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Review

Listening to neuropeptides by microdialysis: Echoes and new sounds?

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ABSTRACT

Neuropeptides represent the largest class of neuromessengers in the central nervous system. They are involved in the regulation of growth processes, reproduction, social behavior, emotion/motivation and cognition. Particularly in subcortical structures, neuropeptides act as neuromodulators, which reach their target sites via diffusion through the extracellular space. This route of information transfer together with the ability of neurons to release neuropeptides from their whole membrane surface predisposes neuropeptides for microdialysis experiments. This review outlines the special characteristics of neuropeptide signaling in relation to other classes of neuromessengers. It further provides a survey of the application of the microdialysis technique for monitoring neuropeptide release patterns in laboratory rodents exemplarily for the two neuropeptides arginine vasopressin and oxytocin, discusses pros and cons of such experiments and outlines perspectives for future neuroendocrine studies in rats and mice.

Contents

1. Neuromessengers

Chemical signaling is a fundamental principle of communication between neurons. The chemical compounds responsible for this information transfer (here called neuromessengers) are known as neurotransmitters or neuromodulators, depending on whether they affect neuronal activity directly (neurotransmitters de- or hyperpolarize target neurons primarily by activating ion channel proteins) or modulate the direct effects caused by neurotransmitter action (neuromodulators alter intracellular signaling primarily via G protein-coupled receptors; Box 1). In a prototypic situation, neuromessengers are stored in vesicles and released from presynaptic terminals. After binding to specific receptors at post- or presynaptic sites, they trigger alterations in intracellular signaling. They are eliminated from the extracellular space either by (i) specific uptake mechanisms with subsequent recycling and/or intracellular degradation, (ii) enzymatic extracellular cleavage or (iii) internalization of the receptor–ligand complex. Today, several different classes of neuromessengers are distinguished (Table 1), including L-amino acids (e.g. glutamate, taurine, glycine, gamma amino butyric acid), D-amino acids (e.g. D-serine), biogenic amines (e.g. dopamine, noradrenaline, serotonin), purines (e.g. ATP, ADP), acetylcholine, gaseous messengers (e.g. nitric

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Box 1
Semantics of neuroendocrinology

Neuroendocrinology stands for the scientific discipline dealing with chemical signals that originate from and/or act within the CNS. There are several different semantic categories, which help to classify those signaling molecules.

Category 1: Chemical nature of the signals

As described in detail in Table 1, several classes of neuromessengers can be distinguished on the basis of their chemical nature (e.g. amino acids, biogenic amines, purines, gaseous messengers, lipid derivatives, neuropeptides).

Category 2: Range of action

Neuromessengers might act locally at their site of release in an *autocrine* (binding to receptors at their neurons of origin) or *paracrine* manner (binding to receptors on other neurons or glial cells in close vicinity to their site of release). If released non-synaptically, i.e. from any part of the neuronal surface, neuromodulators may diffuse over long distances to remote brain areas. Signaling molecules released into the blood are called *hormones*.

Category 3: Mode of action

Neurotransmitters directly alter the activity status of the target neuron, primarily by activating ion channels at postsynaptic sites. *Neuromodulators*, in turn, tune the effects of neurotransmitters primarily via binding to G protein-coupled receptors, without affecting neuronal activity in the absence of transmitter signalling. *Retrograde messengers* originate from postsynaptic sites and control presynaptic activity. Often the term neurotransmitter is used to describe the class of neuromessengers, which are involved in point-to-point signaling (i.e. wired transmission) with presynaptic release and binding to postsynaptically localized receptors. Conversely, neuromodulators stand for neuromessengers involved in volume transmission, i.e. they reach distant target sites via diffusion in the extracellular space. Neuromessengers typically mediate their effects via multiple and variable modes of actions (Landgraf and Neumann, 2004).

oxide), neurotrophins (e.g. brain-derived neurotrophic factor), lipid derivatives (e.g. arachidonic acid, endocannabinoids, endovanilloids) and peptides (i.e. neuropeptides; Table 2).

2. What are neuropeptides?

Coined by David de Wied in the 1970s (for reviews see Klavdieva, 1995; Strand, 2000), the term "neuropeptide" has undergone a variety of interpretations which rather softened than sharpened the distinction of neuropeptides from other signals in the body. We adhere to a more conservative version, according to which *neuropeptides are endogenous peptidergic neuromessengers, which are synthesized by and released from nerve cells and involved in nervous system functions. Neuropeptides may act as precursors for smaller neuroactive peptides, which result from neuropeptide degradation*. Depending on their route of action, neuropeptides might serve as neurotransmitters or neuromodulators within the central nervous system. Furthermore, a number of neuropeptides are also secreted into the blood, where they act as hormones. Neuropeptides are 3 to 100 amino-acid residues long and, thus, much larger than classical neurotransmitters such as amino acids or acetylcholine (Hökfelt et al., 2000, 2003). They are predominantly synthesized in subcortical brain structures, including hypothalamus, bed nucleus of the stria terminalis (BNST) and amygdala complex, as part of large precursor molecules. More than 200 neuropeptides have been described to contribute to brain signaling, with more than 50 neuropeptides frequently studied in the rat and mouse central nervous systems (see Table 2; Merighi, 2002; Hökfelt et al., 2000).

