

The VD₁/RPD₂ α 1-neuropeptide is highly expressed in the brain of cephalopod mollusks

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Abstract In certain gastropod mollusks, the central neurons VD₁ and RPD₂ express a distinct peptide, the so-called VD₁/RPD₂ α 1-neuropeptide. In order to test whether this peptide is also present in the complex cephalopod central nervous system (CNS), we investigated several octopod and squid species. In the adult decapod squid *Idiosepius notoides* the α 1-neuropeptide is expressed throughout the CNS, with the exception of the vertical lobe and the superior and inferior frontal lobes, by very few immunoreactive elements. Immunoreactive cell somata are particularly abundant in brain lobes and associated organs unique to cepha-

lopods such as the subvertical, optic, peduncle, and olfactory lobes. The posterior basal lobes house another large group of immunoreactive cell somata. In the decapod *Idiosepius notoides*, the α 1-neuropeptide is first expressed in the olfactory organ, while in the octopod *Octopus vulgaris* it is first detected in the olfactory lobe. In pre-hatchlings of the sepiolid *Euprymna scolopes* as well as the squids *Sepioteuthis australis* and *Loligo vulgaris*, the α 1-neuropeptide is expressed in the periesophageal and posterior subesophageal mass. Pre-hatchlings of *L. vulgaris* express the α 1-neuropeptide in wide parts of the CNS, including the vertical lobe. α 1-neuropeptide expression in the developing CNS does not appear to be evolutionarily conserved across various cephalopod taxa investigated. Strong expression in different brain lobes of the adult squid *I. notoides* and pre-hatching *L. vulgaris* suggests a putative role as a neurotransmitter or neuromodulator in these species; however, electrophysiological evidence is still missing.

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Introduction

Bioactive peptides are important neurotransmitters, neuromodulators and hormones that are involved in crucial physiological and behavioral processes such as feeding, energy flow, growth and water–ion balance in a number of animals (Bogerd et al. 1991; Kastin 2006). Since mollusks represent the second largest group among bilaterian metazoans, they are attractive models to study these

biological processes (Di Cosmo and Di Cristo 2006; Ponder and Lindberg 2008).

The VD_1 / RPD_2 system with its endogenous α -neuropeptides was first discovered in the two giant neurons VD_1 and RPD_2 in the visceral and the right parietal ganglion of the adult pond snail *Lymnaea stagnalis* (Fig. 1; Bogerd et al. 1991, 1993, 1994; Kerkhoven et al. 1992, 1993). In this species, both neurons innervate other central neurons but also distinct skin areas, heart auricle and the opening of the respiratory cavity (Kerkhoven et al. 1993). Interestingly, they express a gene that has sequence similarities to the *R15* gene, which is expressed by the R_{15} neuron of the sea slug *Aplysia californica* (Buck et al. 1987; Kerkhoven et al. 1991). Moreover, homology has been claimed for the respective prohormone of *L. stagnalis* and two R_{15} prohormones of *A. californica*, which are generated by alternative splicing (Bogerd et al. 1991). In *L. stagnalis*, the ϵ , δ , α_1 and β neuropeptides as well as a single aspartate can be cleavage products of the VD_1 / RPD_2 prohormone (Bogert et al. 1991). VD_1 , RPD_2 and R_{15} — and probably their endogenous neuropeptides — are involved in the regulation of cardiorespiratory phenomena (Kerkhoven et al. 1991). The α_1 -peptide is expressed within the CNS of a number of adult gastropods as well as in two bivalve species (Table 2; Kerkhoven et al. 1993). In *L. stagnalis*, it has been shown that the α_1 -peptide is expressed in a rather restricted manner in the VD_1 and RPD_2 neurons of the visceral and parietal ganglia but also in other cell somata and nerve fibers of the cerebral, pedal and abdominal ganglia, as well as in a few peripheral nerves (Fig. 1b).

Gastropods, scaphopods (tusk shells), bivalves and monoplacophorans possess a rather simple CNS that consists of a few pairs ganglia (Bullock and Horridge 1965). Thus, it appears surprising that a recent phylogenetic study proposes that the cephalopods, with their highly centralized central nervous system, constitute the basal offshoot of a conchiferan clade comprising Cephalopoda, Scaphopoda, Gastropoda and Bivalvia (Kocot et al. 2011).

Homologies of ganglia and brain lobes between molluscan clades are poorly established and thus specific neuronal markers are sought after in order to shed light on the origin of the complex cephalopod CNS from a simple, gastropod-like CNS. Therefore, we characterized the VD_1 / RPD_2 system in selected cephalopods by applying an antibody directed against the α_1 -neuropeptide in developmental stages and adults of the following coleoid cephalopods: the decapods *Idiosepius notoides*, *Loligo vulgaris*, *Euprymna scolopes* and *Sepioteuthis australis* and the octopod *Octopus vulgaris*. Intraspecific similarities and differences in the developing and adult cephalopod CNS are discussed and compared to both gastropod and bivalve conditions.

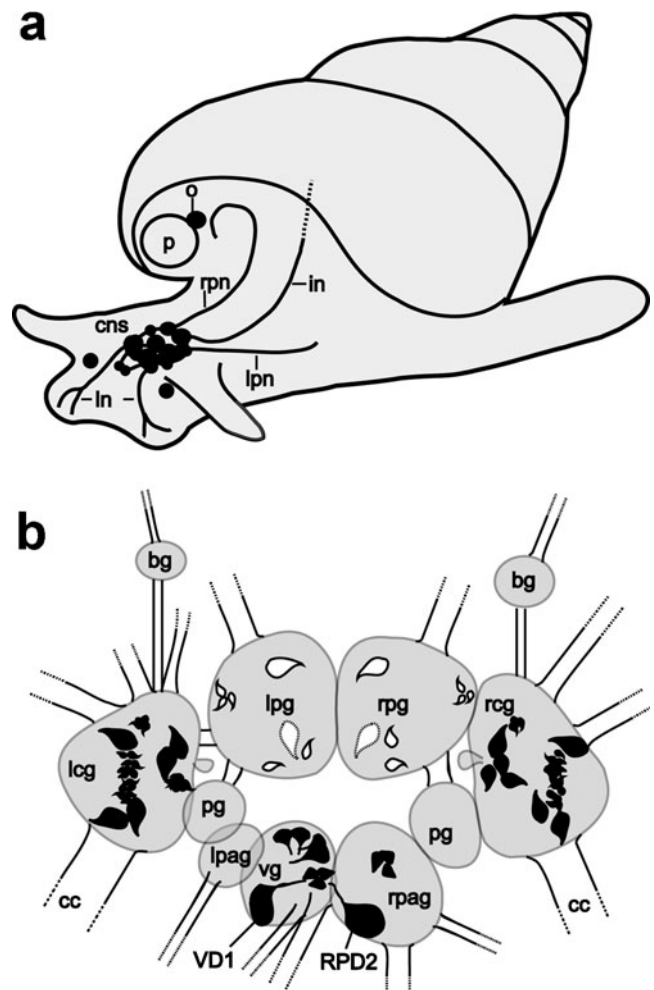


Fig. 1 VR α -ir cell somata within the adult central nervous system of the gastropod *Lymnaea stagnalis*. **a** Schematic outline of the relative position of the CNS. Modified from Kerkhoven et al. (1991). **b** Schematic outline of the relative position of VR α -ir cell somata, including the neurons VD_1 and RPD_2 . Dorsal view on the flat-mounted CNS with the cerebral commissure cut and the left and right cerebral ganglia bent aside. Dorsal cell somata are indicated by *solid lines* and ventral cell somata are labeled with *dashed lines*. See Table 1 for abbreviations. Modified from Kerkhoven et al. (1992)

Material and methods

Animal culture and staging of embryos

Idiosepius notoides

Adult specimens of *Idiosepius notoides* were dip-netted in shallow water of seagrass beds at Moreton Bay, Queensland, Australia. Individuals of *I. notoides* were reared in artificial seawater at 25 °C in tanks in a semi-natural environment. Females attached egg clutches to leaves of seagrass or glass plates. These egg clutches were carefully transferred to six-well plates. During embryonic development, water was changed twice daily and completion took place approximately

9–10 days after laying at 25 °C. All individuals were staged according to Yamamoto (1988).

