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## Substance P and the neurokinin 1 receptor

Hart, Maria Geertrudis Cornelia van der

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## Chapter 5

# **In vitro and in vivo evaluation of neuropeptide microdialysis in the brain of freely moving rats**

Marieke G.C. van der Hart<sup>1,2</sup>, Fokko J. Bosker<sup>1</sup>, Thomas I.F.H. Cremers<sup>2</sup>, Johan A. den Boer<sup>1</sup> and Ben H.C. Westerink<sup>2</sup>

- 1) Department of Biological Psychiatry, University of Groningen, Groningen, the Netherlands
- 2) Department of Biomonitoring and Sensing, University of Groningen, Groningen, the Netherlands

### **Abstract**

Neuropeptides are subject of increased interest for their alleged role in various central nervous system processes. However, *in vivo* assessment of these compounds is complicated because of the generally low concentrations in the brain and the neuropeptides susceptibility to proteolytic degradation. This explains why so few microdialysis studies on neuropeptides have been published in the past. An additional problem is insufficient *in vivo* recovery of the neuropeptides when using microdialysis membranes.

In this study we determined the recovery and dynamic behavior of several microdialysis membranes *in vitro* for three neuropeptides. For this purpose existing radio immuno assays (RIA) were optimized for Substance P (SP), vasopressin and corticotrophin releasing factor quantification in microdialysates. Finally, local potassium stimulation in freely moving rats was used to induce neurotransmitter release in order to assess the dynamic behavior of the membranes *in vivo*. Our results show that recovery of neuropeptides depends on the cut-off, pore-size and physico-chemical properties of the membrane, but the individual physico-chemical properties of neuropeptides also play a role. Polyethersulfon (PES) membranes appeared to be most effective membranes for use *in vitro* and *in vivo*.

## 5.1 Introduction

Measurement of neurotransmitters in brains of freely moving animals has evolved considerably in the past decades. This has enhanced research into brain functioning, pathology and pharmacotherapy to a large extent (L'Heureux *et al.*, 1986; Sharp *et al.*, 1986). Given the complexity of the brain it is not realistic to think that *in vivo* measurements will be replaced by *in vitro* alternatives in the near future.

In the early years, neurotransmitter content of brain tissue was quantified *ex vivo*. These studies gave insight in the synthesis and turnover of neurotransmitters (Westerink & Korf, 1976; Neff & Tozer, 1968), but its relevance for synaptic neurotransmission is questionable (Cheramy *et al.*, 1981).

To monitor synaptic processes more accurately, *in vivo* sampling techniques were developed that allowed measurement of neurotransmitters with improved spatial and temporal resolution. Examples are push-pull sampling (Philipu, 1984), ultrafiltration (Leegsma-Vogt *et al.*, 2003), voltammetry (Robinson *et al.*, 2003), sensing (Burmeister & Gerhardt, 2001; Oldenziel *et al.*, 2006) and microdialysis (Ungerstedt, 1991b; Ungerstedt, 1971). Clearly all these techniques have advantages and disadvantages. Push pull sampling, for instance has a high *in vivo* recovery and is uncritical with respect to the molecular mass of the compounds under study, however, practical problems such as tissue damage have restricted its wide spread use for *in vivo* sampling (Myers *et al.*, 1998). With respect to tissue damage, ultrafiltration is probably superior. In this technique samples are drawn under negative pressure through a large pore dialysis fiber. This technique is also not limited by the molecular weight of the analyte or recovery issues, but low levels of extracellular fluid in the brain restrict its practical use (Kaptein *et al.*, 1998). Voltammetry, in which neurotransmitters are measured locally by an electrode, might seem the ideal sampling technique given its ease of use, absolute recovery and high temporal and spatial resolution. However, the use of this technique is limited to a small number of analytes that can be quantified in a complex brain matrix by voltammetric sweeps. Moreover, because of its moderate selectivity only brain areas with a relatively high concentration of the neurotransmitter under investigation can be studied (Benveniste, 1989). An improvement to this technique is sensing, which uses enzymatic conversions to increase selectivity and increase in signal strength. Moreover, it is also applicable for not electrochemically active compounds (Gardiner & Silver, 1979). Finally, microdialysis is by far the most frequently used technique for sampling of neurotransmitters. Its ease of use, low invasiveness to the animal and brain micro environment makes microdialysis ideal for studying extracellular levels of small molecules in the brain (Westerink, 2000a). In contrast to sensing and voltammetry, analyses are performed after sampling, which extends its applicability

to an almost indefinite number of analytes, including neurotransmitters, hormones and exogenous compounds.

