brings proofs of the existence in the CNS of the leech *E. octoculata* of an amidated AII peptide with a diuretic activity.This peptide possesses the same primary structure as Vertebrate AII, but for a C-terminal amidation. In contrast, the leech AII-like precursor differs in mass from the Vertebrate AII precursor, the Ao [*ca*19 kDa *vs ca* 50 kDa][5, 36].

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BIOCHEMICAL EVIDENCE OF ANGIOTENSIN II-LIKE PEPTIDES AND PRECURSORS IN THE BRAIN OF THE RHYNCHOBDELLID LEECH *Theromyzon tessulatum.*

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Abstract

The peptides contained in angiotensin II-like (AII-like) neurons localized in the brain of the leech *Theromyzon tessulatum*, were purified by a combination of chromatographic separations and dot immunoassays. Three AII-like peptides which exhibited the same retention times and chromatographic behaviors as synthetic AII, fragment 3-8 of AII (AIV) and fragment 6-8 of AII (AVII), were resolved in brain extracts.

An identification and a partial characterization of the AII-like precursor performed at the level of both brain extracts and *in vitro* translated RNA products indicated that the Pro-AII-like and the PrePro-AII-like precursors possessed very close molecular masses (*ca* 18 kDa *vs ca* 19 kDa).

A partial mapping of the AII-like precursor indicated that the Pro-AII-like precursor is also recognized by two other antibodies, a polyclonal one against γ MSH and a monoclonal one called Tt159, which establishes that it is a multi-peptidic precursor.

Key Words : Angiotensin II-like peptide, Angiotensin II-like-precursors, Immunoassays, Leech, Reversed-Phase HPLC, Western Blot

Introduction

Angiotensin II (AII) is an octapeptide discovered *ca* 50 years ago in Mammals by Page and Helmer (1940). It possesses multiple physiological functions *e.g.* regulation of blood pressure and stimulation of mineralocorticoids secretion (at the surrenal level), natriuresis, glomerular filtration and glycogenolysis (Guillon , 1989).

Besides these peripheral actions, AII, which is also synthesized in the brain, exerts at this level different actions *e.g.* regulation of blood pressure, drinking and salt appetite and stimulation of pituitary hormones synthesis (adrenocorticotopic hormone, prolactin, luteinizing hormone) and secretion (vasopressin) [for a review, see Saavedra, 1992].

All these actions, both peripheral and central, give to AII an important physiological role in the control of blood pressure and in the maintenance of the volume of extracellular fluid (Guillon, 1989).

If the role of this molecule has been very well explored in Vertebrates, there exists at our knowledge no data related to Invertebrates but for Hirudinae. In this zoological group, Salzet *et al.* (1992a) have described in *Theromyzon tessulatum ca* 33 cells immunoreactive to anti-AII in the brain (18 in the supraesophageal ganglion and 15 in the subesophageal ganglion) and 6-8 AII-like immunoreactive cells per segmental ganglion.

Among these cells, 4-5 pairs of neurons located in the posterior compartments of the supraesophageal ganglion were remarkable for their size, hence their name of ß giant cells, and the special aspect of their secretion in light microscopy. This histological appearance has not been found in any other cell of the CNS. These ß giant cells are unipolar and one of the branches issued from the cellular process ramifies abundantly at the level of the neurohaemal area (Salzet *et al.*, 1992a).The peptidic secretion of these cells could be released into the circulatory system at the neurohaemal site and exert an hormonal role. Moreover, these cells immunoreact with anti-γ-MSH and with monoclonal antibody Tt159 (Verger-Bocquet *et al.*, 1992).

The injection in *T. tessulatum* of either AII or AII (5-8) or AIII resulted in a decrease of mass expressing a diuretic effect of these molecules. The fragment 5-8 of AII at a very low concentration (10 pmol) seemed to be the most effective. The AII-like substance could thus be a diuretic hormone in leeches (Salzet *et al.*, 1992a). An AII-like molecule has been purified in the leech *Erpobdella octoculata*, it is an AII-amide whose diuretic action was confirmed by injection of this purified peptide to *T. tessulatum.* (Salzet *et al.*, in preparation)

The aim of our work was to characterize biochemically the AII-like material in *T.tessulatum* and its precursors.

Material and Methods

Animals

T. tessulatum (Hirudinea, Rhynchobdellida) at stage 3 *i.e.* having taken their third blood meal (Malecha *et al.*, 1989b), were used in this study.

Dissections

After anaesthetization in 0.01 % chloretone, animals were pinned out, dorsal side up, in leech Ringer (Muller *et al.*, 1981). Brains were carefully dissected, frozen at -180°C in nitrogen and finally stored at -20°C until use for biochemical purification.

Chemicals

3-3'-diaminobenzidine-tetra-hydrochloride (DAB), 4-chloro-1-naphtol, hydrogen peroxide and synthetic peptide (AII) were obtained from Sigma, the trifluoroacetic acid (TFA), sequencer grade, from Pierce, the peroxidase-conjugated goat anti-rabbit IgG from Pasteur Diagnostics and BrCn-Sepharose-4B from Pharmacia. All organic solvents were HPLC grade and were purchased from Merck. Deionized water was obtained from a Milli-Q system (Millipore).

Antibodies

Polyclonal antisera against angiotensin II (a-AII) and γ–MSH (a-γ-MSH) and a monoclonal antibody (Tt159) produced after immunization with *T. tessulatum* supraesophageal ganglia extracts, were used in this study. Characterization of these antibodies was reported respectively in Aguirre *et al.* (1989), Verger-Bocquet *et al.* (1988) and Boilly-Marer *et al.* (1987).

Immunoassays

a - ELISA procedures

- Immunoassays were conducted according to procedures described elsewhere (Salzet *et al.*, 1992b). Polyclonal antibodies (a-AII and a-γ-MSH) were used at a dilution of 1:1000, monoclonal antibody Tt159 was employed under the form of undiluted hybridoma supernatant.

- Quantification of AII-like peptides in brain extracts was done in direct ELISA according to Salzet *et al.* (1992b).

b - Dot immunoassay (DIA)

An aliquot of 1 µl of each HPLC fraction was spotted onto a nitrocellulose membrane (0.45 µm pore size, Schleicher and Schuell) which was then baked 30 min at 110°C. The membrane was blocked, under gentle agitation at room temperature, 1 hr with PBS (50 mM Phosphate buffer, 150 mM Sodium chloride, pH 7.4) including 0.05 % Tween 20 and 5 % Skilm milk and then incubated overnight at 4°C with a-AII [diluted 1:1000 in PBS/0.05 % Tween 20 (PT)]. After the primary incubation, the membrane was washed for four 5 min periods with PT and incubated for 1 hr with peroxidase-conjugated goat anti-rabbit IgG diluted 1:1000 in PT. Washing in PT was repeated (4 x 5 min). Bound antibody was revealed with a solution comprising 45 mg of DAB and 20 µl of hydrogen peroxide in 100 ml of PT.

Purification of angiotensin II-like peptide

Step I : Sep-Pak prepurification - Brains in batches of 200 were homogenized in 200 µl of 1 M acetic acid with a Dounce homogenizer and then sonicated (30 sec) twice. Homogenates were centrifuged at 12,000 rpm for 30 min at 4°C. After reextraction of the pellet, the two supernatants were combined. Then, they were applied on C_{18} Sep-pak cartridges (500 µl/cartridge, Waters) and eluted with 5 ml of 50 % acetonitrile (ACN). Sep-pak eluted fractions were reduced 20-fold by freeze-drying. Total amount of AII-like was quantified in ELISA.

Step II : High pressure gel permeation chromatography (HPGPC) - Five hundred microliters of Sep-pak eluted fractions were injected into a HPGPC column (Ultraspherogel, 7.5 x 300 mm, Sec2000, Beckman) associated to a precolumn (Ultraspherogel, 7.5 mm x 40 cm, Beckman). Samples were eluted with 30 % ACN at a flow rate of 1 ml/min and the fractions immunoreactive in DIA were then concentrated.