Euprymna scolopes

Egg clutches were shipped from a rearing facility at the Department of Medical Microbiology & Immunology of the University of Wisconsin (Madison, WI, USA) and were transferred to a recirculating aquarium with artificial sea water (24 °C, 32 ppt) at New Mexico State University, where the developmental stages of *E. scolopes* were reared until hatching (approximately 25 days).

Sepioteuthis australis

Egg clutches were collected from shallow water of seagrass beds at Moreton Bay, Queensland, Australia. Embryos were reared in artificial seawater at 25 °C in tanks in a semi-natural environment until hatching.

Loligo vulgaris

Eggs were harvested from the cages of crab-fishermen in Roscoff, France, in spring 2007. They were transferred to an aquarium with an ambient flow-through-seawater system and were reared until hatching.

Octopus vulgaris

Adults of *Octopus vulgaris* were captured off the coast of Galicia, Spain, in the area Cies–Toralla–Cabo Silleiro, in March 2009. Individuals were transferred to suspended cages in the Ria of Vigo. The specimens were individually weighed and placed in a galvanized cage (3 × 1.5 × 1.5 m) that was suspended from a raft used for mussel production (25 × 25 m). The submerged cages were accessible from the top. Adult octopuses (120 individuals with an initial weight of 880 ± 150 g) were fed mainly fish such as Atlantic horse mackerels (*Trachurus trachurus*), blue whittings (*Micromesistius poutasou*) and occasionally Mediterranean mussels (*Mytilus galloprovincialis*) until they weighed approximately 2.5 kg. Female octopuses were isolated when they laid eggs to allow undisturbed embryonic development. One single female was maintained for up to approximately 1.5 months in order to sample different developmental stages until shortly after hatching. Individual developmental stages were staged according to Naef (1928). For *O. vulgaris*, stages XI to hatchlings (i.e., older than stage XX) were investigated.

Immunocytochemistry

Developmental stages 18 to 30 (cf. Yamamoto 1988) and adults of *Idiosepius notoides*, pre-hatchlings of *Loligo vulgaris*,

hatchlings of *Sepioteuthis australis* and various developmental stages of *Euprymna scolopes* [stage XVI to XIX; cf. Naef 1928; i.e., 14 to 17 days post laying (dpl)], as well as *Octopus vulgaris* (stages X–XIII, XV, XVIII to XX; cf. Naef 1928) were slowly chilled and anesthetized with 7.14 % MgCl₂ in seawater and fixed in 4 % paraformaldehyde in 0.1 M phosphate buffered saline (PBS) at room temperature (RT) for 2 h. Fixed specimens were stored in PBS with 0.1 % NaN₃ at 4 °C. Alternatively, developmental stages and adults of *I. notoides* were fixed in 4 % paraformaldehyde in 0.1 M 3-(N-morpholino) propane sulfonic acid (MOPS), pH 7.5 and rinsed thrice in 70 % EtOH for 15 min. Fixed specimens were stored in PBS with 0.1 % NaN₃ at 4 °C or in 70 % EtOH at –20 °C respectively. Prior to further processing, the fixed animals were rehydrated into PBS.

Developmental stages of *O. vulgaris*, *L. vulgaris*, *S. australis*, *E. scolopes* and the CNS of adult *I. notoides* were embedded in a gelatin–albumine solution. The embedded samples were stored in PBS with 10 % formaldehyde for 12 h at 4 °C and sectioned into 50–150 μm-thick sections using a vibratome (VT1000S, Leica Microsystems, Wetzlar, Germany). Developmental stages of *I. notoides* were processed as whole-mount preparations. The latter and the vibratome sections were rinsed thrice for 15 min each in PBS and another three times for 15 min each in PBS with 2 % Triton X-100 (PBT) to increase tissue permeability. Samples were blocked for 4 to 15 h in PBT with 5 % normal swine serum (NSS; Jackson ImmunoResearch, West Grove, PA, USA) at RT. A primary polyclonal antibody against the VD₁/RPD₂ α₁-peptide (published amino acid sequence: CDMYEGLAGRCQHHPNCPGFN; see Bogerd et al. 1991), raised in rabbit (CASLO Laboratory, Lyngby, Denmark), was applied in a 1:300 to 1:800 dilution. In addition, a primary monoclonal antibody against acetylated α-tubulin raised in mouse (Sigma, Brøndby, Denmark) was diluted 1:800 in blocking medium. Samples were incubated for 48 h at RT in a cocktail containing both primary antibodies. Specimens were rinsed six times for 20 min each in PBS at RT. Subsequently, both secondary fluorochrome-coupled antibodies were diluted in a blocking solution with 1% NSS and applied in a 1:400 to 1:800 dilution. For VD₁/RPD₂ α₁-peptide visualization, Alexa Fluor 594 (anti-rabbit; Invitrogen, Taastrup, Denmark) and for acetylated α-tubulin visualization, Alexa Fluor 488 (anti-mouse; Invitrogen) were chosen as secondary antibodies. After 15–20 h in the dark at RT, 5 % 4', 6-diamidin-2-phenylindol (DAPI, Invitrogen) was added for 2 h to label the cell nuclei in the developing CNS (see Wollesen et al. 2009). Subsequently, samples were rinsed in PBT three times for 20 min each and another three times for 1 h in PBS. Finally, whole-mount preparations and vibratome sections were mounted on glass slides, either in Fluoromount G (Southern Biotech, Birmingham, Alabama, USA) or in Elvanol (Rodriguez and Deinhard 1960) according to Wollesen et al. (2009). Samples were stored in the dark at 4 °C for at least 3 days

prior to examination. Previously described neurons reactive against the VD_1/RPD_2 $\alpha 1$ -peptide in the adult CNS of *Lymnaea stagnalis* served as positive control (Wollesen, unpublished observation; Kerkhoven et al. 1992).

Negative controls (same procedure but omitting either the primary or the secondary antibody) were performed to assess specificity of the labeling experiments and yielded no signal. Preabsorption experiments employing the $\alpha 1$ -neuropeptide and the antibody directed against the latter rendered no signal in any of the species investigated.

Confocal laser scanning microscopy and data analysis

All preparations were examined with a Leica DM IRBE microscope equipped with a Leica TCS SP2 confocal unit (Leica Microsystems, Wetzlar, Germany). Optical sections with a Z-step size of 0.5 μm to 1 μm were generated and digitally merged to yield maximum projection images. These projection images were further processed with Photoshop

9.0.2 software (Photoshop 9.0.2 software (Adobe Systems, Inc., San Jose, CA, USA) to adjust contrast and brightness.

Results

Terminology

The terminology of the CNS applied in this paper is in accordance with Young (1971; 1979), Shigeno and Yamamoto (2002), Yamamoto et al. (2003) and Wollesen et al. (2010a) (Table 1). For consistency with previous neuroanatomical literature, all developmental stages are described with respect to their adult swimming posture. Accordingly, the head is considered anterior, the mantle apex posterior, the funnel ventral and its opposite side dorsal. Elements immunoreactive against the VD_1/RPD_2 $\alpha 1$ -peptide are referred to as $VR\alpha$ -ir elements or $VR\alpha$ -immunoreactivity-expressing elements respectively.

Table 1 List of abbreviations used in figures

abl	anterior basal lobe	og	optic gland
acl	anterior chromatophore lobe	ol	optic lobe
alpl	anterior lateral pedal lobe	oo	olfactory organ
asm	anterior subesophageal mass	p	pneumostome
apl	anterior pedal lobe	pcl	posterior chromatophore lobe
bg	buccal ganglion	pdl	peduncle lobe
bm	buccal mass	pg	pleural ganglion
cc	cerebral commissure	plpl	posterior lateral pedal lobe
cns	central nervous system	pml	posterior magnocellular lobe
dbl	dorsal basal lobe	pn	pallial nerve
dll	dorso-lateral lobe	ppl	posterior pedal lobe
dml	dorsal magnocellular lobe	prl	precommissural lobe
e	esophagus	psm	posterior subesophageal mass
ey	eye	pvl	palliovisceral lobe
fl	fin lobe	rcg	right cerebral ganglion
ibl	inferior buccal lobe	rpag	right parietal ganglion
ibrl	intra-brachial lobe	rpg	right pedal ganglion
ifl	inferior frontal lobe	rpn	right parietal nerve
in	intestinal nerve	sbl	superior buccal lobe
itl	interbasal lobe	sfl	superior frontal lobe
iy	internal yolk	sg	stellate ganglion
lbl	lateral basal lobe	spl	subpedunculate lobe
lcg	left cerebral ganglion	spm	supraesophageal mass
ln	labial nerve	stc	statocyst
lpag	left parietal ganglion	svl	subvertical lobe
lpg	left pedal ganglion	vg	visceral ganglion
lpn	left parietal nerve	vml	ventral magnocellular lobe
mbl	median basal lobe	vsn	visceral nerve
msm	middle subesophageal mass	$VR\alpha$ -ir	immunoreactive against the VD_1/RPD_2 $\alpha 1$ -peptide
o	osphradium	vsl	visceral lobe
ofl	olfactory lobe	vtl	vertical lobe