The primary focus of pharmacotherapy of brain pathology has been on classical neurotransmitters such as the monoamines and acetylcholine (Gardier *et al.*, 1996; Di *et al.*, 2004; Bruno *et al.*, 1999). Lately, there is increased interest in neuropeptide systems as potential targets for treating central nervous system diseases (Holmes *et al.*, 2003). However, most research has been targeted toward the effects of neuropeptide receptor antagonists and agonists on monoamine, amino acid and acetylcholine levels. Consequently, relatively little information is available concerning neuropeptide transmission. Further reasons for this lack of information are the physico-chemical properties of neuropeptides and their low abundance in the brain, which prevent reliable *in vivo* assessments. Technically, voltammetry and sensing techniques are unsuitable for measuring neuropeptides. While sampling based techniques are hard to apply because neuropeptides are non-polar and high in molecular weight, which will limit *in vivo* recovery and facilitate non-specific binding to the sampling system. Clearly, any sampling technique must be extensively validated in terms of dynamic behavior and *in vivo* recovery, because these parameters will crucially influence the *in vivo* results.

Within the group of neuropeptides, the variation of size, mass (see Table 1), charge, pKa and other physical and chemical properties is considerable. This might necessitate a specific approach for each neuropeptide when optimizing microdialysis conditions. Generally in microdialysis studies of neuropeptides the technique is not extensively validated, which makes it difficult to judge how accurate the *in vivo* assessment reflects the extracellular situation in the brain.

**Table 1** *Molecular weights of four monoamine neurotransmitters and the three neuropeptides measured in the present study*

Analyte	Molecular weight (Da)
Serotonin	177
Acetylcholine	147
Norepinephrine	169
Dopamine	154
Substance P	1348 (11 AA's)
[Arg] <sup>8</sup> -vasopressin	1084 (9 AA's)
CRF	4757 (41 AA's)

In the current study we evaluated several microdialysis membranes with respect to their *in vitro* and *in vivo* dynamics for Substance P, vasopressine (VP) and cortico releasing factor (CRF). To this end, the absolute recoveries of the membranes were studied, but also their response to sudden changes in external concentrations.

## 5.2 Materials and methods

### 5.2.1 *In vitro* experiment

The artificial cerebrospinal fluid (aCSF) used as perfusion medium was the same in all the experiments performed in this study, except for the bovine serum albumine (BSA) concentration (see example CRF table 2), which was dependant of the neuropeptide under investigation. aCSF used was composed of: 142 mM NaCl, 3 mM KCl, 1.2 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>. The BSA concentration was 0.025%, 0.5% or 0.2% for SP, CRF or VP respectively. BSA was used to reduce non-specific binding of the neuropeptides to the sampling system.

**Table 2** *Recovery of CRF with different concentrations of BSA in the perfusion medium*

BSA Concentration	Recovery
0.2%	1.15 ± 0.48
0.3%	2.98 ± 2.40
0.5%	3.15 ± 0.94

The microdialysis probes used to investigate the recovery for the neuropeptides were custom made by Brainlink, the Netherlands. All the probes were  $\gamma$ -shaped and had the same dimensions, 2 mm of free dialyzable membrane and a shaft length of 6 mm. The only difference was the type of dialysis membrane; the following membranes were used (see Table 3).

**Table 3** *Overview of different characteristics of the membranes used in this study*

Membrane	Material	ID	OD	Cut-off
Hospal AN 69	Polyacrylonitril	200 µm	300 µm	6 kD
Cuprophane	Regenerated cellulose	200 µm	208 µm	9 kD
Hollow fibre	Cellulose	200 µm	216 µm	18 kD
Hemophan	Synthetically modified cellulose	200 µm	208 µm	6 kD
PES	Polyethersulfon	200 µm	280 µm	20 kD

The following protocol was used to determine the recovery over the tested dialysis membranes. One *in vitro* setup consisted of a beaker containing only aCSF with the appropriate BSA concentration, a second was prepared with the same bath solution but now containing the neuropeptide under investigation at  $10^{-9}$  M. A microdialysis probe with a membrane of interest was placed in the first beaker and a flow of 1.5 µl/min was established through the probe. Samples were collected during 30 minutes interval for three hours in vials already containing 65µl RIA-buffer. During the first sample, at  $t = 23$  minutes, 7 min prior to sample change (to correct for lag time) the probe was placed in the second bath. The samples were collected by an automated fraction collector (CMA 142, Sweden) and stored at  $-80$  °C pending analysis. Recovery for CRF and VP was only tested using the PES membrane.