Step III : Affinity column - Positive fractions evaporated and dried with a Speed-Vac (SVH, Savant) and resuspended in 0.1 M Sodium Carbonate, 0.5 M NaCl buffer (pH 9.6) were chromatographed on an affinity column (1 x 10 cm, a-AII coupled to BrCn-Sepharose-4B) prealably equilibrated in 200 mM Phosphate, 150 mM NaCl buffer (pH 7.4). The sample went back over continuously the column at a flow rate of 300 µl/min, overnight at 4°C. The column was then washed with 25 ml of the equilibrated buffer. Elution

was performed with 4 ml of 1 N Propionic acid (pH 3) at a flow rate of 200 µl/min. Eluted fractions (200 µl) were rapidly neutralized and concentrated 10-fold in Speed-Vac.

Step IV : Reversed-phase HPLC - Immunoreactive fractions were pooled and separated on a reversed-phase HPLC with a C_{18}Vydac Protein Peptide column (250 x 4 mm) associated to a RP_{18} precolumn (Merck). The mobile phase consisted of a gradient established with 0.1 % TFA in 100% of water (Solvent A) and 0.1% TFA in 100% of ACN (Solvent B). The gradient of solvent B was 0 to 15 % over a 10 min period, then 15 to 45 % during 30 min. The flow rate was 1ml/min, one min fractions were collected and then concentrated 5-fold. Peptide peaks were detected by monitoring absorbance at 226 nm. One to five microliters aliquots of each fraction were assayed in DIA. Immunoreactive fractions were pooled and then re-injected on the C_{18} Vydac column, but eluted with an isocratic gradient (10 % solvent B) at a flow rate of 1ml/min. Collected fractions were concentrated to a final volume of 50 µl and a 0.5 µl aliquot of each fraction was tested by DIA.

Step V : Final purification -Immunoreactive peaks were finally loaded on a C_{18} narobore column (250 x 2 mm) (Beckman). Elution was performed with an isocratic solvent B gradient (10 %) at a flow rate of 300 µl/min. Fractions were collected, assayed by DIA, titrated in ELISA and stored at -20°C. Synthetic peptides were analyzed by chromatography in the same conditions at each purification step.

Protein identification

a - Brain protein extracts

Brains in batches of 200 or 400 were homogenized in 200 or 400 µl of 50 mM Tris/HCl pH 7.4, 150 mM NaCl (TBS) with a Dounce homogenizer and then sonicated (30 sec) twice. Each homogenate was centrifuged at 12,000 rpm for 30 min at 0°C. The pellet was dissociated from the supernatant and reextracted as before. The two supernatants were finally combined, subjected to a HPGPC, eluted with 30 % ACN at a flow rate of 300 µl/min and monitored with diode array at both 215 nm and 280 nm. Eluted fractions were concentrated 5-fold by freeze-drying and tested in ELISA. Positive fractions were then subjected to an electrophoresis and to an immunoblot analysis.

b- *in vitro* translated products of brain RNA extracts

Brains in batches of 400 were subjected to a total RNA extraction by the guanidium isothiocyanate method (Sambrook *et al.*, 1989). Total RNA were then subjected to a translation in a mixture containing 30 µl of rabbit reticulocyte lysate and 20 µl of a solution containing 30 µg of total RNA for 1 hr at 30°C. Translation was stopped on ice. The translated products underwent the same procedures as brain protein extracts and in addition a C3 reversed-phase HPLC.

Samples after HPGPC were injected on a C3 reversed-phase HPLC (75 x 4.6 mm) column (Beckman).The solvent program consisted of an initial hold with 100 % of solvent A [0.1 % TFA in water] then gradients of solvent B [0.1 % TFA in 100 % of ACN]. The flow rate was 1 ml/min. Proteins peaks were detected by monitoring absorbance at 215 nm and 280 nm with a diode array.

Polyacrylamide gel electrophoresis and Immunoblot analysis

Sodium dodecyl sulphate/polyacrylamide gels (SDS-PAGGE) were prepared according to Laemmli (1970) except that the separating gel consisted of a 10-25 % polyacrylamide gradient slab gel. Molecular mass standards were : serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrate (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa). After SDS-PAGGE, proteins were electroblotted 5 hr at 250 mA on a poly(vinylidene difluoride) (PVDF) transfer membrane (Immobilon-P, Millipore) in a transfer buffer consisting of 25 mM Tris, 192 mM glycine, 17 % methanol and 0.005 % SDS. After transfer, membranes were blocked with 2 % dried skilm milk in TBS containing 0.05 % Tween 20 [TT]. Incubations with a-AII (diluted 1:1000 in TT) [12 hr)] and with peroxidase-conjugated goat anti-rabbit IgG (diluted 1:1000 in TT) [2 hr] were performed on rocking at room temperature. Peroxidase activity was detected by incubating blots successively in an oxidative medium [3 ml of solution of 4-chloro-1-naphtol (10 mg in 5 ml of methanol) + 10 µl of H_2O_2 in 50 ml TBS] and then, after washing in TBS, in a solution of 45 mg DAB and 15 µl H_2O_2 in 100 ml of TBS]. Control of specificity was realized by preadsorbing a-AII with the AII peptide (100µg/ml of pure a-AII).

Other methods

A comparison between scans provided from synthetic peptides and the endogenous immunoreactive peptides and first derivates of the scans, was realized with a previous normalization thanks to the diode array and the Gold system (Bekman), which can scan from 200 to 300 nm with step wise 2 nm increase, each 0.5 s.

Results

Peptide isolation

Results of HPGPC analysis of Sep-Pak pre-purified brain extracts combined with DIA are presented in Fig. 1. Retention times (RT) of the immunoreactive peaks were between 8 and 12 min and corresponded to peptides with a molecular mass ranging from *ca* 0.5 to 2 kDa.

The positive fractions were loaded to an antibody affinity column and then immunoreactive eluted fraction were separated on a C18 RP-HPLC column. Immunoreactivity to a-AII, determined by DIA, was detected in fractions with a RT of 9-12 min (Fig. 2).In the same conditions, RT of Vertebrate AII peptide was 11.5 min.

These positive fractions were further separated on a C18 reversed-phase HPLC and the isocratic gradient of 10 % ACN used in this study allowed to resolve three a-AII immunoreactive peptides (P1, P2, P3) contained in three peaks with RT of respectively 7.4 min for P1, 8.2 min for P2 and 8.4 min for P3 (Fig. 3).

Finaly, these three peptides were purified on a narrowbore column. P1, P2 and P3 presented RT of respectively 8.4 min, 9.4 min and 10.2 min which corresponded respectively to RT of synthetic Vertebrate peptides AVII (fragment 6-8 of AII), AIV (fragment 3-8 of AII) and AII (Figs 4a, b, c).

In order to confirm that P3 and AII or AII-amide have the same chromatographic behavior, pools of either P3 and AII or of P3 and AIIamide were injected on a C_{18} reversed-phase HPLC narrowbore column. Results indicated the presence of a single immunoreactive peak with a RT of 10.2 min. Moreover, the spectral scan of P3 and vertebrate AII between 200-300 nm showed, after comparison and normalization, a total spectral overlapping and a ratio of 0.999 (Fig. 5). With a same procedure, it was demonstrated that P1 and P2 have the same chromatographic behavior as respectively AVII and AIV peptides.