Gross anatomy of the adult and the developing cephalopod CNS

The CNS of adult coleoid cephalopods is composed of a brain with two laterally positioned optic lobes (Fig. 2). The brain is penetrated by the esophagus and is composed of a dorsal supraesophageal mass that connects laterally to the ventral subesophageal mass. The periesophageal mass (all magnocellular lobes) constitutes the postero-lateral-most part of the brain close to the statocyst (Fig. 2). For detailed descriptions of the development of the cephalopod CNS, see Yamamoto et al. (2003) and Wollesen et al. (2010a) for *Idiosepius*, Shigeno et al. (2001) for *Sepioteuthis*, Meister (1972) for *Loligo* and Marquis (1989) for *Octopus*.

In general, the neurogenic placodes of the cerebral, intra-brachial, optic, palliovisceral, pedal and stellate ganglia are formed by ingress, migration and accumulation of neuroblasts, when the majority of the yolk syncytium of the embryo is covered by the ecto- and mesentoderm. Subsequently, neuropil develops within the core regions of all ganglia. The palliovisceral ganglia develop into the posterior subesophageal mass

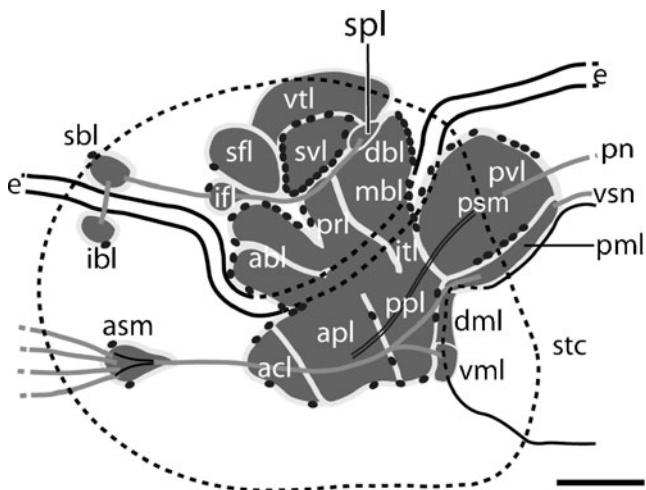
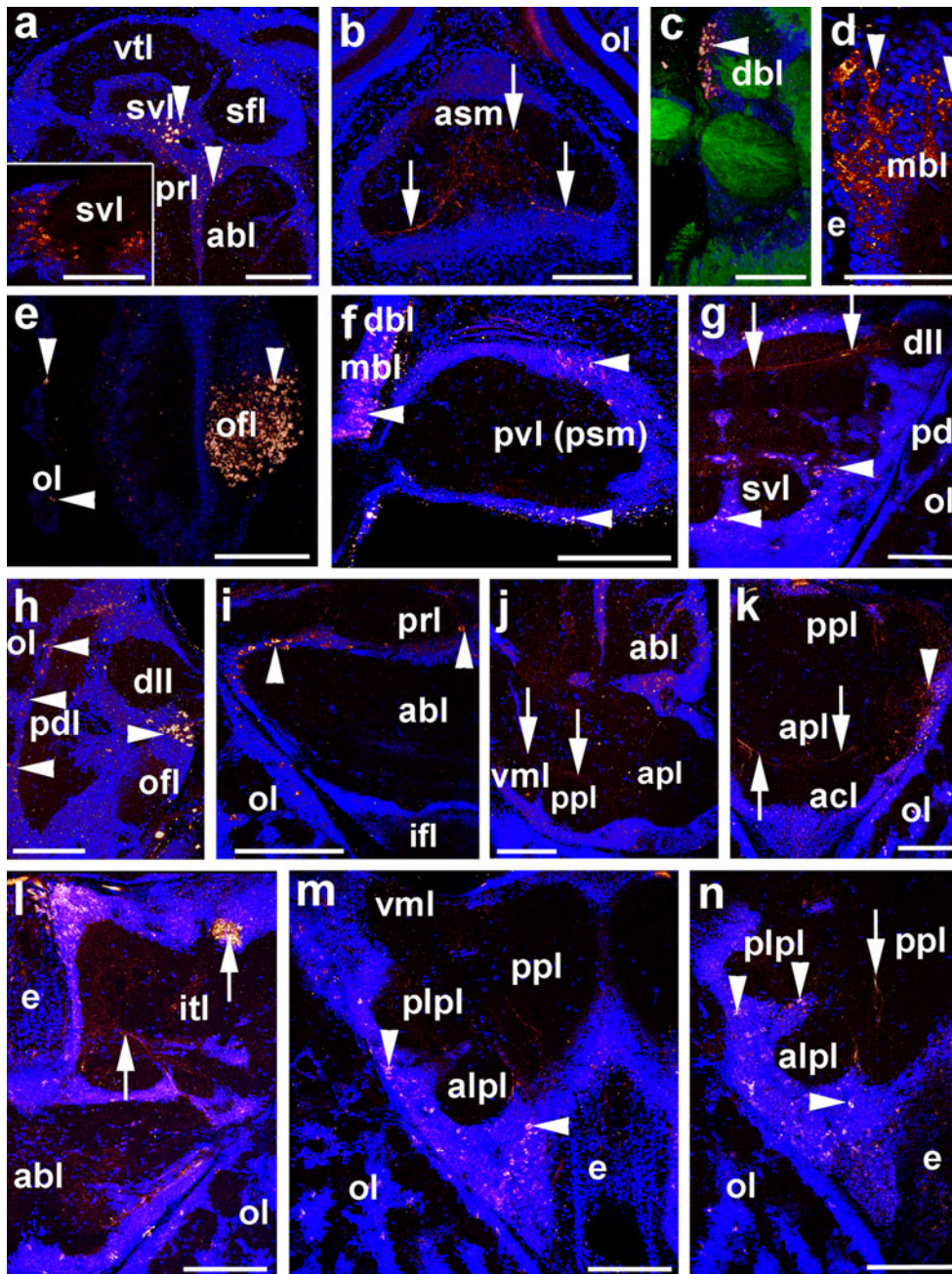


Fig. 2 Schematic outline of the distribution of VR α -ir elements in the CNS of the adult pygmy squid *Idiosepius notoides*. Sagittal section through the middle of the brain with anterior to the left. One optic lobe (dashed circle) attaches on each side to the central brain. The brain is divided by the esophagus into a supraesophageal and subesophageal mass. The boundaries of the various brain lobes are often continuous and therefore arbitrary. VR α -ir cell somata (black dots) and neurites (black lines) are plotted on the CNS with respect to their relative abundance and distribution. In general, faintly fluorescent VR α -ir neurites are distributed throughout the various lobes. Here, only strongly fluorescent VR α -ir neurites are marked. The stellate ganglia, the visceral lobes, the posterior chromatophore lobes, the posterior and anterior lateral pedal lobes, the dorso-lateral lobes, the lateral basal lobes, the fin lobes, the optic tract region and most connectives and commissures are omitted for clarity. The periesophageal mass comprises the ventral magnocellular lobes, the dorsal magnocellular lobes and the posterior magnocellular lobes. All of them are located close to the statocyst (outlined by a solid black line). See Table 1 for abbreviations. Scale bar=300 μ m

and the pedal ganglia give rise to the anterior and the middle subesophageal mass. The cerebral ganglia are the precursors of the supraesophageal mass, while the optic ganglia are termed optic lobes after stage 23. Further development is characterized by increased neuropil growth.