3. Modes of interneuronal communication

With deeper insights into the characteristics of the different neuromessenger systems, it has become evident that "classical" neurochemical signaling between neurons from the pre- to the postsynapse represents the exception rather than the rule. Only the class of amino-acid messengers seems to be largely restricted to this type of point-to-point signaling (also called wired transmission; Fig. 1). Other classes of neuromessengers (e.g. lipid derivatives, gaseous transmitters or neurotrophins) originate from postsynaptic sites and transfer information to presynaptic targets in a retrograde manner (Fig. 1; retrograde messengers, Table 1). Strikingly enough, they are often synthesized and released on demand and, thus, not necessarily stored in vesicles. The observation that biogenic amines (i.e. dopamine) are released in a "fuzzy" manner from extrasynaptically localized axonal swellings (varicosities) and reach potential distant target sites via diffusion in the extracellular space (also called volume transmission; Fig. 1; Table 1; Agnati et al., 1995; Zoli et al., 1999) was surprising. But even more so was the discovery that neuropeptides might be released not only from axon terminals and axons *en passant*, but almost from the whole surface of a neuropeptidergic neuron (Pow and Morris, 1989). Similar to biogenic amines, neuropeptides may reach distant target sites via diffusion through the extracellular space, what most likely explains the mismatch between the sites of synthesis/release and the localization of respective binding sites (Herkenham, 1987). Other than in wired transmission, where signaling is defined by topology and efficient uptake and/or degradation processes that prevent spill-over of transmitters to neighboring synapses, the specificity of neuropeptide signaling is achieved by their chemical complexity and the high binding affinity to their receptors (Landgraf and Neumann, 2004). This explains why a few transmitters are sufficient for the myriads of wired pathways seen in cortical structures, whereas a large number of different neuropeptides act as neuromessengers in phylogenetically older brain structures in any species (Hökfelt et al., 2003; Landgraf and Neumann, 2004).

4. Listening to neuropeptides

Although there are different approaches for neurochemical monitoring of neuropeptide signaling within the brain, only few tools provide an appropriate insight into its spatial and temporal dynamics. Today, brain punches are disregarded as adequate tools as they provide snap-shots of peptide concentrations at best, but are unable to detect dynamic changes in neuropeptide release. Moreover, they cannot differentiate between intra- and extracellular origin, thus rendering it impossible to identify the proportion of biologically active (i.e. extracellular) vs. inactive (i.e. intracellular) neuropeptides. Samples obtained from the cerebrospinal fluid (CSF) may allow to draw conclusions about changes in neuropeptide release. However, neuropeptide concentrations within the CSF only provide a rough temporal and spatial integration over release processes throughout the entire central nervous system. The physiological significance of neuropeptides present in the CSF is far from being clear, in particular as neuropeptide concentrations are considerably lower in the CSF than in the extracellular fluid, and neuropeptides reach the CSF by diffusion and bulk flow (Landgraf and Neumann, 2004). Hence, despite its value for diagnostic purposes in human patients, the CSF, from a

Table 1
Classes of neuromessengers selected according to their chemical nature

Class	Examples	Messenger characteristics
L- and D-amino acids	Glutamate, GABA, glycine, taurine, D-serine	Prototypic neurotransmitters of wired transmission; activate ion channels and G protein-coupled receptors; range of action confined to less than 100 μm around release sites; inactivation by uptake into neuronal terminals or glial cells via specific transporters and subsequent degradation/recycling
Acetylcholine		Binds to ion channels and G protein-coupled receptors; inactivation by extracellular degradation
Biogenic amines	Dopamine, noradrenaline, serotonin	Synaptic and extrasynaptic release; wired and volume transmission; inactivation by uptake into neuronal terminals or glial cells via specific transporters and subsequent degradation/recycling
Gaseous messengers	Nitric oxide (NO)	On-demand synthesis in postsynaptic terminals (no vesicular storage); retrograde messengers from post- to presynaptic sites; spatially restricted range of action; activate intracellular targets; degradation in presynaptic terminals
Lipid derivatives	Anandamide, 2-arachnoidyl glycerol	On-demand synthesis in postsynaptic terminals (no vesicular storage); retrograde messengers from post- to presynaptic sites; spatially restricted range of action; activate G protein-coupled receptors; intracellular inactivation by pre- or postsynaptically localized enzymes
Neuropeptides	See Fig. 4 and Table 2;	Storage in large vesicles; release from the whole neuronal surface; dissociated release from axon terminals and dendrites/soma possible; high receptor affinity (G protein-coupled receptors) and large range of action; inactivation by extracellular degradation by peptidases or by internalization of the receptor–ligand complex followed by intracellular degradation.
Purines, pyrimidines	ADP, ATP UDP, UTP	Release from neurons and glial cells; binding to G protein-coupled receptors or ligand-gated ion channels.

physiological perspective, might simply serve as “sewerage”, which helps to clear the brain from neuromessengers including neuropeptides and their neuroactive fragments.

Two major technical advances have enabled the monitoring of neuropeptides at the site of their release, thus revealing their participation in intercellular neuropeptidergic communication: first, the development of radioimmunoassays as highly sensitive analytical tools for the detection of subtle amounts of neuropeptides and, second, the implementation of *in vivo* sampling techniques (push–pull perfusion, microdialysis), which allowed collecting neuropeptides from the extracellular space of defined brain structures in freely-behaving animals over intervals of minutes to days (Ungerstedt and Hallstrom, 1987; Benveniste and Hüttemeier, 1990). For both, push–pull perfusion and microdialysis, small cannulae are chronically implanted into a given brain area. In case of push–pull perfusion, a perfusion medium (typically artificial CSF or Ringer's solution) is pumped through the push cannula, which extends beyond the pull cannula (Fig. 2A), through which the perfusion medium is constantly pulled back to be collected outside of the organism for subsequent analysis. The principle of a constant fluid flow, which gathers potential extracellular signaling molecules, applies also to the microdialysis technique. However, in microdialysis probes, the two cannulae are connected by a semipermeable membrane (Fig. 2B), which separates the dialysis medium from the surrounding tissue. This construction makes a separate pull line dispensable, because the dialysis medium is only pumped (pushed) through the probe into a collecting tube. Neuromessengers enter the dialysis medium by diffusion through the membrane according to the concentration gradient (high concentration in the extracellular space vs. low concentration in the dialysis medium). Physico-chemical characteristics of the neuromessengers (e.g. size, hydrophobia), the dialysis membrane (e.g. material, pore diameter and cut-off, respectively), diffusion characteristics in the brain tissue surrounding the microdialysis membrane (e.g. tortuosity; Nicholson and Sykova, 1998) and the perfusion speed restrict the entry of the neuromessengers into the dialysis medium (Plock and Kloft, 2005). From the amount of the neuromessenger gathered under calibrated *in vitro* conditions, the recovery of the probe can be estimated (ratio of messenger concentration within the dialysis medium and outside). However, the relative recovery of the messenger in the dialysate is considerably lower under *in vivo* conditions because of the different tortuosity in the extracellular space of the brain tissue. Nevertheless, sophisticated and laborious approaches including the methods of zero-net-flux and dynamic no-net-flux have been suggested to converge to the real value, (Plock and Kloft, 2005). For neuropeptides, the relative recovery in the dialysate collected with microdialysis probes with cut-offs between 6 and 20 kDa is below 5%. Generally, the sampling interval for neuropeptides has to be in the range of 30 min at a speed of 2 to 3 μl/min to ensure detectability in the radioimmunoassay. The yield of push–pull perfusion is considerably higher, because of the unrestricted exchange of molecules between perfusion medium and extracellular fluid. However, this technique is highly sensitive to destruction and erosion of brain tissue during sample collection due to the direct contact of the perfusion medium with the brain parenchyma, possible pressure imbalances between the push and pull lines and blockade of the pull cannula by tissue debris.