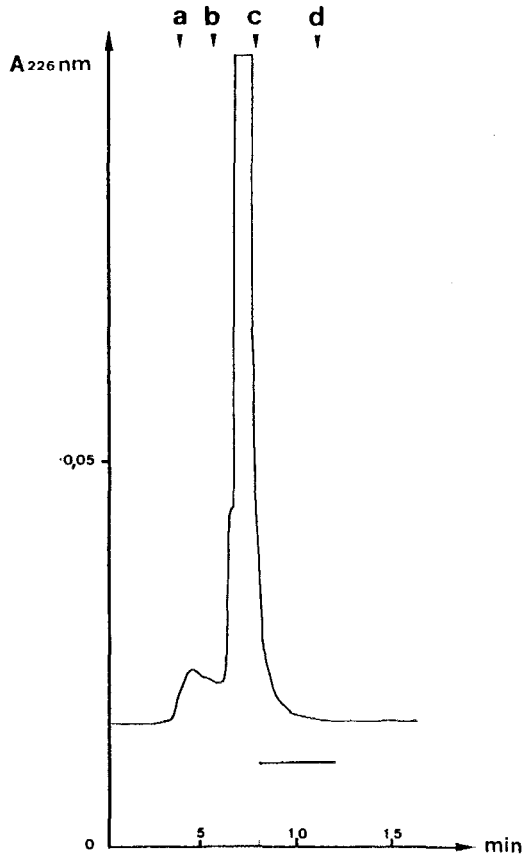

Figure 1 : *HGPC conducted on a C18 Sep-Pak prepurified extract of 400 brains of T. tessulatum.*
- Elution rate : 1 ml/min, solvent : ACN 30 %
- Arrows indicate the eluted position of standards in identical conditions. (a : hirudin, b : ACTH, c : angiotensin II, d : L-Tryptophane).
- The solid line indicates absorbance.
- The immunoreactive AII-like zone is indicated by a solid bar.
- Absorbance was monitored at 226 nm (A226 nm).

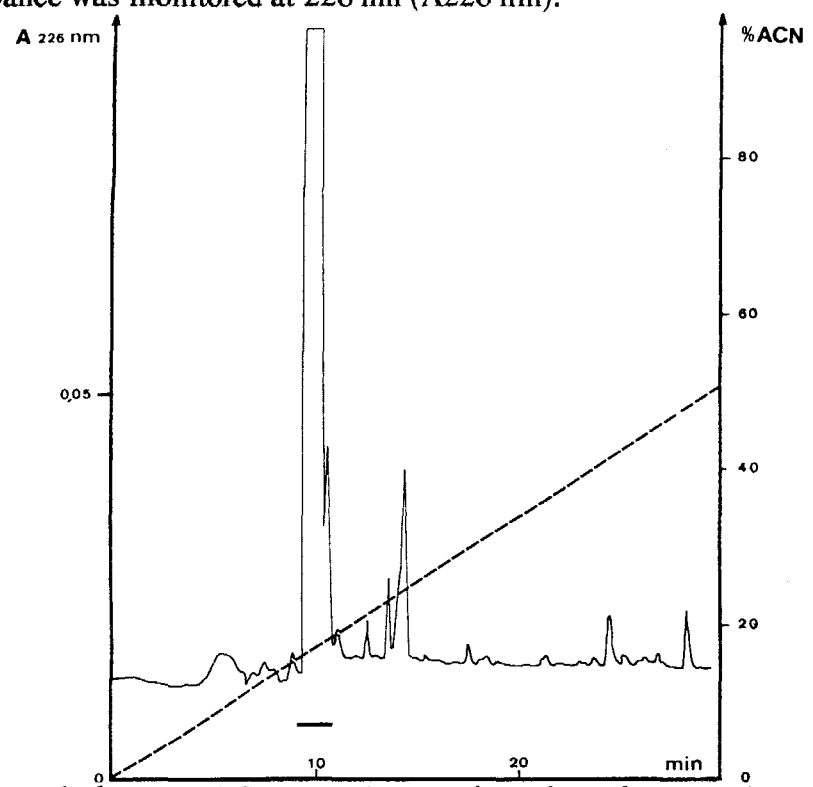

Figure 2 : *C18 reversed-phase HPLC separation conducted on the a-AII immunoreactive fractions after affinity column purification.*
- Elution rate: 1 ml/min; solvent A : 0.1 % TFA in water ; solvent B : 0.1 % TFA in 100 % ACN ; solvent program : gradients of B at 1.5 %/min from 0 to 50 %.
- The solid line indicates absorbance ; the dotted line indicates the gradient.
- The immunoreactive AII-like zone is indicated by a solid bar.
- Absorbance was monitored at 226 nm (A226 nm).

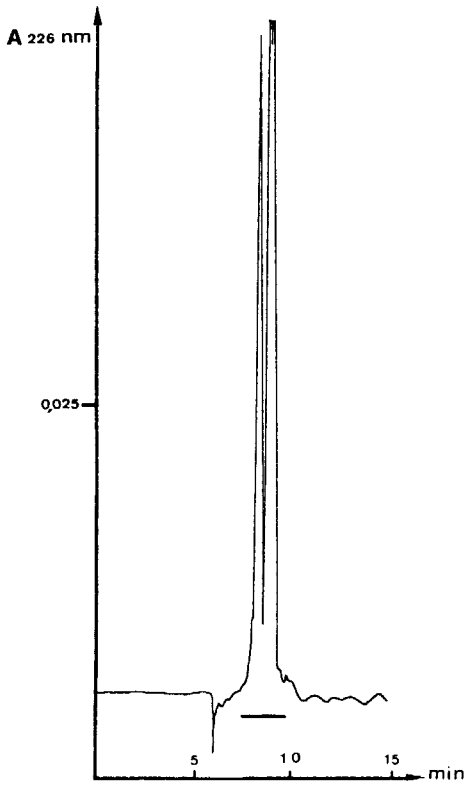

Figure 3

C_{18} reversed-phase HPLC of the AII-like immunoreactive fractions, previously separated on C_{18} reversed-phase HPLC column.

- Elution rate : 1 ml/min; solvent A : 0.1 % TFA in water; solvent B : 0.1 % TFA in 100 % ACN; solvent program : 10 % of B.
- The solid line indicates absorbance.
- The immunoreactive AII-like zone is indicated by a solid bar.
- Absorbance was monitored at 226 nm (A226 nm).

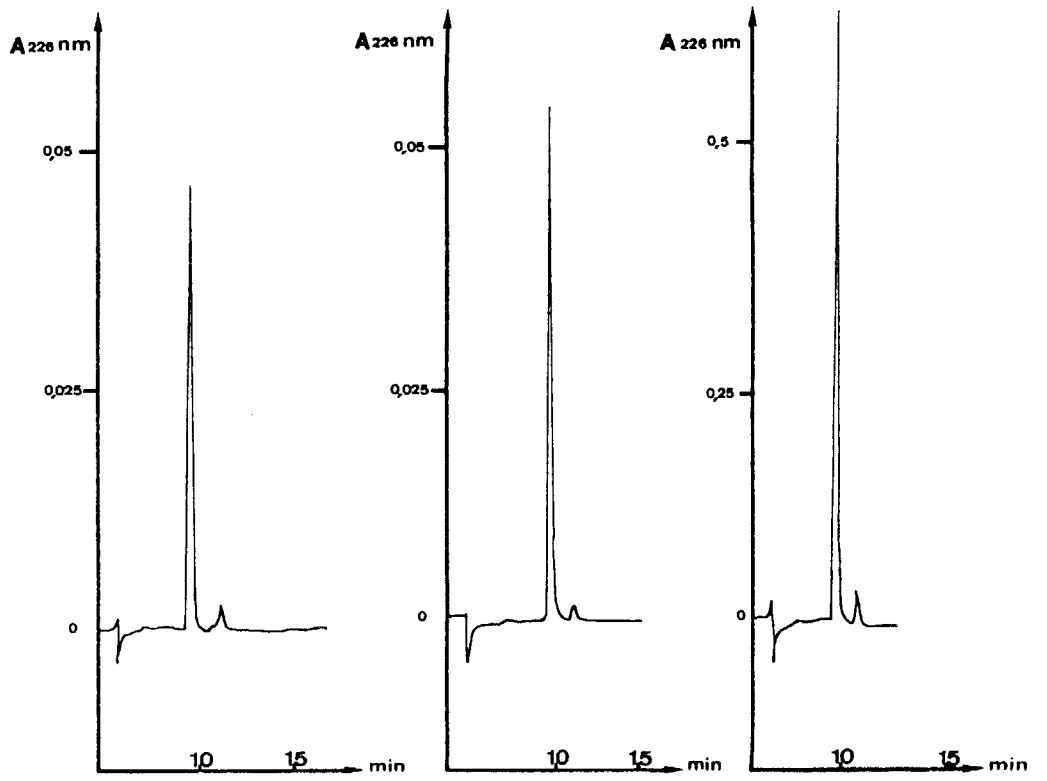

Figure 4 : *C_{18} reversed-phase HPLC on a narobore column of the three AII-like immunoreactive peptides (a, b, c) previously separated on a C_{18} reversed-phase HPLC column.*