The VD₁/RPD₂ system in the adult CNS of *Idiosepius notoides*

VR α -ir elements were found in various lobes of the CNS (Figs. 2, 3 and 4; Table 3). In general, VR α -ir cell somata of the various lobes were intensely immunolabeled (Fig. 3a), in contrast to neurites, which were rather faintly stained (Fig. 3b,g). Labeled neurites do not necessarily connect to labeled cell somata but few immunopositive somata send immunostained neurites into the neuropils (Fig. 3d). Larger populations of VR α -ir somata are present in the subvertical lobes (Figs. 2; 3a,g; 4a), the posterior basal lobes (Figs. 2; 3c,d; 4b,c), the optic lobes (Figs. 3e, 4b), and the olfactory lobes (Figs. 3e; 4b). In the vertical lobe system of the supraesophageal mass, the subvertical lobe, but not the vertical lobe itself, exhibits VR α -ir cell somata (Figs. 2; 3a,g; 4a). Few VR α -ir perikarya are also present in the precommissural lobes (Figs. 2; 3a; 4b). The inferior and superior frontal lobes exhibit only very few VR α -ir cell somata and are devoid of neurites (Figs. 2; 3a,i; 4a,b). Further VR α -ir cell somata are present in the posterior basal lobes such as the dorsal basal (Figs. 2; 3c,f; 4a), the median basal (Figs. 2; 3d,f; 4b), the dorso-lateral (Figs. 3h, 4a) and the lateral basal lobes (Fig. 4c; Table 3). No VR α -ir cell somata or neurites were observed in the subpedunculate lobes (Figs. 2; 4a). The interbasal lobes exhibit no VR α -ir cell somata but VR α -ir neurites (Figs. 2; 3i; 4c). Few VR α -ir neurites are visible in the neuropils of the posterior basal lobes and in those of the anterior basal lobes (Figs. 3c,d,f,h, i,j; 4a–c). The latter possess another population of VR α -ir cell somata (Figs. 2; 3i; 4c). The optic tract region connects both optic lobes to the central brain (Fig. 4b). The peduncle and olfactory lobes comprise VR α -ir cell somata (Figs. 3e,h; 4b; Table 3). In the periesophageal mass, few VR α -ir cell somata are found in the dorsal magnocellular lobe (Figs. 2; 4c; Table 3). In the posterior and ventral magnocellular lobes, no VR α -ir cell somata were observed (Figs. 2; 3m; 4c,d). Individual VR α -ir cell somata are distributed in the posterior subesophageal mass, e.g., the palliovisceral lobe (Figs. 2; 3f; 4b). VR α -ir fibers are part of a nerve strand interconnecting the posterior subesophageal and the middle subesophageal mass (Figs. 2; 3j; 4b,d). The anterior and posterior pedal lobes as well as the anterior and posterior lateral pedal and the chromatophore lobes possess VR α -ir cell somata in their perikaryal layers and neurites in their neuropils (Figs. 2; 3j,k,m,n; 4d; Table 3). VR α -ir neurites are abundant in the neuropil of the anterior subesophageal mass, however,



only very few cell somata are present (Figs. 2; 3b; 4d; Table 3). Few VR α -ir cell somata are present in the buccal ganglia (Fig. 2; Table 3).

The VD₁/RPD₂ system during cephalopod CNS development

In the following, the spatio-temporal distribution of VR α -ir elements in the CNS of coleoid cephalopods is described.

Idiosepius notoides

The earliest VR α -ir elements are cell somata located in the olfactory organ, which is ventrally attached to the optic lobes of stage 27 individuals (Fig. 5c,d; Table 3). Few individuals exhibit VR α -ir cell somata in the labial region and the intra-brachial lobes (Fig. 5e). In stage 29 individuals VR α -ir cell somata are located in the anterior subesophageal mass (not shown), the middle subesophageal mass and the anterior and

◀ **Fig. 3** VR α -ir elements (red) within the CNS of the adult pygmy squid *Idiosepius notoides*. Neuropil is stained green (acetylated α -tubulin) and cell somata are labeled blue (DAPI). Sagittal vibratome sections with anterior facing to the right if not stated otherwise. **a** VR α -ir cell somata (arrowheads) within the subvertical and precommissural lobes of the vertical lobe system. Note the absence of VR α -ir elements within the vertical lobe. *Inset*: Horizontal section through the right portion of the subvertical lobe (anterior faces down). **b** Horizontal section with anterior facing down, showing rather faintly stained VR α -ir neurites (arrowheads) in the anterior subesophageal mass. **c** VR α -ir cell somata (arrowhead) within the dorsal basal lobes. **d** Horizontal section through the left medial basal lobe close to the esophagus with VR α -ir cell somata (arrowheads) (anterior faces down). **e** The optic lobes and the olfactory lobe house VR α -ir cell somata (arrowheads). **f** The posterior basal lobes and the posterior subesophageal mass exhibit VR α -ir cell somata (arrowheads) (anterior faces to the left). **g** The dorso-lateral lobes are connected via VR α -ir neurites with each other (arrows). VR α -ir cell somata are located in the subvertical lobes (arrowheads). **h** The left peduncle lobe, the olfactory lobe and the dorso-lateral lobes exhibit VR α -ir cell somata (arrowheads). **i** The anterior basal lobe and the precommissural lobes house many VR α -ir cell somata (arrowheads), in contrast to the inferior frontal lobes with only very few somata (not shown) (horizontal section with anterior facing down). **j** The middle subesophageal mass comprises the anterior pedal and posterior pedal lobes, as well as the anterior chromatophore lobes (not shown). Faintly stained VR α -ir neurites (arrows) project from the middle subesophageal mass into the posterior subesophageal mass. **k** Horizontal section of the left portion of the middle subesophageal mass, exhibiting VR α -ir neurites (arrows). **l** Horizontal section of the left portion of the middle subesophageal mass showing VR α -ir neurites (arrows) and an accumulation of VR α -ir neurites in the posterior interbasal lobes. **m** Horizontal section of the right portion of the middle subesophageal mass with the anterior lateral pedal lobes, posterior lateral pedal lobes and VR α -ir cell somata (arrowheads). **n** Horizontal section of the right portion of the middle subesophageal mass with VR α -ir neurites (arrow) and cell somata (arrowheads). See Table 1 for abbreviations. Scale bars 150 μ m (except **d**: 75 μ m)

posterior basal lobes (Fig. 5g). Stage 30 individuals (hatchlings) possess VR α -ir neurites in the anterior and posterior basal lobes and in the posterior middle subesophageal mass (Fig. 5f).

Euprymna scolopes

In stage XVI and XVII individuals, VR α -ir cell somata are present in the periesophageal, posterior, middle and anterior subesophageal masses (Fig. 5c,h; Table 3). In the periesophageal mass, cell somata are located in the perikaryal layers of the ventral magnocellular lobe (Fig. 5c). Further VR α -ir cell somata were found in the stellate ganglia (Fig. 5c). In stage XVIII and XIX individuals, VR α -ir cell somata are also situated in the olfactory organ (Fig. 5c,i) and in the posterior basal lobes, most likely in the perikaryal layers of the dorsal basal lobes (Fig. 5a,j; Table 3). VR α -ir cell somata were also observed in the dorsal magnocellular lobe of the periesophageal mass (Fig. 5c). Further VR α -ir

cell somata are situated in the dorsal perikaryal layer of the inferior or superior frontal lobe (Fig. 5a; arrow that points to supraesophageal mass in Fig. 5j).

Octopus vulgaris

It is not until stage XIX and XX that the pre-hatching paralarvae of *O. vulgaris* exhibit VR α -ir cell somata. These are restricted to the posterior olfactory lobule of the optic tract region (Fig. 5b,k; Table 3).

Sepioteuthis australis

In hatchlings of *S. australis*, VR α -ir cell somata are located in the dorsal perikaryal layer of the posterior subesophageal mass and in the dorsal magnocellular lobe (Fig. 5c,l; Table 3). Further VR α -ir cell somata are present in the optic tract region, most likely in the optic gland and in the posterolateral portion of the subpedunculate lobes (Fig. 5b,m; Table 3).