In the recent 25 years, both techniques have been employed for monitoring the release of different neuropeptides (Table 2) with the number of studies using microdialysis clearly outweighing those using push–pull perfusion (Fig. 3). This development becomes plausible if one considers the following points:

(1) Only distinct neuronal populations synthesize and release neuropeptides, thus leading to relatively high local concentrations in the extracellular fluid of individual brain areas.
(2) Signal transfer by neuropeptides mostly involves volume transmission, which is more readily monitored by microdialysis compared to the difficulties of monitoring the release of neurotransmitters, which is largely confined to the synaptic cleft with little spill-over into the extracellular space (see Fig. 1).
(3) After their intracerebral release and diffusion through the extracellular space, neuropeptides bind to their receptors and/or are degraded. There are no specific transporters mediating a neuropeptide uptake. As a consequence, extracellular neuropeptide concentrations remain more or less unaffected by non-exocytotic processes such as inversion of transporter activity and, thus, are supposed to reflect the signal strength even under pharmacological conditions.
(4) There are no reports that glial cells may synthesize neuroactive peptides that could be deemed neuropeptides. This is particularly important with respect to astroglial or microglial responses activated by tissue traumatization due to the implantation of microdialysis probes and perfusion of the membrane with artificial cerebrospinal fluid or Ringer's solution (Benveniste and Diemer, 1987).

Table 2
Selection of the most commonly investigated neuropeptides

Neuropeptide	Abbreviation	Amino acids	MD/PPP
5-HT-moduline		4	–
Agouti gene-related protein	AGRP	131	–
Amylin	Islet amyloid polypeptide (IAPP)	37	+
Bombesin-like peptide	BLP (neuromodulin)	10	+
Brain natriuretic peptide	BNP	32	–
Calcitonin gene-related peptide	α- or β-CGRP	37	+
Cholecystokinin	CCK	8	+
Cocaine and amphetamine regulated transcript/peptide (family)	CART, fragments CART(55–102), CART(62–102)	Up to 116	–
Corticostatin		14/29	–
Corticotropin-releasing hormone (~ factor)	CRH (CRF)	41	+
Dynorphin	Dyn	17	+
Endomorphins	Endomorphin 1 or 2	4	+
β-Endorphin		30	+
Enkephalins	Met- or Leu-Enkephalin	5	+
Galanin	GAL	29/30	+
Gastrin-releasing peptide	GRP	27	+
Growth hormone-releasing hormone	GHRH	44	+
Hypocretins/orexins	Hcrt	29/39	+
Melanin concentrating hormone	MCH	19	–
α-Melanocyte-stimulating hormone	α-MSH	13	+
Neurokinin	NKA (Substance K), NKB	10	+
Neuropeptide FF	F8Fa	8	–
Neuropeptide K	NPK	24	–
Neuropeptide S	NPS	20	–
Neuropeptide Y	Neuropeptide Tyrosine, NPY	36	+
Neurotensin	NTS	13	+
Nocistatin		17 (bovine), 30 (human)	–
Orphanin FQ/nociceptin	OFQ	17	+
Oxytocin	OXT or OT	9	+
Pancreatic polypeptide	PPY	36	–
Peptide hisidine leucine	PHI	27	–
Peptide tyrosine tyrosine	PYY	36	–
Pituitary adenylate cyclase activating peptide	PACAP	27/38	–
Prolactin-releasing peptide	PRP	20/31	+
Secretoneurin	SN	33	–
Somatostatin	SS (SOM)	14	+
Substance P	SP	11	+
Thyrotropin-releasing hormone	TRH	3	+
Urocortins	Ucn	40	–
Vasoactive intestinal polypeptide	VIP	28	+
Vasopressin (arginine ~)	AVP or VP	9	+

+ release investigated by microdialysis (MD) or push–pull perfusion (PPP); – release not investigated by MD or PPP.

(5) Sampling and analytical tools have been refined, thus decreasing the detection thresholds of the neuropeptides in the microdialysate and enabling the detection of larger neuropeptides (e.g. Duo et al., 2006). For example, antibodies have been included into the dialysis medium to "catch" endogenous corticotropin-releasing hormone (CRH) as soon as it passes the dialysis membrane, thereby preventing unspecific adherence of CRH to plastic surfaces and increasing the sensitivity of subsequent analyses (Merlo Pich et al., 1995). Others developed an immunosensor that enabled the measurement of neuropeptides collected via microdialysis with an outstanding sensitivity (Cook, 2001).