-Elution rate : 1 ml/min; solvent A : 0.1 % TFA in water; solvent B : 0.1 % TFA in 100 % ACN; solvent program : 10 % of B.
- The solid line indicates absorbance.
- Absorbance was monitored at 226 nm (A226 nm).

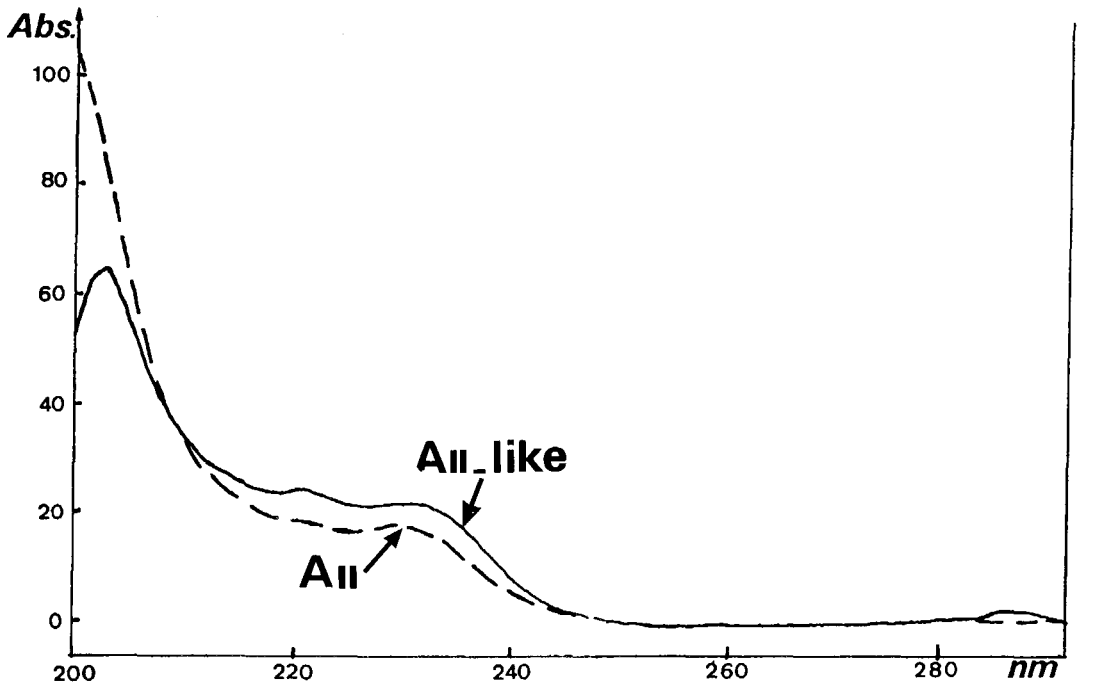

Figure 5 : *Comparison of normalized first derivatives profiles (between 200-292 nm) of purified AII-like peptide and synthetic AII peptide.*

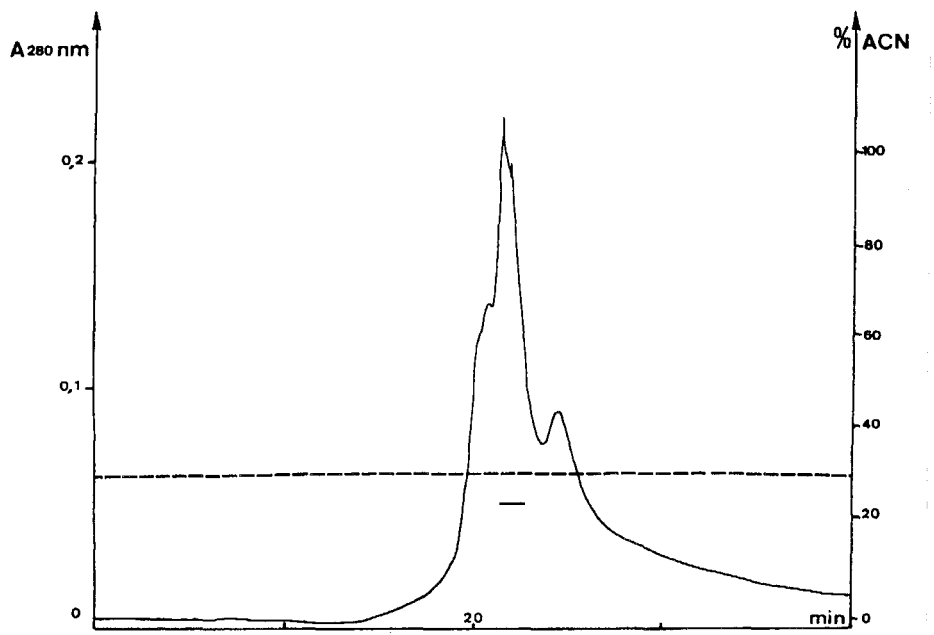

Figure 6 : *HGPC of an extract of 400 brains of T. tessulatum*
- Elution rate : 0,3 ml/min, solvent : ACN 30 %.
- The solid line indicates absorbance.
- The immunoreactive AII-like zone is indicated by a solid bar.
- Absorbance was monitored at 280 nm (A 280 nm).

Estimation of AII-like peptide amounts in brain extracts

From the amounts of AII-like peptide determined with ELISA in each isolated peak (Table I), it can be seen that P3 which represents 80 % of the total AII-like amount, is the major peptide.

Table I : Total amount of AII-like peptides after Sep-Pak prepurification and amounts of each identified AII-like peptide isolated at the final step of purification present in *T. tessulatum* brains.

		Amount (fmol/brain)
Total AII-like peptide		15.69 ± 5.65
Peptide (P#)		
AVII	(P1)	0.95 ± 0.67
AIV	(P2)	1.35 ± 0.86
AII	(P3)	10.08 ± 3.08

Values are expressed as a mean ± SD (from 4 determinations)

Identification of the AII-like precursors

In brain extracts

Brain extracts were subjected to a HPGPC. Collected fractions were assayed in ELISA with a-AII (Fig. 6). After evaluating the difference between the values obtained in direct and in inhibiting ELISA, an immunoreactive zone [Z1] corresponding to proteins eluted from the column with a molecular mass ranging between 25-10 kDa [Z1] was considered as specific. Proteins contained in Z1 were then subjected to SDS-PAGGE in presence of β mercaptoethanol (reducing conditions) before being transferred to PVDF membrane. After immunodetection with a-AII, a protein with a molecular mass of about 18 kDa was detected (Fig. 7, lane a), we emit the hypothesis that it is the Pro AII-like (PAII).