Loligo vulgaris

For *L. vulgaris*, only the immunohistochemical expression patterns of paralarvae close to hatching were investigated. Within the vertical lobe complex, the vertical lobe houses the largest subset of VR α -ir cell somata and neurites (Figs. 5a; 6a, b; Table 3). The inferior frontal lobes possess some VR α -ir cell somata and neurites, while only a few VR α -ir neurites and no cell somata were observed in the precommissural lobes (Figs. 5a,b; 6b,d; Table 3). The same is true for the subvertical and superior frontal lobes (Figs. 5a; 6a,c; Table 3). The lobes of the posterior basal lobe system do not comprise many VR α -ir elements (Figs. 5a,b; 6c,d; Table 3). The majority of VR α -ir cell somata are associated with the subpedunculate lobes, which exhibit strong VR α -immunoreactivity (Figs. 5a; 6a,c; Table 3). Dorsal basal and dorso-lateral lobes exhibit numerous VR α -ir neurites but no cell somata were observed (Figs. 5a; 6e; Table 3). Some VR α -ir cell somata were observed in the posterior perikaryal layer of the median basal lobes (Figs. 5b; 6d; Table 3). Very few VR α -ir neurites and no cell somata were observed in the lateral basal and interbasal lobes (Fig. 6f; Table 3). The anterior portion of the anterior basal lobes exhibits only few VR α -ir cell somata or neurites compared to the posterior portion of the anterior basal lobes with many VR α -ir cell somata and neurites (Figs. 5b; 6d; Table 3). Among the lobes of the optic tract region, the peduncle lobes possess only few VR α -ir neurites; no VR α -ir cell somata were observed (Figs. 5b; 6d; Table 3). The olfactory lobes house many VR α -ir cell somata and exhibit strong VR α -immunoreactivity in their neuropils (Figs. 5b; 6d; Table 3).

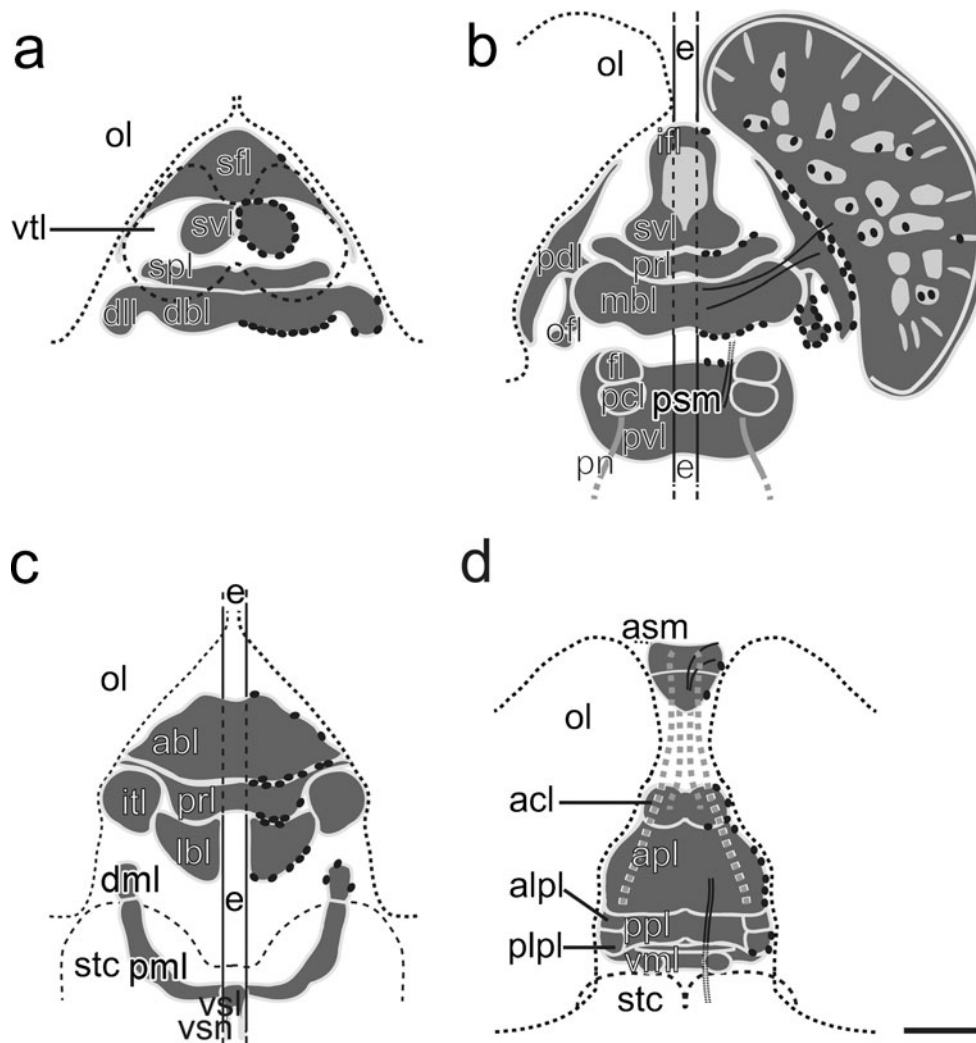


Fig. 4 Schematic outline of the distribution of VR α -ir elements within the CNS of the adult *I. notoides*. VR α -ir cell somata (black dots) and neurites (black lines) are plotted onto the right hemisphere of the CNS with respect to their relative abundance and topographical distribution. The boundaries of the various brain lobes are often continuous and therefore arbitrarily chosen. In general, faintly fluorescent VR α -ir neurites are distributed throughout the various lobes. Here, only strongly fluorescent VR α -ir neurites are marked. The buccal lobes, stellate ganglia, most connectives and commissures are omitted. **a** The most dorsal portion of the supraesophageal mass. The shape of the vertical

lobe is outlined by a dashed line. Optic lobes are omitted. **b** Lower portion of the supraesophageal mass and the posterior subesophageal mass. **c** Parts of the supraesophageal mass surrounding the esophagus. The periesophageal mass comprises the posterior, dorsal and the ventral magnocellular lobe (shown in **d**). **d** The anterior subesophageal mass is located anterior to the middle subesophageal mass, which comprises the anterior chromatophore lobe, anterior pedal lobe, posterior pedal lobe, anterior lateral pedal lobe and posterior lateral pedal lobe. See Table 1 for abbreviations. Scale bar 500 μ m

Within the subesophageal and the periesophageal mass, the largest populations of VR α -ir cell somata are present within the magnocellular lobes and palliovisceral lobe of the posterior subesophageal mass (Figs. 5c; 6f; Table 3). VR α -ir neurites are not very abundant in the middle subesophageal mass (Figs. 5c; 6g–j; Table 3). The majority of VR α -ir neurites are located in the posterior pedal lobes (Figs. 5c; 6g,i; Table 3). VR α -ir neurites are abundant in the neuropil of the anterior subesophageal mass; no VR α -ir cell somata were observed (Figs. 5c; 6j; Table 3).

The superior and inferior buccal lobes exhibit VR α -ir neurites within their neuropils; however, VR α -ir cell somata

only appear to be associated with the superior buccal lobes (Figs. 5a; 6a and j; Table 3). The optic lobes contain no or only very few VR α -ir elements (Fig. 6a,d,g,h,j; Table 3).

Discussion

Expression of the VD₁/RPD₂ α 1-peptide within the adult cephalopod CNS

VR α -ir cell somata and neurites are distributed throughout the entire CNS of the adult decapod squid *Idiosepius*