(6) Of similar importance as measuring extracellular neuropeptide concentrations is the possibility of local drug administration. By reversing the principle of microdialysis (retrodialysis), agonists, antagonists or other drugs may be included into the dialysis medium, from where they diffuse into the dialyzed brain area, thus avoiding pressure injections. This offers an elegant approach to administer drugs (e.g. neuropeptide agonists or antagonists) over prolonged periods of time without providing additional stress to the animals during behavioral tests and without acute tissue traumatization. Moreover, administration of synthetic neuropeptides via this route is likely to mimic endogenous neuropeptide release more closely than intracranial pressure injections.

(7) Microdialysis can be efficiently combined with other *in vivo* techniques, including (i) simultaneous measurements of intracerebral neuropeptide release and hormone secretion into the blood by either a specialized microdialysis probe (Neumann et al., 1993a) or by chronically implanted jugular venous catheters (Wotjak et al., 2002) and (ii) electrophysiological recordings (Ludwig and Leng, 1997).

5. Vasopressin and oxytocin and the Rosetta stone for neuroendocrinology

A detailed analysis of the number of studies employing the microdialysis technique for monitoring intracerebral neuropeptide release revealed two molecules that outnumber by far all other candidates: the nonapeptides arginine *vasopressin* (AVP) and *oxytocin* (OXT; Fig. 3). Both AVP and OXT are the primary messengers of the hypothalamo-neurohypophysial system (HNS), which is comprised by magnocellular neurons of the supraoptic (SON) and hypothalamic paraventricular nuclei (PVN), both of which send their axons to the neurohypophysis. Numerous fundamental processes of neuropeptidergic signaling have been discovered in this system, including the principle of neurosecretion (Scharrer, 1928, 1933; Bargmann, 1949), basics of neuropeptide synthesis from precursor molecules (Mohr and Richter, 1993), neuron–glia communication (Hatton, 2004; Oliet et al., 2004), principles of stimulus-secretion coupling, dendritic/somatic neuropeptide release (Landgraf and Neumann, 2004; Ludwig and Leng, 2006) and its functional distinction from axon terminal release (Engelmann et al., 2004a). The growing evidence that these processes apply also to other neuropeptide systems led Harold Gainer and co-workers to conclude that the HNS represents a "veritable Rosetta stone for neuroendocrinology and neuroscience" (Gainer et al., 2002).

The application of microsampling techniques to the HNS revealed that both AVP and OXT are released not only from axon terminals in the neurohypophysis into the blood, but also within their nuclei of origin from somata and/or dendrites of magnocelllular neurons (i.e. into the extracellular fluid of the SON and PVN in a process called intranuclear release). This has been a breakthrough as it allowed to directly link the activity of neuroendocrine cells to behavioural performance of the animals.

6. Neuroendocrine correlates of behavior

Several studies used the microdialysis technique for monitoring OXT release within both the SON and PVN during different reproductive stages. For instance, it could be shown that parturition and suckling is accompanied not only by an increase in OXT secretion into the blood, but also by increased release of the neuropeptide within the SON and PVN (Neumann et al., 1993b). This intranuclear release of OXT turned out to be essential for the synchronization of the firing of OXT neurons, involving both direct influences on neuronal activity (Neumann et al., 1994; Moos and Richard, 1989) and neuron–glia communication with subsequent structural reorganizations within the SON (Theodosis et al., 1986; for review see Theodosis, 2002).

Other studies focused on the role of AVP and OXT in anxiety and behavioral stress coping. Rats, which have been selectively bred to

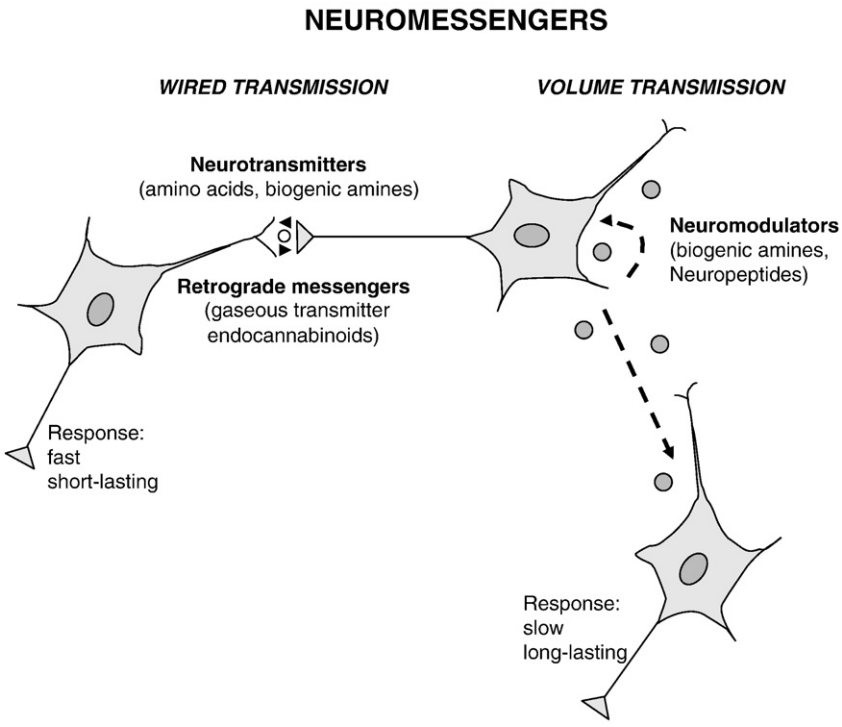

Fig. 1. Classes of neuromessengers differ in range and mode of action. The classical way of chemical signaling in the CNS involves the release of neuromessengers from axon terminals into the synaptic cleft and subsequent binding to postsynaptically localized receptors (wired transmission). Most neurotransmitters directly control neuronal activity of postsynaptic neurons in this manner. At the same time, retrograde messengers originating from the postsynaptic site may reach presynaptic terminals, where they control synaptic transmission. As a third form of neuromessengers, neuromodulators are released non-synaptically. They reach even remote target sites via diffusion through the extracellular space, thus modulating neuronal activity triggered by neurotransmitters.