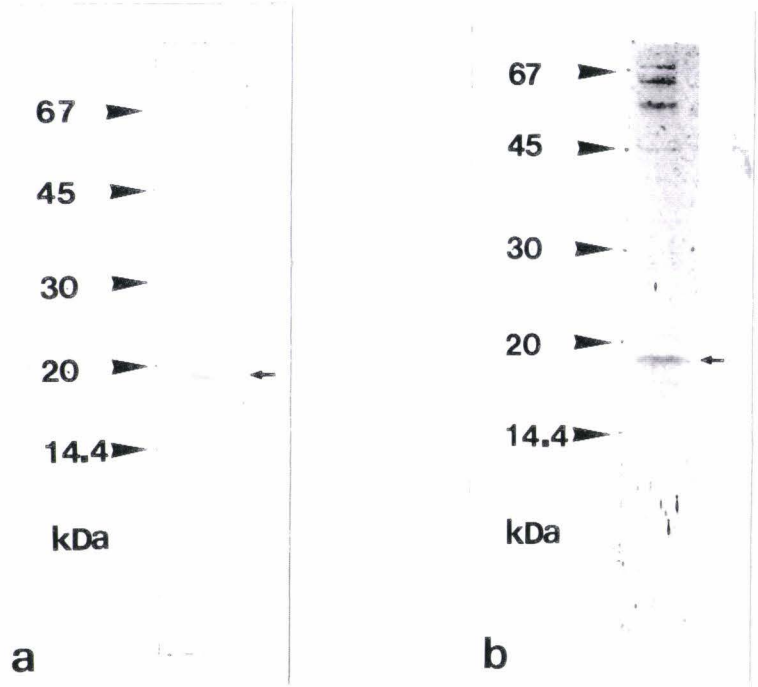

Figure 7

Immunoblot analysis after HPGPC separation of a brain extract from T. tessulatum.

-Total proteins from brains were separated by SDS-PAGGE with (a) or without (b) ß mercaptoethanol, transferred to poly(vinylidene difluoride) membrane and immunoblotted with a-AII.

-Small arrows indicate the position of the AII-like precursor.

A similar result was obtained after SDS-PAGGE in absence of ßmercaptoethanol. However in this case, the immunoreactive bands ranging from 45 to 68 kDa which were observed under reducing conditions (Fig. 7, lane a) were no more detected (Fig. 7, lane b). Since the bands ranging between 45 to 68 kDa were also immunodetected when only the sample buffer in presence of ß mercaptoethanol was loaded on the gels, it thus appears that a-AII interacts with artefactual proteins associated with the presence of ß mercaptoethanol, proteins which according to Tasheva and Dessev (1983) and Ochs (1983) could be interpreted as being of a keratin-type.

In brains mRNA translated products

After extraction of total RNA and transcription in rabbit reticulocyte lysate, translated proteins were treated as the brain extracts. Results, illustrated in Fig. 8, indicated an immunoreactive zone [Z2] corresponding to proteins with molecular masses ranging from 25 to10 kDa. After western blot, a protein with a size of *ca* 19 kDa was detected (Fig. 9, lane a), it was slightly larger than the one detected in the brain extracts (*ca* 18 kDa) (Fig 9, lane b). It is likely the PrePro-AII-like (PPAII)

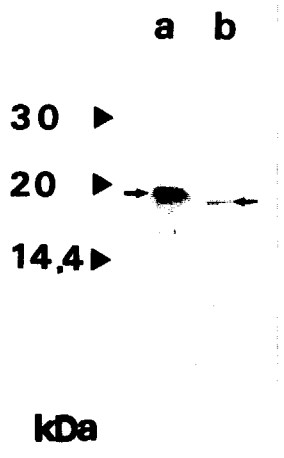

Figure 9

Immunoblot analysis after HPGPC separation of a brain extract (b) or translated total RNA extracted from brains of T. tessulatum (a).

- Total proteins from brains were separated by SDS-PAGGE in presence of ß mercaptoethanol, transferred to poly(vinylidene difluoride) membrane and immunoblotted with a-AII.
- Small arrows indicate the position of the Pro-AII-like and the PrePro-AII-like.

Partial characterization of the pro-angiotensin II-like

A HPGPC was conducted on brain extracts, and the eluted fractions were assayed in ELISA with two antibodies (Tt159 and a-γ-MSH) known to react with epitopes localized in the same neurons (Verger-Bocquet *et al.*, 1992).The HPGPC zone (25-10 kDa) reacting with a-AII, was also recognized by these two antibodies (data not shown). A western blot analysis was realized on the fraction immunopositive to a-AII, using a-γ-MSH (Fig. 10, lane a) or Tt159 (Fig. 10, lane b). Only one polypeptide with a molecular mass similar

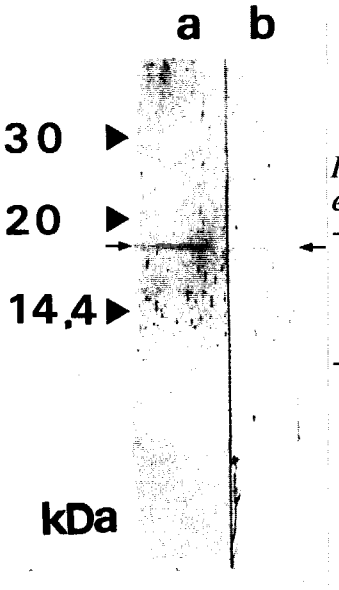

Figure 10

Immunoblot analysis after HPGPC separation of a T. tessulatum brain extract

- Total proteins from brains were separated by SDS-PAGGE in presence of ß mercaptoethanol, transferred to poly (vinylidene difluoride) membrane and immunoblotted with either a-g MSH (a) or Tt159 (b).

-Small arrows indicate the position of the Pro-AII-like molecule.

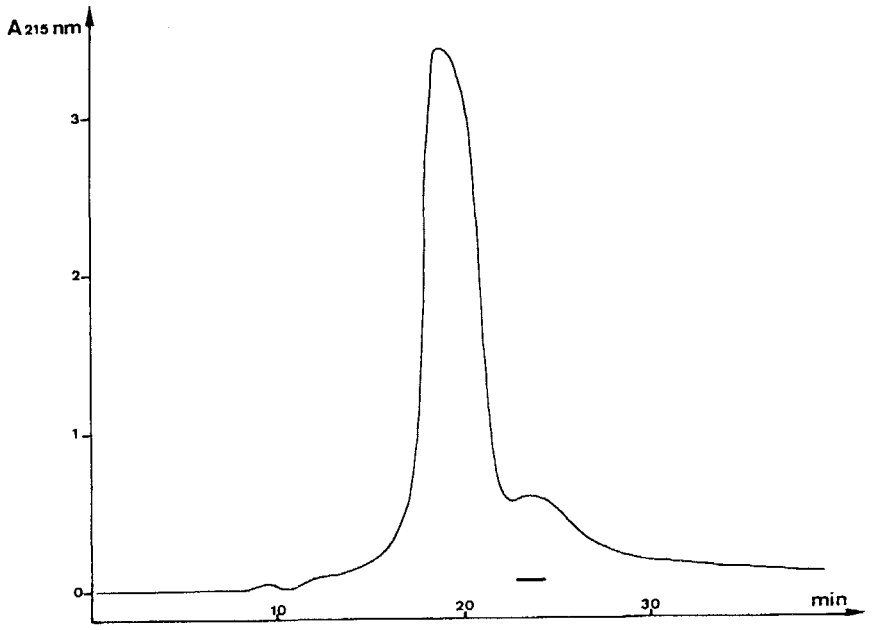

Figure 8
HPGPC of translated total RNA extracted from brains of T. tessulatum
- Elution rate : 0.3 ml/min, solvent : ACN 30 %.
- The solid line indicates absorbance.
- The immunoreactive AII-like zone is indicated by a solid bar.
- Absorbance was monitored at 215 nm (A 215 nm).