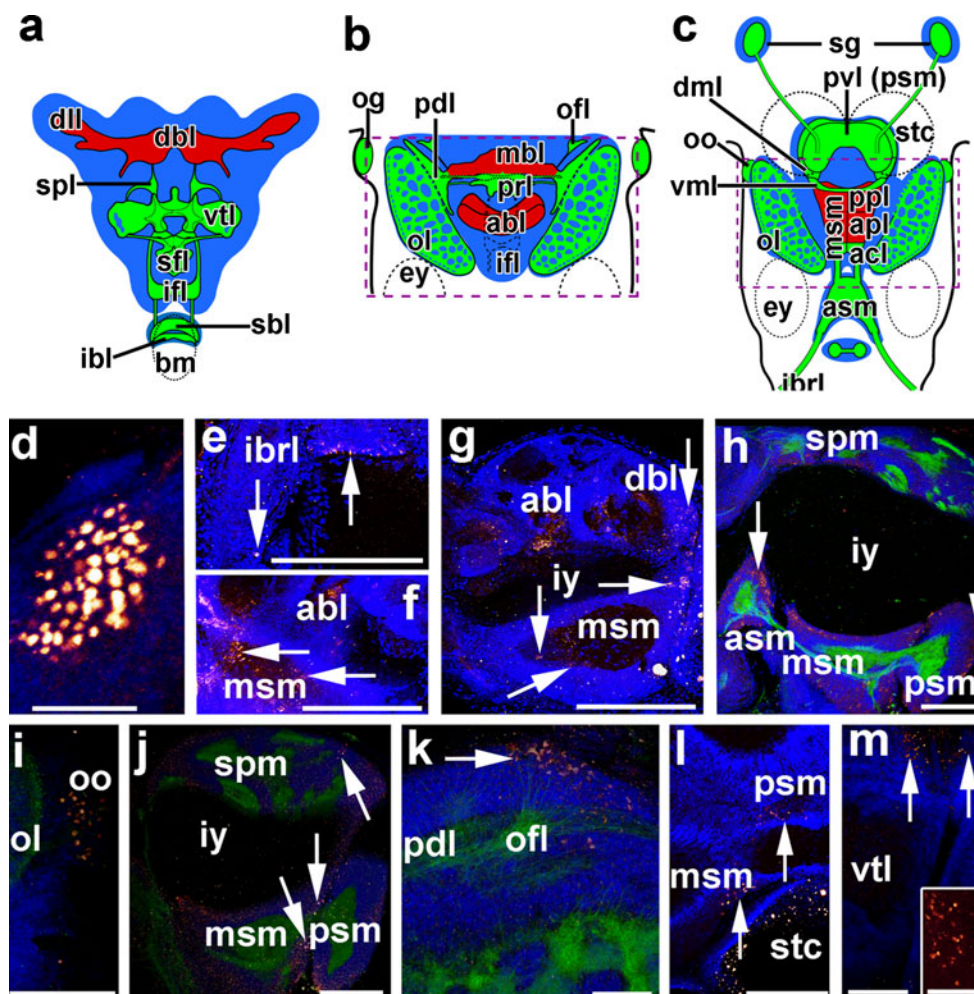


Fig. 5 VR α -ir elements within the developing CNS of the decapods *Idiosepius notoides*, *Sepioteuthis australis*, *Euprymna scolopes* and the octopod *Octopus vulgaris*. **a–c** Schematic drawings of horizontal optical sections through the cephalopod CNS, exemplified by a stage 30 individual (hatchling) of *I. notoides* (anterior faces down; not to scale). Brain lobes that exhibit VR α -ir elements are stained red, the neuropil is labeled green and perikaryal layers are blue. The dashed pink box in **b** corresponds to the one in **c**. Modified from Wollesen et al. (2010a). Immunocytochemistry was performed on whole-mount preparations (**d–g**) or vibratome sections (**h–m**). **a** The dorsal portion of the supraesophageal mass (dorsal third of the brain) includes the vertical and the superior frontal lobes. The inferior frontal lobes are also indicated in **b** (dashed outline). The posterior basal lobes comprise the subpedunculate, the dorso-lateral and the dorsal basal lobes. **b** The median portion of the supraesophageal mass comprises, among others, the anterior basal lobes. The peduncle lobe, the olfactory lobe and the optic gland belong to the optic tract region that connects to the optic lobe. **c** The subesophageal mass (ventral third of the brain) is composed of an anterior, middle and a posterior portion. The dorsal and ventral magnocellular lobes belong to the periesophageal mass. The palliovisceral lobe is the largest portion of the posterior subesophageal mass. The olfactory organ is ventrally connected to the optic lobe. **d–m** Confocal micrographs showing VR α -ir elements (red), neuropil (green; acetylated α -tubulin) and cell somata (blue; DAPI). All images are sagittal sections with anterior to the left, if not stated otherwise. **d** The earliest VR α -ir elements within the CNS of *I. notoides* are cell somata in the olfactory organ of stage 27 individuals (cf **c**; horizontal section and anterior faces down). **e** In a few stage 27 individuals of *I.*

notoides VR α -ir cell somata are present in the labial region and in the intrabrachial lobes (horizontal section and anterior faces down). **f** Stage 30 individuals (hatchlings) of *I. notoides* exhibit VR α -ir elements in the anterior basal lobes, the posterior basal lobes and the posterior middle subesophageal mass (sagittal section with anterior facing to the right). **g** Stage 29 individuals of *I. notoides* exhibit VR α -ir elements in the middle subesophageal mass, the anterior basal lobes and the posterior basal lobes (sagittal section with anterior facing to the left). **h** In stage XVI and XVII individuals of *E. scolopes*, the first VR α -ir cell somata (arrows) are present in the posterior, middle and the anterior subesophageal masses (cf **c**) but not in the supraesophageal mass. **i** VR α -ir cell somata within the olfactory organ in a stage XIX individual of *E. scolopes* (cf **c**; horizontal section and anterior faces down). **j** VR α -ir cell somata (arrows) within the posterior basal lobes and the middle and posterior subesophageal masses of a stage XIX individual of *E. scolopes* (cf **a** and **c**). **k** VR α -ir cell somata (arrow) within the posterior olfactory lobule of a stage XIX *O. vulgaris* (cf **b**; horizontal section and anterior faces to the left). **l** In the CNS of *S. australis* hatchlings, VR α -ir cell somata (arrows) are present in the dorsal perikaryal layer of the posterior subesophageal mass and in the dorsal magnocellular lobe (cf **c**). **m** Left hemisphere of the supraesophageal mass of a *S. australis* hatchling (horizontal section with anterior facing down). VR α -ir cell somata are located within the optic tract region, most likely in the optic gland (right arrow; cf **b**). Further VR α -ir cell somata are present in the postero-lateral subpedunculate lobes (left arrow; cf **a**). Inset: Magnified group of cell somata labeled by left arrow. See Table 1 for abbreviations. Scale bars 150 μ m (except **a,c**: 50 μ m)

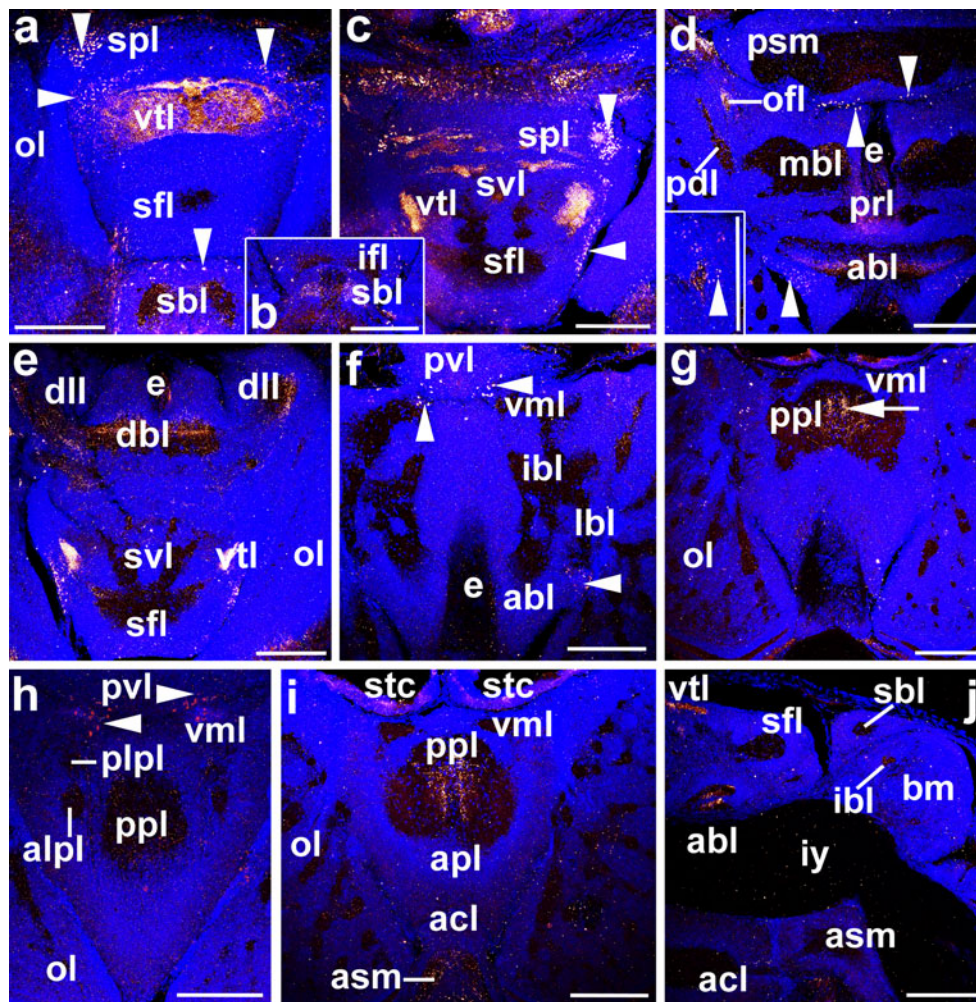


Fig. 6 VR α -ir elements within the CNS of pre-hatchlings of the decapod *Loligo vulgaris*. Brain lobes that exhibit VR α -ir elements are stained red and perikaryal layers are stained blue. Horizontal vibratome sections and anterior faces downwards if not stated otherwise. VR α -ir cell somata are labeled with arrowheads and neurites with arrows. **a** The vertical lobe comprises the largest subset of VR α -ir cell somata and neurites. Other large subsets are associated with the subpedunculate lobes and the superior buccal lobes. No VR α -ir elements are located in the superior frontal lobe. **b** The inferior frontal lobes and the superior buccal ganglia comprise VR α -ir cell somata and neurites. **c** No VR α -ir cell somata and only very few neurites are located in the superior frontal lobe and the subvertical lobes. The subpedunculate lobes also possess many VR α -ir cell somata and neurites. **d** The precommissural lobes contain no or only few VR α -ir elements. Note the subset of VR α -ir cell somata in the anterior perikaryal layer of the