show exaggerated anxiety-related behavior on the elevated plus maze (for review see Landgraf et al., 2007), revealed increased levels of AVP within the PVN under basal conditions and in response to a forced swimming session (Wigger et al., 2004). Importantly, local administration of an AVP V1a/b receptor antagonist ameliorated the anxiety-like symptoms and decreased passive stress coping (Wigger et al., 2004), similarly to chronic antidepressant treatment (Keck et al., 2003). Noteworthy, there is still the option that neuropeptides released within PVN or SON might reach remote extrahypothalamic target sites such as the septum and amygdala, where they could trigger, at least partially, their behavioral effects (Engelmann et al., 2004a). In fact, as revealed by microdialysis and retrodialysis, AVP and OXT seem to regulate anxiety (Liebsch et al., 1996), stress coping behavior in the forced swim test (Ebner et al., 1999, 2002, 2005) and maternal aggression (Bosch et al., 2005) within both brain structures. However, it still remains to be shown, whether AVP and OXT concentrations measured in microdialysates collected from septum and amygdala reflect volume transmission resulting from intrahypothalamic release or whether they originate from axonal release of extrahypothalamic vasopressinergic and oxytocinergic nerve fibers.

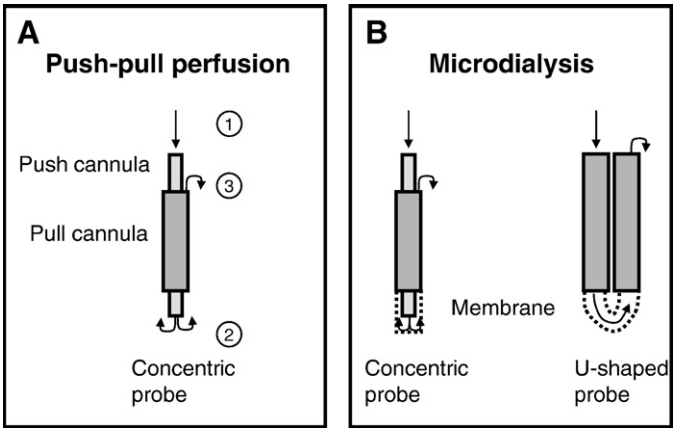

Fig. 2. Microsampling techniques employed for measuring neuropeptide release within the CNS. (A) The concentric push–pull probe consists of a smaller inner (push) cannula, which extends beyond a wider outer (pull) cannula. The perfusion medium is pumped into the push cannula (1), gets into direct contact with the brain tissue and is immediately pulled back into the pull cannula (2). The perfusate is collected outside the animal for subsequent analysis (3). (B) Concentric microdialysis probes are similar to push–pull probes except for the fact that the dialysis medium does not get into direct contact with the brain tissue, from which it is separated by a semipermeable membrane. Furthermore, the dialysis medium has not to be actively pulled back. The principle of U-shaped microdialysis probes is essentially the same. However, the two cannulae are aligned to each other and the membrane is U-shaped.

Microdialysis and retrodialysis have been applied not only in the context of reproduction and emotionality, but also in learning and memory paradigms. For example, after microdialysis experiments had revealed a correlation between the release of AVP within the SON and the ability of rats to remember conspecific juveniles, retrodialysis of AVP or its receptor antagonist into this brain structure was used to either facilitate or impair social recognition in male rats (Engelmann et al., 1994). Similar approaches have been employed for studying the impact of AVP release within septum and hippocampus in terms of spatial learning (Engelmann et al., 1992a) and active avoidance learning (Engelmann et al., 1992b; for review see Engelmann et al., 1996). The fact that swivels and tethers were deliberately omitted in the experimental setups and microdialysates were, instead, directly collected in Eppendorf vials placed at the animals' head (Horn and Engelmann, 2001) proved to be essential for the success of microdialysis in the respective behavioral studies.

7. Regulation of neuropeptide release

Magnocellular neurons are osmosensitive (Bourque and Oliet, 1997). Accordingly, both local and systemic osmotic challenges are