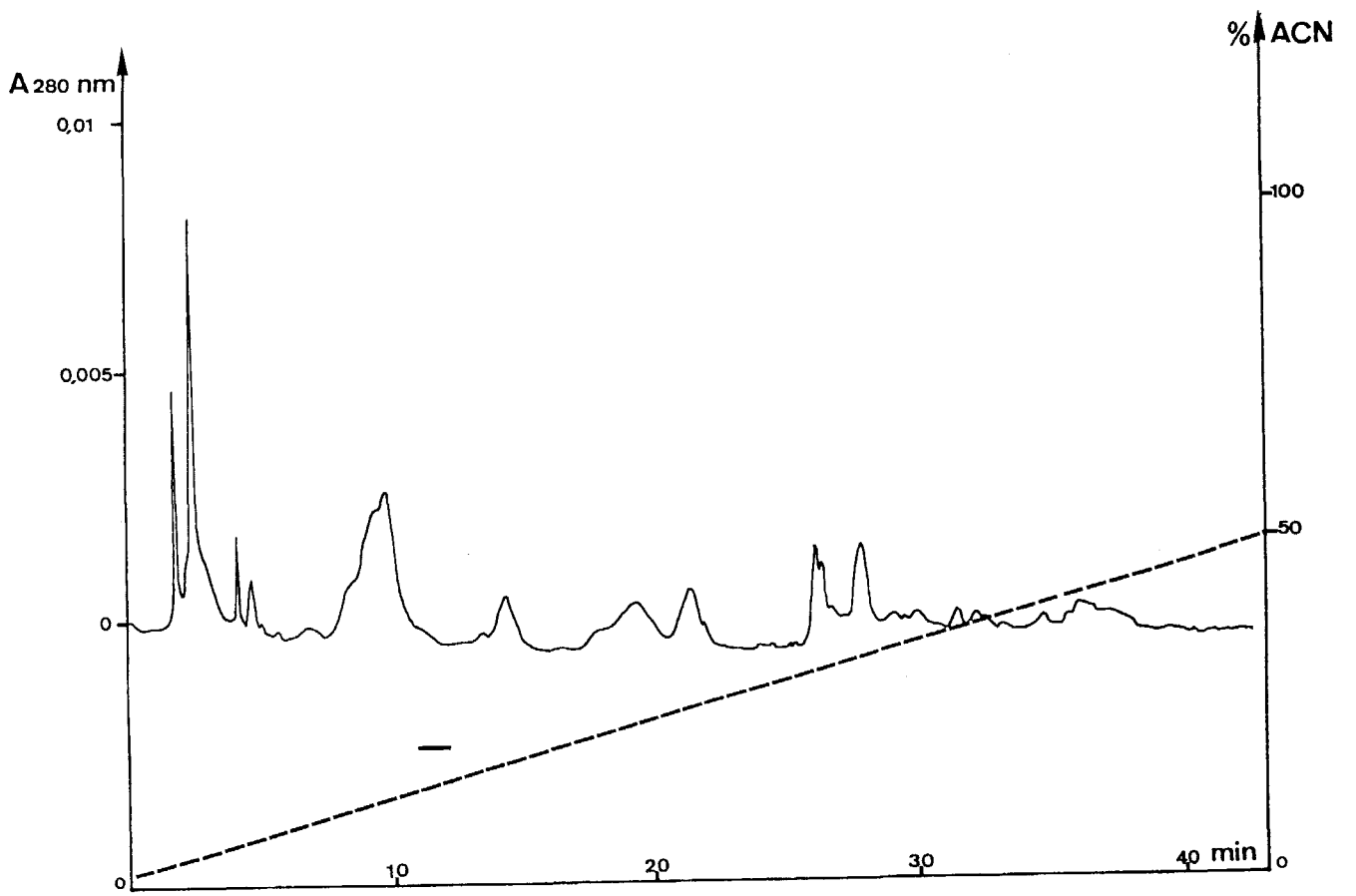

Figure 11 *C3 reversed-phase HPLC elution profile of an extract of 400 brains of T. tessulatum.*
- Elution rate : 1 ml/min ; solvent A : 0.1 % TFA in deionized water : solvent B : 0.1 % TFA in 100 % ACN; solvent program : gradients of B at 1 %/min from 0 to 50 % followed by 3 %/min from 50 to 80 %.
- The solid line indicates absorbance; the dotted line indicates the gradient.
- The immunoreactive AII-like zone is indicated by a solid bar.
- Absorbance was monitored at 280 nm (A280 nm).

to the one detected with a-AII (ca 18 kDa), was recognized. In order to determine if the three antibodies (a-AII, a-γ-MSH and Tt159) recognized the same precursor, a C3 reversed-phase HPLC was conducted on the immunoreactive HPGPC fractions. A peak with a RT of 12-13 min was recognized by the three antibodies (Fig. 11) ,which demonstrated that these antibodies recognize the same protein: the Pro-AII-like.

Discussion

Three AII-immunoreactive peptides (P1, P2, P3) contained in three peaks were isolated in the brain of *T. tessulatum* after reversed-phase HPLC. One of these three peptides (P3) had exactly the same retention time as Vertebrate AII and as the AIIamide recently isolated from the leech *Erpobdella octoculata* (Salzet *et al.*, in preparation). Moreover, an injection on a narobore column of a pool of P3 and either AII-amide or Vertebrate AII revealed in both case a single identical immunoreactive peak to a-AII. Thus, P3 could be an AIIamide as in *E. octoculata* (Salzet *et al.*, in preparation). The two other peptides (P1, P2) recognized by a-AII possessed the same biochemical behavior as respectively AVII and AIV, which allows to postulate that they are similar to Vertebrate AVII and AIV. Considering the fact that AVII and AIV come from the catabolism of AII (Abhold and Harding,1988), such a process of degradation of P3 in P2 and P1 might exist in leeches. This hypothesis was strenghtened by the quantification of the three *T. tessulatum* AII-like peptides after purification, which established that P3 was the major product. Nevertheless, a degradation of AII due to the techniques employed cannot be excluded.

After HPGPC fractionation and immunoblot analysis with a-AII, a protein with a molecular mass of *ca* 18 kDa was detected, it was designated as Pro-AII-like hormone (PAII). Using the same procedure, a polypeptide of *ca* 19 kDa (Pre-Pro-AII-like : PPAII) was immuno-revealed in the *in vitro* translated products of brain RNA extracts. The registered difference of 1 kDa between PAII and PPAII and the observation of the slight increase of RT between the two products after C3 reversed-phase HPLC (data not shown), led to think that a signal peptide was associated to PAII in PPAII. So, in *T. tessulatum* brain, the precursor of AII is a protein with a size of *ca* 19 kDa, size different from the one of the Vertebrate AII precursor (the angiotensinogen: Ao) which is a protein of *ca* 60 kDa (Wei *et al.*, 1988 ; Campbell *et al.*, 1991).

Immunocytochemical observations in *T. tessulatum* having demonstrated that three antibodies (a-AII, a-γ-MSH, Tt159) recognize three distinct epitopes in identical cells located in compartment 4 of the supraesophageal ganglion (Verger-Bocquet *et al.*, 1992), these three antibodies were tested on PAII. They all recognized a protein of *ca* 18 kDa molecular mass with an identical chromatographic behavior in C3 reversed-phase HPLC. These results allowed to think that three epitopes are borne by the same precursor. This postulation was strenghtened by results obtained after an immunoprecipitation with a-AII followed by an immunoblot analysis with Tt159 and a-γ-MSH which demonstrated that the same protein was immunologically detected with the three antibodies (data not shown).

Our results suggest that the AII-like gene codes for several distinct peptides. The presence of large neuropeptide precursors has already been reported in several species of Invertebrates (De Loof *et al.*,1988, 1990). The peptides generated from these large precursors are often involved in asame physiological control. Concerning the peptide recognized by a-γ MSH, sequencing of one of the two purified MSH peptides indicated that it was a degrading product with a sequence of three amino acids residues (FRW) common to the MSH family (Salzet *et al.*, unpublished data). Only the sequencing and the *in vivo* injection to *T. tessulatum* of the second purified MSH-like peptide could inform us if the MSH-like peptide interacts or not with the AII-like peptide on a same physiological function. Nevertheless, it has to be noted that an α-MSH has already been detected in other groups of Invertebrates *e.g.* in the Insect *Locusta migratoria* (Schoof *et al.*, 1988) or in the Mollusc *Aplysia californica* (Taussing and Scheller, 1986).