posterior subesophageal mass. The olfactory lobes contain numerous VR α -ir cell somata (*inset*). **e** The dorsal basal and dorso-lateral lobes exhibit VR α -ir fibers but lack cell somata. **f** VR α -ir cell somata in the ventral magnocellular lobes, the palliovisceral lobe and the latero-ventral anterior basal lobes (*arrowheads*). No VR α -ir elements were observed in the lateral basal and interbasal lobes. **g** A few neurites exhibit strong VR α -immunoreactivity in the posterior pedal lobes of the middle subesophageal mass. **h** VR α -ir cell somata in the ventral magnocellular lobes and the palliovisceral lobes (*arrowheads*). No VR α -ir elements were observed in the anterior and posterior lateral pedal lobes. **i** VR α -ir neurites in the neuropils of the posterior pedal lobes and the anterior subesophageal mass. **j** This sagittal brain section shows VR α -ir neurites in the vertical lobe, the anterior basal lobes, the superior lobes and the inferior buccal lobes. See Table 1 for abbreviations. Scale bars 150 μ m

notoides (Figs. 2 and 4; Table 3). Cell somata show intense VD₁/RPD₂ α 1-peptide expression, while neurites are less abundant and stain rather weakly. The vast majority of VR α -ir cell somata are located in the perikaryal layers of the various lobes, except for very few cell somata, which are situated in the inner neuropils of the lobes. VR α -ir cell somata are particularly abundant in the subvertical, posterior basal and optic lobes. In *Loligo vulgaris*, the posterior basal lobes, which house a large number of VR α -ir cell somata in

adults of *I. notoides*, act as control centers for steering and jet movements (dorsal and median basal lobes) and for reproduction (dorso-lateral lobes) (Young 1977). They also innervate the chromatophores (lateral basal lobes) (Young 1977), although the presence of VR α -ir neurites innervating the chromatophores of *I. notoides* remains to be proven. Nevertheless, these data suggest the involvement of this neuropeptide in important and complex behavioral traits of cephalopod squids.

The subvertical lobes possess one of the largest populations of VR α -ir cell somata and connect the vertical lobe to the remaining CNS. The vertical lobe is the highest integration center and is involved in learning and memory (Young 1979). Further VR α -ir cell somata are present in the optic tract region, including the peduncle lobe and the adjacent optic lobes. The peduncle lobe receives visual input from the optic lobe and a “labyrinthine” input from the statocyst (Messenger 1979).

Expression of the VD₁/RPD₂ α 1-peptide during cephalopod CNS development

In our study, VR α -ir elements were found in different but also corresponding regions of the developing CNS. In the decapod *Idiosepius notoides* and the octopod *Octopus vulgaris*, neuropeptide expression commences when the neuropils of the various lobes are already differentiated to a high degree. In both species, the neuropeptide is first expressed in late paralarvae, i.e., in individuals that are close to hatching. Shared expression domains are lobes or organs associated with the

optic tract region, such as the olfactory lobe or the olfactory organ. This is also the case for prehatchlings of the decapods *Sepioteuthis australis*, *Euprymna scolopes*, *Loligo vulgaris* and, as already mentioned, for the adult CNS of *I. notoides*. The olfactory organ, which possesses numerous VR α -ir cell somata in two cephalopod species examined, has been suggested to be of chemoreceptive function (Wildenburg 1997). This may be an important aspect for prey location, mate identification and reception and avoidance from predators, all key behaviors commonly observed in adult cephalopods.

In *Loligo vulgaris*, *E. scolopes* and *S. australis*, prehatchlings possess VR α -ir cell somata in the periesophageal mass. Additional cell somata are present in the perikaryal layers of the posterior subsophageal mass of *S. australis*, *L. vulgaris* and *E. scolopes*. Compared to other neuropeptides or neurotransmitters such as FMRFamide-related peptides or serotonin (5-HT), the α 1-neuropeptide is expressed relatively late during ontogenesis and not stringently first in the subsophageal mass of the CNS (Wollesen et al., 2010a, 2010b).

Table 2 Expression of the VD₁/RPD₂ α 1-peptide within the CNS of the molluscan groups investigated so far, compiled from several sources (Bogerd et al. 1991, 1993, 1994; Kerkhoven et al. 1992, 1993). VR α -ir elements or entire neural structure absent (-)

Ganglion Species Gastropoda Pulmonata	Caudal regions of visceral (vsg) and right parietal ganglia (rpg)	Cerebral ganglia	Pedal ganglia	Abdominal ganglion	Peripheral nervous system
<i>Bulinus truncates</i> , <i>Lymnaea stagnalis</i> ("Basommatophora")	single giant cell soma & 2–5 medium-sized cell somata	3–5 medium-sized cell somata & 1 cluster of small cell somata	2–4 small cell somata	-	neurites, connectives, peripheral nerves
All Stylommatophora species investigated	2–4 giant cell somata in partly or fully fused vsg and rpg	-	Medium and/or small sized cell somata	-	-
<i>Achatina fulica</i> (Stylommatophora)	1 giant cell soma in caudal margin of rpg near the vsg. 1 giant cell soma in ganglion located at the caudal margin of the vsg. 10–15 medium- sized cell somata in vsg & rpg.	-	-	-	-
<i>Limax maximus</i> (Stylommatophora)	3 giant & 5–8 medium-sized cell somata in rpg & vsg.	-	2–4 medium sized cell somata	-	-
<i>Cepea sp.</i> (Stylommatophora)	2 pairs of adjacent giant cell somata (1 in vsg & 1 in rpg)	-	-	-	small & medium- sized cell somata in different ganglia
<i>Eobania vermiculata</i> (Stylommatophora)	2 giant cell somata in fused vsg/ rpg. Medium-sized cell somata close to them & 1 giant cell somata in rpg	-	-	-	innervation of pedal ganglia
<i>Helix aspersa</i> , <i>H. pomatia</i> (Stylommatophora)	2 giant & several small cell somata in subsophageal ganglion	-	-	-	innervation of pedal ganglia
"Opisthobranchia"					
<i>Aplysia brasiliiana</i> , <i>A.</i> <i>californica</i>	-	-	-	1 giant cell soma on caudal margin of right hemiganglion, 10–15 medium-sized cell somata in medial part of fused ganglion	-
Caenogastropoda					
<i>Viviparus viviparus</i> , <i>Nassarius reticulata</i>	-	small cell somata & neurites	-	-	other ganglia not studied
Bivalves					
<i>Mytilus edulis</i> , <i>Anodonta sp.</i>	40–50 small cell somata & neurites	several cell somata	-	-	-

VR α -ir elements or entire neural structure absent (-)

Table 3 Expression of the VD₁/RPD₂ α1-peptide within cell somata of the adult and the developing CNS of the cephalopod species investigated in this study. In the literature, the anatomy and terminology of the octopod and decapod CNS differ in parts from each other. Since expression of the VD₁/RPD₂ α1-peptide in octopods is restricted to CNS regions for which the same terms are used as in their decapod counterparts, this common terminology is followed herein