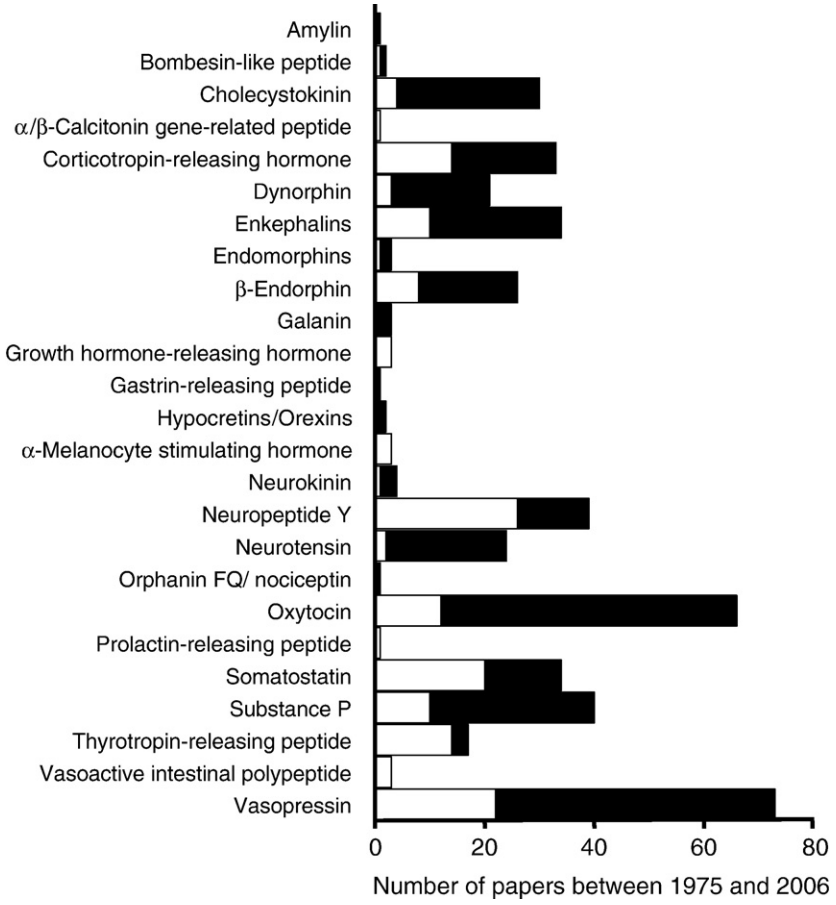

Fig. 3. Number of studies measuring neuropeptide release by means of push–pull perfusion (open bars) or microdialysis (filled bars). A selection of the most commonly studied neuropeptides (cf. Hökfelt et al., 2000; for details see Table 2) has been screened for their measurement by microdialysis or push–pull perfusion. Only original articles published between 1975 and 2006 have been considered, which measured the release of the respective neuropeptide. Studies employing retrodialysis (i.e. local application of neuropeptide by adding it to the dialysis medium) without concomitant measurement of neuropeptide release are not included. Analyses are based on PubMed searches.

accompanied by an increase (in case of hypertonic solutions; Neumann et al., 1993a; Ludwig et al., 1994) or decrease (in case of hypotonic solutions; Hussy et al., 1997) in neuronal activity and neuropeptide release. Although being somehow artificial, local hypertonic stimulation by increasing the osmolality of the dialysis medium, in combination with local drug administration via retrodialysis proved to be of high value for gaining deeper insights into the regulation of intrahypothalamic AVP and OXT release (for reviews see Ludwig, 1998; Landgraf and Neumann, 2004).

Another "milestone" has been the discovery that AVP and OXT are released within the SON and the PVN in response to certain stressors (Engelmann et al., 2004a). By combining microdialysis, retrodialysis and *in vivo* electrophysiology, we could demonstrate that amino acids differentially control the release of AVP and OXT within the SON and from axon terminals into the blood. At level of the SON, for instance, GABA (Engelmann et al., 2004b) and taurine (Engelmann et al., 2001) selectively constrain the activity of OXT and AVP neurons, respectively. The comparison of individual release patterns with the localization of the respective microdialysis probes helped to identify potential sources of the inhibitory amino acids, with GABA likely originating from perinuclear interneurons and taurine from the ventral glial lamina (Decavel and Hatton, 1995; Hussy et al., 2000), thus underscoring the importance of a careful histological verification of the microdialysis sites. Therefore, data of both correctly implanted and misplaced microdialysis probes should be reported to unequivocally identify a distinct brain area as site of neuropeptide release, with the assumption that the neuropeptide concentration within the dialysates decreases with increasing distance from the release sites.

8. Dissociation of central and systemic neuropeptide release

The combination of microdialysis within the SON/PVN and simultaneous monitoring of AVP and/or OXT levels within the blood enabled the direct comparison of dendritic release with axon terminal secretion in the same neuronal population. For example, simultaneous microdialysis within brain and blood of anesthetized rats revealed striking differences in central and systemic release of AVP and/or OXT in response to suckling and intraperitoneal injection of hypertonic solution (Neumann et al., 1993a,b; Ludwig et al., 1994). These data provided direct evidence for a dissociation of central and systemic neuropeptide release. In addition, the two to four orders of magnitude higher local concentration of AVP and OXT in the extracellular fluid of SON and PVN, as compared to plasma levels, rendered it unlikely that blood-derived hormones contaminate neuropeptide release measured within the brain (Box 2).

Other studies demonstrated that dendritic release of OXT and AVP is differentially regulated within SON and PVN during physical and/or emotional stress (Wotjak et al., 1996, 1998; Engelmann et al., 1999). Particularly striking has been that, under physiologically relevant conditions, local release of AVP and OXT within one or both hypothalamic nuclei may occur in the absence of concomitant secretion of the respective neuropeptide from axon terminals into the blood (Wotjak et al., 1996, 1998; Engelmann et al., 1999, 2001).

Box 2

Neuropeptide concentration in the brain extracellular fluid

Central release patterns finally determining the concentration in the extracellular fluid (ECF) of distinct brain areas are crucial in the cascade of events from neuropeptide *de novo* synthesis to receptor-mediated effects. Importantly, due to the anatomical discontinuity between sites of release and potential targets and, consequently, their more homogeneous distribution in the ECF, neuropeptides primarily acting as neuromodulators are more reliable to be monitored by microdialysis than classical transmitters. Microdialysis, in other words, integrates temporal and spatial gradients and fluctuations; this certainly creates less problems for analysis of somato-dendritic (typical of neuropeptides) than presynaptic (typical of classical transmitters) release patterns.

The amount of neuropeptide collected in the microdialysate depends on many variables, including species used, actual location of probe in the brain tissue, type of probe, collection interval and speed, diffusion and tortuosity characteristics, etc. Due to their high variability, absolute dialysate levels are rarely presented. Instead, many authors prefer relative comparisons to demonstrate the neuropeptide response to a given stimulus as per cent change relative to basal. While such approaches eliminate or at least reduce the influence of variables, they impede the comparison of release patterns between different substances, brain regions, compartments (central vs. peripheral), and species.