This work confirms, by a biochemical approach, the presence of a molecule related to mammalian AII in the leech *T. tessulatum*. The most abundant molecule is very likely identical to the AIIamide recently isolated in *E. octoculata* (Salzet *et al.*, in preparation). In contrast, the two other molecules (AIV and AVII) are either natural products of AII catabolism or artefactual products coming from the degradation of AII during the purification. The AII-like precursor (PAII) could be a molecule with a molecular mass of 18 kDa and the preprohormone a protein with a molecular mass of 19 kDa. The PAII bears two different epitopes recognized respectively by an anti-γ MSH and by monoclonal antibody Tt159, it is thus a multi-peptidic precursor which greatly differs from the Vertebrate AII precursor, the angiotensinogen.

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CONCLUSION

La localisation expérimentale dans les follicules paramédians postérieurs du ganglion supra-œsophagien (follicules 4) de la sangsue *T. tessulatum* de cellules productrices d'un facteur hormonal régulateur de la balance hydrique (Malecha, 1979, 1983), nous a conduit dans un premier temps à réaliser une cartographie immunocytochimique de ces follicules. Pour cela, nous avons utilisé des anticorps polyclonaux dirigés contre des peptides de Vertébrés et monoclonaux spécifiques de substances du système nerveux central de *T. tessulatum* . C'est ainsi qu'ont été caractérisés près de la moitié de la trentaine de neurones constitutifs du follicule 4. Ils se répartissent en sept types cellulaires, parmi lesquels quatre ont retenu notre attention, car immunoréactifs à des anticorps dirigés contre des peptides de Vertébrés contrôlant l'osmorégulation. Il s'agit de quatre ou cinq cellules immunoréactives à l'anti-AII , à l'anti-γ-MSH et aux anticorps monoclonaux Tt 7 et Tt 159, d'une cellule immunoréactive à l'anti-vasopressine et à l'anticorps monoclonal Tt 9, d'une cellule immunoréactive à l'anti-ocytocine et à l'anticorps monoclonal Tt 1 et de six cellules immunoréactives à l'anti-FMRF-amide.

Avant l'isolement biochimique des subtances reconnues par ces anticorps, nous avons mis au point une méthode rapide, sensible et répétitive de quantification de ces molécules. Trois tests ELISA ont été conçus : direct, d'inhibition et compétitif. Ces tests nous ont aussi permis de préciser le site de reconnaissance de l'anti-OT utilisé pour une partie dans ce travail. Il s'est révélé être spécifique de la partie C-terminale de l'ocytocine (OT) *i.e.* la séquence Prolyl-X-Glycinamide (X étant un résidu non polaire). Grâce à ces tests de quantification et aux données relatives à l'anti-OT, nous avons entrepris la caractérisation de la molécule apparentée à l'OT (OT-like).

L'hypothèse d'un rôle hormonal de la substance OT-like du cerveau de *T. tessulatum* est soutenue par nos résultats montrant d'abondantes arborisations au niveau de la zone neurohémale des prolongements de neurones exprimant cette substance dans le ganglion supra-œsophagien et une synthèse accrue de cette substance OT-like au cours du stade physiologique 3B, correspondant à la phase de rétention d'eau vraisemblablement nécessaire à l'accumulation des vitellogénines dans le liquide cœlomique (Baert *et al.*, 1991, 1992).

Les différentes voies expérimentales que nous avons suivies pour vérifier l'effet anti-diurétique de la substance OT-like cérébrale ont confirmé les preuves indirectes apportées par Malecha *et al.* (1989a). La comparaison des résultats des injections d'extraits de cerveau de *T. tessulatum* préadsorbés ou non par l'anti-OT mettent, en effet, en évidence l'action anti-diurétique de la molécule OT-like. De

plus, l'injection du peptide PLGa, partie C-terminale de l'OT reconnue par l'anti-OT, entraîne une rétention d'eau, variant en fonction de la dose injectée.

La nécessité de disposer de quantités suffisantes d'OT-like purifiée pour son séquençage, nous a conduit à réaliser son extraction à partir du système nerveux central d'*E. octoculata*, espèce chez laquelle l'OT-like est en moyenne 10 fois plus abondante que chez *T. tessulatum*. Nous avons en outre recherché les stades physiologiques et les parties du système nerveux les plus riches en cette substance. Suite à la découverte de nombreuses cellules surnuméraires (*ca* 150) immuno-réactives à l'anti-OT dans les ganglions génitaux d'*E. octoculata* (SG5 et SG6) (Verger-Bocquet *et al.*, 1991), nous avons réalisé la purification de l'OT-like à partir de ganglions génitaux. Au niveau de ces ganglions, l'OT-like colocalisée avec un épitope immunoréactif à l'anti-FMRF-amide, est en quantité 10 fois supérieure (5 pmol) à celle trouvée dans les autres ganglions segmentaires de la chaîne nerveuse ventrale (0,5 pmol). De plus, celle-ci est trois fois plus abondante chez les animaux immatures que chez les animaux matures.

Après avoir vérifié l'existence d'une substance anti-diurétique dans ces ganglions par l'injection d'extraits de ganglions génitaux d'*E. octoculata* à des *T. tessulatum*, la purification de la substance OT-like des ganglions génitaux d'*E. octoculata* à maturité sexuelle a été engagée. Le séquençage a donné pour la substance OT-like la formule suivante : IPEPYVWD. Ce peptide n'appartient pas à la famille des OT/VP mais possède par contre 100 % d'homologie avec le fragment N-terminal de la myohéméthrine du Sipunculien *Themiste zostericola* (Klippenstein *et al.*, 1976). Apparenté à l'OT sur des critères immunologiques, mais n'ayant pas la structure d'une OT, ce peptide sera désormais appelé peptide IPEP.

Comme nous l'avons exposé précédemment, de nombreux arguments sont en faveur de l'existence du peptide IPEP comme neuropeptide endogène des ganglions génitaux. Cependant, seules des études immunocytochimiques en utilisant un anticorps spécifique et en hybridation *in situ* en utilisant une sonde oligonucléotidique réalisée à partir de la séquence connue, permettront de conclure. S'il s'agit d'un peptide biologiquement actif, cette activité pourrait être due à son extrémité N-terminale, reconnue par l'a-OT et possédant une certaine analogie avec la partie C-terminale de l'OT (PLGa).

La recherche des précurseurs de la substance OT-like a permis de mettre en évidence un homodimère de *ca* 34 kDa pouvant être la Pro-hormone et un monomère de *ca* 19 kDa pouvant être la PréPro-hormone. Bien qu'un épitope apparenté au FMRF-amide soit localisé au niveau de ces ganglions génitaux dans

les neurones reconnus par l'anti-OT, celui-ci n'est pas porté par le même précurseur. De même, le précurseur de l'OT n'est pas reconnu par un anticorps anti-neurophysine, ce qui tend à prouver que le peptide IPEP, comme son précurseur, n'a aucune parenté avec la famille des OT/VP.