Species, developmental stage CNS region	Idiosepius notoides adult	Idiosepius notoides stage 30 hatchlings	Euprymna scolopes stages XVI–XIX	Sepioteuthis australis hatchlings	Loligo vulgaris almost hatching	Octopus vulgaris stages XIX–XX almost hatching	
Subesophageal mass	Anterior	+	-	-	-	-	
	Middle	Anterior chromatophore lobes	++	+++ only in stages XVI and XVII	-	-	-
		Anterior pedal lobes	++	+++ only in stages XVI and XVII	-	-	-
	Posterior	Posterior pedal lobes	+	+	-	++	-
		Palliovisceral lobe	+	-	++	++	-
		Posterior chromatophore lobes	+	-	+++ only in stages XVI and XVII	-	-
		Visceral lobes	+	-	+++ only in stages XVI and XVII	-	-
	Periesophageal mass	Fin lobe	+	-	+++ only in stages XVI and XVII	-	-
		Ventral magnocellular lobes	-	-	-	++	-
		Dorsal magnocellular lobes	+	-	++ only in stages XVIII and XIX	-	-
Posterior magnocellular lobes		-	-	-	-	-	
Vertical lobe		-	-	-	-	-	
Subvertical lobes		+++	-	-	-	+++	-
Superior frontal lobes		+	-	++ (?) only in stages XVIII and XIX	-	-	
Inferior frontal lobes		+	-	++ (?) only in stages XVIII and XIX	-	-	
Precommissural lobes		++	-	-	-	-/+	-
Dorsal basal lobes		+++	++	++ (?) only in stages XVIII and XIX	-	-	-
Suprasophageal mass	Vertical lobe complex	Dorso-lateral lobes	++	-	-	-	
		Median basal lobes	+++	-	-	+	-
	Lateral basal lobes	++	++	-	-	-	-
	Interbasal lobes	-	++	-	-	-	-
	Subpedunculate lobes	-	++	-	+++ (?)	+++	-
	Posterior basal lobes	Anterior basal lobes	++	++	-	+++	-
		Optic tract region	++	-	-	-	-
	Optic lobes	Superior buccal lobes	Superior buccal lobes	+++	-	+++	+++
			Inferior buccal lobes	+	-	-	+/-
		Stellate ganglia	Stellate ganglia	n.i.	-	n.i.	n.i.
Optic gland			n.i.	-	+++ (?)	-	-
Peripheral lobes, ganglia, and tissues		Optic gland	n.i.	-	-	-	-
		Olfactory organ	n.i.	-	+++ only in stages XVIII and XIX	-	-

High (+++), medium (++) and low density (+) of VRα-ir cell somata. No VRα-ir cell somata present (-). Not investigated (n.i.). Identity of brain lobe or tissue ambiguous (?).

Paralarvae of *L. vulgaris* close to hatching exhibit the largest population of VR α -elements (Fig. 6). The largest subset of VR α -cell somata is located in the vertical lobe, a region that does not exhibit VR α -immunoreactivity in any of the other cephalopod species investigated so far (Figs. 2, 4, 5 and 6 in present study). In adults of the cuttlefish *Sepia officinalis* and *I. notoides*, as well as in developmental stages of *I. notoides* and *O. vulgaris*, none or only very few cell somata in the vertical lobe express serotonin (5-hydroxytryptamine) (Boyer et al. 2007; Wollesen et al. 2010b; Wollesen et al. 2012).

In *L. vulgaris*, VR α -ir elements are present in the majority of brain lobes but appear to be entirely missing in the superior frontal lobe (Fig. 6a,b). In developmental stages of all other cephalopod species observed, the superior frontal lobe does not appear to house VR α -ir elements (present study). The same is true for the neurotransmitter serotonin, which is expressed in none or only very few cell somata and neurites in the superior frontal lobe in developmental stages of *I. notoides* and *O. vulgaris*, as well as in adults of *I. notoides* and the cuttlefish *Sepia officinalis* (Boyer et al. 2007; Wollesen et al. 2010b; Wollesen et al. 2012).

Prior to hatching, cephalopods may undergo crucial neurophysiological modifications in order to cope with different environmental regimes as juveniles. In addition, interspecific neuroanatomical and physiological differences exist due to the adaptation to different habitats, i.e., benthic vs planktonic life history strategies. Both facts may account for differences in timing and location of VR α -expression in the specimens investigated.

Comparison of the expression of the VD₁/RPD₂ α 1-peptide in the gastropod, bivalve and cephalopod CNS

Table 2 summarizes the different expression domains of the VD₁/RPD₂ α 1-peptide in gastropods and bivalves, i.e., all molluscan taxa investigated so far. In all cephalopods investigated, except for developmental stages of *I. notoides* and *O. vulgaris*, VR α -ir cell somata are present in different regions of the supraesophageal mass, which is considered a counterpart of the cerebral ganglia of other mollusks (Table 3; Bullock and Horridge 1965). Homology of the various brain regions of cephalopods and other mollusks, however, is difficult to assess due to the unique degree of complexity of the cephalopod CNS. Only in “prosobranch” and “basommatophoran” gastropods as well as in bivalves, VR α -ir cell somata are present in the cerebral ganglia (Table 2). Except for the “prosobranchs”, the largest groups of VR α -ir cell somata are usually present in the partially or fully fused parietal and visceral ganglia of gastropods (Table 2; Fig. 1b).

The posterior subesophageal mass of cephalopods and possibly the visceral ganglia of bivalves, are thought to correspond to the visceral/ pleurovisceral ganglia of

gastropods (Bullock and Horridge 1965). In bivalves as well as cephalopods, with the exception of the developmental stages of *I. notoides* and *O. vulgaris*, these ganglia house many VR α -ir cell somata (Tables 1 and 2). Kerkhoven et al. (1993) report VR α -ir cell somata in the visceral ganglia of *Mytilus edulis* and *Anodonta spec.* Since such ganglia are, however, absent in bivalves, these somata may belong to the visceral ganglia of these species instead (cf Bullock and Horridge 1965; Ellis and Kempf 2011). The middle subesophageal mass of adult *I. notoides* and stage XVI and XVII individuals of *E. scolopes* contains another subset of VR α -ir cell somata (Table 3). This portion of the cephalopod CNS is assumed to correspond to the pedal ganglia of other mollusks (Bullock and Horridge 1965). Previous studies have only described VR α -ir cell somata in the pedal ganglia of “basommatophoran” and “stylommatophoran” gastropods (Table 2).

Interestingly, in cephalopods, VR α -ir cell somata are particularly abundant in many brain lobes and organs that are considered evolutionary innovations of this clade, such as the stellate ganglia, optic lobes, lobes of the adjacent optic tract region, vertical lobes, subvertical lobes and olfactory organs and optic glands (Table 3). This demonstrates the high potential of the VR α -ir system for recruitment into novel neural structures. It thus calls for further comparative investigations in other mollusks and the Lophotrochozoa to better understand how neuropeptides are recruited for cognitive processes and behavior in developmental stages and adults.

In summary, VR α -ir elements are abundant and widely distributed throughout the CNS of the adult pygmy squid *Idiosepius notoides* and in *Loligo vulgaris* specimens close to hatching. Future electrophysiological experiments may contribute to assessing whether or not the α 1-neuropeptide acts as a neurotransmitter and/or a neuromodulator. Moreover, VR α -ir cell somata are present in high densities in brain lobes that are unique for coleoid cephalopods and thus do not appear to have a counterpart in other mollusks.

The species-specific distribution of VR α -ir cell somata in developmental stages of all cephalopods investigated suggests a specific recruitment of the α 1-peptide into the neural control of certain behaviors ascribed to these specific brain regions. The overall spatio-temporal plasticity of α 1-peptide expression during cephalopod ontogeny indicates that the expression domains are evolutionary and ontogenetically labile among the various cephalopod species (cf Table 3). Since only adults of a single cephalopod species were investigated, any conclusions concerning the evolutionary conservation of the α 1-peptide expression domains in adult cephalopods would be premature. This study and previous investigations on cephalopods, gastropods and bivalves, demonstrate that VR α -ir cell somata are numerous in the diverse ganglia and brain lobes that are assumed to be

homologs of each other (Bullock and Horridge 1965). Although data on scaphopods and monoplacophorans are still missing, it is probable that VR α -ir elements are part of the conchiferan ground pattern. Future work on the VD₁/RPD₂ system in the relatively simple CNS of basal molluscan groups such as polyplacophorans and the worm-shaped neomeniomorph and caudofoveate aplacophorans will allow comparisons with gastropod and cephalopod conditions. These future studies should clarify whether the distribution of the VD₁/RPD₂ α 1-peptide was part of the ancestral molluscan neural bodyplan, or whether this system evolved at a later stage in more derived mollusks.

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