In a first attempt to roughly calculate vasopressin concentrations in the ECF of the hypothalamic supraoptic and paraventricular nuclei of the rat brain, we demonstrated that, in addition to substance diffusion, an exchange occurs between ECF and dialysis medium with ca. 8 μl ECF being detectable in a 30-min microdialysate (ca. 22 μl in a 30-min push–pull perfusate). Based on (i) these data, (ii) pg amounts of vasopressin measured in subsequent radio-immunological assay and (iii) both *in vitro* and *in vivo* recoveries, we suggested vasopressin concentrations in the ECF in the 10^{-9} M range, being up to 1000 times higher than basal levels in plasma or cerebrospinal fluid (for review see Landgraf, 1992). This suggestion has recently been confirmed by Ludwig and Leng (2006). Focusing on somato-dendritic vasopressin release in the rat brain, these authors based their calculation on how many vasopressin molecules are contained in hypothalamic nuclei, a single neuron within the supraoptic nucleus or even a large dense-core vesicle from which vasopressin is released by exocytosis. Interestingly, receptors at which neuropeptides act have affinities in the nanomolar range. Microdialysis studies in rats revealed that basal OXT levels were similar in the SON and PVN. In contrast, the concentration of AVP was higher in the SON than in the PVN (Wotjak et al., 1998).

It remains to be shown whether basal concentrations in the 10^{-9} M–10^{-10} M range in the ECF and in the 10^{-11} M–10^{-12} M range in plasma and cerebrospinal fluid, typical of vasopressin, may be generalized to other neuropeptides. Similarly, their species-dependency remains unclear. In mice, for example, vasopressin levels in microdialysates resemble those collected in rats (Keßler et al., 2007), despite plasma concentrations being 100 times higher in the former.

It is of note in this context that – in addition to the blood-brain barrier – it is the brain/plasma concentration gradient which makes a transport of endogenous neuropeptides from plasma to the brain parenchyma (i.e. against this gradient) unlikely to occur. Under pharmacological conditions, however, a limited transport (according to the then inverse gradient), predominantly in circumventricular organs, is conceivable.

These data provided conclusive evidence for a functional separation between neuropeptide action as hormone and that as neurotransmitter/neuromodulator. The striking dissociation between central and peripheral neuropeptide release could be confirmed and substantially extended in studies combining microdialysis/retrodialysis with electrophysiological approaches. Ludwig et al. (2002), for instance, succeeded in showing that the application of agents that mobilize intracellular Ca^{2+} induce oxytocin release from dendrites without affecting both the electrical activity of the cell bodies and OXT secretion from nerve terminals. Conversely, electrical activity of the cell bodies can cause axonal secretion of OXT with little consequences on dendritic OXT release (Ludwig et al., 2002). In an add-on study, stimulation of the SON by α-melanocyte-stimulating hormone (α-MSH) led to hyperpolarization of oxytocinergic neurons, while it facilitated dendritic OXT release at the same time (Sabatier et al., 2003).

Altogether, the microdialysis technique played a crucial role in the discovery and characterization of non-synaptic communication channels and, thus helped to substantially extend our knowledge about the communicatory capacities of peptidergic neurons (Ludwig and Leng, 2006).

9. Lessons learned

More than 15 years of experience with measuring neuropeptide release by means of microdialysis taught us several lessons. There is a set of remarkably positive results, which argue in favor for a broad application of this method also to other neuropeptides: First, the microdialysis technique is robust and reliable, even if self-customized and chronically implanted probes are employed and experiments are performed in unrestraint animals (Horn and Engelmann, 2001). Second, in rats, a recovery period from surgery of 48 h appears to be sufficient for getting stable baseline release patterns. Third, combined microdialysis and blood sampling via chronically implanted jugular venous catheters have revealed that even sensitive endocrine parameters such as plasma ACTH and corticosterone concentrations remain virtually unaffected by ongoing microdialysis (Wotjak et al., 2002). Accordingly, ongoing microdialysis *per se* has no disturbing consequences on stress sensitive behaviors such as maternal care (Neumann et al., 1994).

Despite the advantages listed above, there are also several shortcomings of the microdialysis technique, which limit its application for monitoring intracerebral neuropeptide release. Firstly, constraints in the relative recovery of the microdialysis probe limit the ability to detect neuropeptides within the dialysate. This holds particularly true, if volume transmission to remote target sites or the release from axon terminals are being monitored. One possibility to cope with this limitation is the prolongation of the sampling intervals. However, the resulting decrease in temporal resolution will mask dynamic fluctuations in neuropeptide release, which are likely to be of biological relevance (cf. dynamic changes in acetylcholine release, which have been unraveled by *in vivo* amperometry only; Parikh et al., 2004).

Secondly, it has to be considered that the implantation of microdialysis probes may destroy axons, which rapidly reseal and

establish new pseudo-terminal release sites. Under these circumstances it is virtually impossible to assign altered levels of neuropeptides within the dialysate to dendritic vs. pseudo-axon terminal release. One possibility could be to apply the criteria established for synaptic transmission also to neuropeptide release These criteria include (i) stimulation of neuropeptide release by high K^+, (ii) blockade of neuropeptide release by using Ca^{2+}-free microdialysis media containing Ca^{2+}-buffering substances, and (iii) the requirement of action potentials, which can be blocked by tetrodotoxin. However, part of these criteria might also apply to dendritic neuropeptide release (c.f. Ludwig and Landgraf, 1992).

Thirdly, mechanical destruction of neurons due to insertion of the microdialysis probe and perfusion of the dialysis membrane *per se* may cause the formation of glial scarves (Benveniste and Diemer, 1987), which provide diffusion barriers for neuropeptides and, thus, may lead to increased tortuosity and a reduced recovery. For example, we could show that microdialysis on three consecutive days, interrupted by microdialysis arrest, hampered the ability to detect stressor-induced release of AVP and OXT within the SON (Wotjak et al., 1998). Therefore, for long-lasting experiments, microdialysis probes should be permanently perfused without interruption, because stopping the flow seems to favor the activation of glial cells within the dialysis area (most likely due to swelling and shrinking of the dialysis membrane; Wotjak and Engelmann, unpublished observation). Accordingly, continuous microdialysis has been successfully employed for monitoring circadian rhythms of AVP release within the suprachiasmatic nucleus over a time period of 36 h (Kalsbeek et al., 1995). In case of monoaminergic transmission, continuous microdialysis revealed basal levels which remained stable for more than a week (Wotjak, unpublished observations). In case of neuropeptides, however, even with continuous dialysis reductions in neuropeptide content in the dialysate are likely to occur with the passage of time, which renders a physiological reduction in neuropeptide release difficult to detect (Engelmann et al., 2004a). One possible explanation for this effect might be that the dialysis medium does not exactly mimic the chemical composition of the extracellular fluid. This may trigger migration and proliferation of glial cells which are attracted by the dialysis medium and impede the passage of molecules through the dialysis membrane.