L'absence chez les sangsues d'une molécule ayant une structure proche de celle de l'OT et l'existence dans le cerveau d'une molécule apparentée à la lysine-vasopressine (LVP-like) chez *T. tessulatum* (Malecha *et al.*, 1986), nous ont conduit à rechercher une molécule apparentée à la vasopressine chez les sangsues. L'isolement de la LVP-like chez *E. octoculata* ont abouti à la purification d'une lysine-conopressine (LC). Une telle molécule a déjà été purifiée chez les Mollusques (Van Kesteren *et al.*, 1992 ; Cruz *et al.*, 1987 ; McMaster *et al.*, 1992). Sa présence chez une sangsue confirme l'hypothèse de Van Kesteren *et al.* (1992) selon laquelle une molécule de la famille des OT/VP devait être présente chez les Archaemétazoaires, groupe à partir duquel ont divergé les Vertébrés et les Invertébrés. La structure de la PréPro-conopressine du Gastéropode *L. stagnalis* présente 49 % d'homologie avec les MSEL-neurophysines et 45 % d'homologie avec les VLDV-neurophysines (Van Kesteren *et al.*, 1992) ce qui montre qu'à ce niveau de l'évolution, il n'y a pas encore eu différenciation pour la lignée des OT ou pour celle des VP. La présence de LC chez une sangsue , animal appartenant au groupe le plus primitif des Métazoaires cœlomates, permet d'avancer l'hypothèse que la LC pourrait être la molécule ancestrale de la famille des peptides OT/VP. A l'heure actuelle, l'activité biologique de la LC est inconnue. Néanmoins, si on se réfère au rôle des peptides de la famille des OT/VP dans la régulation de la balance hydrique chez les Vertébrés (Guillon, 1989) et chez l'insecte *Locusta migratoria* (Proux *et al.*, 1987), il est possible d'envisager qu'un tel rôle ait été conservé au cours de l'évolution (Mühlethaler *et al*; 1984 ; Vincent et Simmonet, 1986).

Concernant les molécules de la famille des RFa, quatre peptides ont été isolés des ganglions génitaux d'*E. octoculata*. Deux de ceux-ci (le FMRF-amide et le FLRF-amide) avaient déjà été purifiés à partir de chaînes nerveuses d'*Hirudo medicinalis* par Evans *et al* (1991). Par contre, le FM(O)RF-amide et le GDPFLRF-amide n'avaient jusqu'à présent jamais été isolés chez les Hirudinées. Certaines activités biologiques des RFa sont connues chez les sangsues dont un rôle majeur sur la contraction musculaire (Maranto et Macagno, 1984). Cependant leur intervention dans le contrôle d'autres fonctions n'est pas à exclure. En effet, un rôle anti-diurétique des RFamides a récemment été mis en évidence chez le Rat (Majane et Yang, 1991) et chez le Mollusque *Helisoma duryi* (Saleuddin *et al.*, 1992). Une telle action pourrait exister chez les sangsues.

En ce qui concerne la molécule apparentée à l'angiotensine II (AII-like), les résultats de dosages ELISA chez *T. tessulatum* ont montré une augmentation du taux d'AII-like juste après un repas de sang (Salzet *et al.*, 1992d). Ce résultat est à rapprocher de ceux obtenus par Zerbst-Boroffka et Wenning (1986) chez *H. medicinalis* qui montraient une augmentation de l'excrétion urinaire (x 8) immédiatement après un repas. L'AII-like était donc une bonne candidate comme molécule à activité diurétique. Son isolement chez *E. octoculata* a révélé une séquence en acides aminés identique à celle de l'AII de Vertébré avec cependant l'addition d'une amide du côté C-terminal. Son action diurétique a pu être confirmée par injection chez *T. tessulatum*. Ces résultats sont les premiers mettant en évidence une angiotensine chez un Invertébré. De plus, c'est la première fois que la présence d'une amidation est décelée au niveau de l'angiotensine dans le règne animal. Cette AIIamide est également présente chez *T. tessulatum* où elle est accompagnée de deux peptides de dégradation. Leur présence permet de supposer l'existence chez *T. tessulatum*, d'un système enzymatique de catabolisme de l'AIIamide comparable à celui des Vertébrés (Abhold et Harding, 1988). Cette analogie au niveau de la structure primaire de l'AII-like de sangsue et de l'AII de Vertébré ne se retrouve pas quand on se place au niveau des précurseurs. En effet, le précurseur de l'AII-like se présente chez *E. octoculata* et *T. tessulatum*, sous la forme d'une protéine majeure de *ca* 19 kDa pour la PréPro-AII-like et *ca* 18 kDa pour la Pro-AII-like, accompagnée (cas d'*E. octoculata*) ou non (cas de*T. tessulatum*) d'une seconde protéine de *ca* 15 kDa, suspectée être un produit de dégradation. D'autre part, ce précurseur serait multipeptidique, il porterait en plus de l'AIIamide, au moins une molécule apparentée à la MSH et un peptide reconnu par l'anticorps monoclonal Tt159. Il diffère en cela de celui des Vertébrés qui est une α-globuline d'une masse moléculaire de *ca* 60 kDa (Campbell *et al.*, 1991).

La localisation au niveau d'un même précurseur de trois déterminants antigéniques différents, reconnus respectivement par l'anti-γ-MSH, l'anti-AII et le Tt 159 est un résultat important pour la suite de notre travail, en vue de la caractérisation de ce précurseur par les techniques de biologie moléculaire.

En conclusion, nos recherches ont conduit à la détermination de la structure primaire de cinq neuropeptides nouveaux chez les Hirudinées : l'AIIamide, la lysine-conopressine, le GDPFLRF-amide, le FM(O)RF-amide et le peptide IPEP. L'étape suivante de notre travail sera de tester ces peptides naturels ou de synthèse sur notre moldéle biologique, ceci afin d'établir ou de préciser leurs rôles physiologiques et notamment leur incidence sur l'osmorégulation.

La preuve de l'existence de molécules identiques à la fois chez *E. octoculata* et de *T. tessulatum* (cas de l'AII et du peptide IPEP) confirme que les sangsues constituent un groupe zoologique très homogéne dans lequel les caractéristiques du système nerveux central sont conservées d'une espèce à une autre tant d'un point de vue morphologique que fonctionnel.

Chez *T. tessulatum*, sont localisés dans un même follicule du ganglion supra-œsophagien (follicule 4), de l'AIIamide, de la lysine-conopressine, de la MSH-like, des peptides de la famille des RFamides ainsi que de la méthionine-enképhaline et de la leucine-enképhaline (opioïdes récemment séquencés, Salzet *et al.*, inédit). Il est bien connu chez les Vertébrés que de nombreux peptides peuvent interagir les uns sur les autres dans le contrôle d'une même fonction. Par exemple, au niveau du complexe hypothalamo-hypophysaire, l'AII est connue pour stimuler le relargage de la vasopressine (Phillips *et al.*, 1991), qui elle même agit sur l'α–MSH (Howe et Ray, 1985). Le FMRF-amide, qui stimule la libération de la vasopressine (Majane et Yang, 1991), serait sous le contrôle des opioïdes (Raffa, 1988). Il sera donc intéressant de rechercher s'il existe chez les sangsues de telles interactions, notamment en ce qui concerne la régulation de la balance hydrique. Cette recherche de peptides intervenant dans l'osmorégulation devra néanmoins être complétée en prenant en compte des molécules isolées chez d'autres Invertébrés et agissant sur cette fonction, par exemple le Sodium Influx Stimulating peptide (SISP) isolé chez le Mollusque *L. stagnalis* (De With *et al.*, 1991) ou le peptide diurétique purifié chez l'Insecte *L. migratoria* (Kay *et al,.* 1991). Une étude préliminaire chez *T. tessulatum* a montré l'existence chez cet animal d'une molécule apparentée au SISP (Salzet *et al.*, inédit).

L'ensemble de nos résultats est en accord avec l'existence chez les Invertébrés de deux catégories de molécules. Certaines (peptide IPEP, SISP) sont propres à certains phyla et résultent d'une adaptation à certaines contraintes physiologiques, elles n'ont pas été conservées au cours de l'évolution. D'autres (familles des RFa, des OT/VP et peut être des angiotensines) possédent une fonction qui doit être fondamentale puisqu'on les retrouve tout au long de l'évolution.

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