10. Ways to go

The number of publications employing microdialysis for studying the role of neuropeptides under physiological and pathological conditions illustrates the success of this research tool (Fig. 4A). However, after having reached its maximum in the mid-90s, the number of published papers stagnates and even tends to decline. Does this mean that this technique has passed its zenith? We don't believe so. Already the fact that only 60% of the most commonly studied neuropeptides have been monitored by microdialysis so far (Fig. 3; Table 2) speaks against this assumption. As a matter of fact, it seems that the switch from rats to mice as preferred subjects in neuroscience research, initiated by the enormous progress in mouse genomics, has slowed down the application of microdialysis. Mice are clearly inferior to rats for neuroendocrine research. They do not allow to repeatedly take blood samples, and, in microdialysis experiments, they take much longer to recover from surgery (at least one week). Nevertheless, it is unlikely that solely technical difficulties with adopting microdialysis to mice (Fig. 4B) or a certain hesitation in using this species in neuroendocrine research (Fig. 4C) account for the decrease in the number of microdialysis studies on neuropeptides. It is more likely that in mice the invasive microdialysis technique has to be restricted to a few larger brain structures (e.g. hippocampus, nucleus accumbens, striatum, prefrontal cortex), in which the tissue trauma caused by insertion of the microdialysis probe leads to less severe malfunction than in the substantially smaller primary targets of neuropeptide release (e.g. hypothalamic or amygdaloid nuclei). This might explain why, according to a PubMed search, only 4 studies monitored neuropeptide release in mice by microdialysis between 1975 and 2006, compared to 260 studies performed in rats during the same period. Upon first view, smaller microdialysis probes would help to further reduce tissue destruction. However, the inevitable resulting decrease in the recovery of the microdialysis probe (Plock and Kloft, 2005), often precludes detectability of the neuropeptides in subsequent radioimmunoassays. How can we overcome these shortcomings? Firstly, the recovery could be increased by including affinity-based trapping agents (e.g. cyclodextrins, antibodies, bovine serum

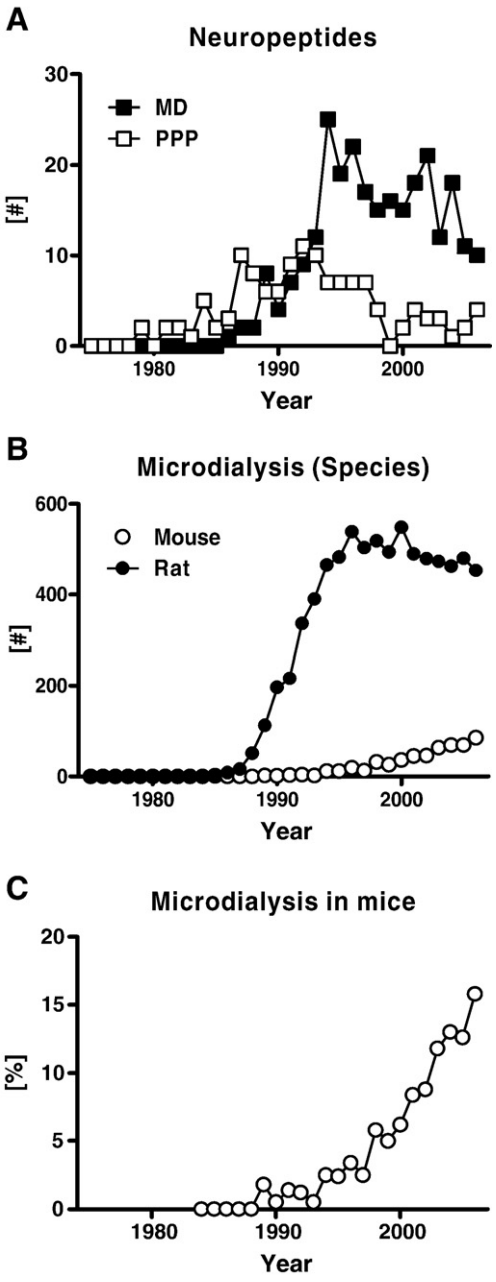

Fig. 4. Time course of microdialysis experiments performed in rats and mice between 1975 and 2006. On the basis of the articles selected in Fig. 3, we analyzed the temporal distribution of (A) microdialysis (MD) and push–pull perfusion studies (PPP) employed for measuring neuropeptide release between 1975 and 2006, irrespective of the species under study, (B) microdialysis studies performed in rats and mice irrespective of the neuromessenger under study, and (C) the percentage of microdialysis studies performed in mice on any neuromessenger with respect to the total number of microdialysis studies performed in mice and rats (cf. B). Analyses are based on PubMed searches.

albumin–heparin conjugates, etc.; Duo et al., 2006). Secondly, refinements of analytical tools, for instance by employing capillary electrophoresis in combination with LC-MS (Haskins et al., 2001), would not only increase the sensitivity (fmol range for RIA vs. amol range for CE/MS; Haskins et al., 2001), but also allow for monitoring the neuroactive fragments of neuropeptides along with different other neuromessengers and their metabolites at once. To those of us, who take the challenge of performing microdialysis experiments in mice, this could be the beginning of a new era, in which analyses of dynamic changes in the "neurosecretome" might be combined with the advantages of mouse genomics. Then the microdialysis technique will remain a valuable tool for listening to neuropeptide communication by detecting harmonies and disharmonies in the neuroendocrine signaling underlying behavioral regulation.

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