### 5.2.2 *In vivo* experiment

Rats (280-350 g; Harlan, Horst, The Netherlands) were used for the *in vivo* experiments. The animals were individually housed in plastic cages (30 x 30 x 20 cm) and had access to food and water *ad libitum*. Experiments were conducted in accordance with the declarations of Helsinki and were approved by the Institutional Animal Care and Use Committee of the University of Groningen.

Rats were anesthetized using isoflurane (2%, 1000 ml/min O<sub>2</sub>). Bupivacain / norepinephrine were used for local anesthesia. The animals were placed in a stereotaxic frame (Kopf instruments, USA), and I-shaped probes (PES Fresenius membrane, 4 mm exposed surface; BrainLink, The Netherlands) were inserted into ventral hippocampus. Coordinates for the tips of the probes were for the ventral hippocampus: posterior (AP) = -5.3 mm to bregma, lateral (L) = +4.8 mm to midline and ventral (V) = -8.0 mm to dura according to Paxinos and Watson, (1982). Experiments were performed 24-48 hours after surgery. On the day of the experiment, the probes were connected with flexible PEEK tubing to a microperfusion pump (Syringe pump UV 8301501, Univentor, Malta) and perfused

with aCSF, as described above. Microdialysis samples were collected during 30 min periods into mini-vials containing 65 µl of SP RIA buffer. Microdialysis samples were collected by an automated fraction collector (CMA 142, Sweden) and stored at -80 °C pending analysis.

After the experiment, rats were sacrificed and their brains removed. The brains were stored for 3 days in a 4% (w/v) solution of paraformaldehyde. The position of each probe was histologically verified according to Paxinos and Watson (1982) by making coronal sections of the brain.

### 5.2.3 Analysis

For the determination of Substance P, VP and CRF concentration, a RIA was used, which was optimized for each neuropeptide. Calibration curves samples were prepared in 45% aCSF and 65% appropriate RIA-buffer mixture, the same matrix as for the collected samples. Calibration curves were prepared in the following range;  $1 \times 10^{-9}$  M -  $5 \times 10^{-14}$  M for the Substance P (SIGMA, St Louis, Missouri, USA) and CRF (SIGMA, St Louis, Missouri, USA) and  $5 \times 10^{-10}$  M -  $1 \times 10^{-14}$  M for VP (SIGMA, St Louis, Missouri, USA) respectively.

Substance P was measured using the following protocol: Standards and samples were preincubated with 100 µl of diluted Substance P-antiserum (Euro-Diagnostica, AB Malmö, Sweden) for 48 h at 4 °C. A cross reaction of Substance P antiserum was 100% to Substance P and less than 0.1% to other peptides. After addition of [ $^{125}$ I]Bolton-Hunter-SP [8000 cpm in 100 µl (Amersham Biosciences, Buckinghamshire, UK), samples were further incubated for 48 h at 4 °C. Antibody-bound radioligand was separated from unbound radioligand by addition of 0.6% charcoal slurry (SIGMA, St Louis, Missouri, USA) in RIA buffer (1 ml) and centrifugation. The supernatant was removed, and the bound radioactivity was counted in a gammacounter (5000 Riastar, Packard, Illinois, USA). Control samples, either without Substance P or without antibodies, were incubated simultaneously to measure maximal tracer binding and unspecific binding, respectively. The detection limit of the assay was 0.5 fmol per sample.

VP concentration was determined in the same way as the Substance P concentrations, except, standards and samples were preincubated with 100 µl of diluted VP-antiserum (Acris, Hiddenhausen, Germany) for 24 h at 4°C. A cross reaction of VP antiserum was 100% to VP and less than 0.1% to other peptides. After addition of [ $^{125}$ I] Vasopressin [Arg8] (Amersham Biosciences, Buckinghamshire, UK), samples were further incubated for 24 h at 4°C. Antibody-bound radioligand was separated from unbound radioligand by using a second antibody. 100 µl of goat anti rabbit IgG 1:1000 in RIA buffer (Linco Research, St Charles, Missouri, USA) 100 µl and 100 µl of 1% Normal rabbit serum in RIA buffer (Jackson Immuno Research, Cambridgeshire, UK) were added. After an additional two hour incubation, to allow for an antibody complex forming, the samples were centrifuged and the supernatant was removed. The bound radioactivity was

counted in a gammacounter (5000 Riastar, Packard, Illinois, USA). Control samples, either without standards or without antibodies, were incubated simultaneously to measure maximal tracer binding and unspecific binding, respectively. The detection limit of this assay was 0.3 fmol per sample.

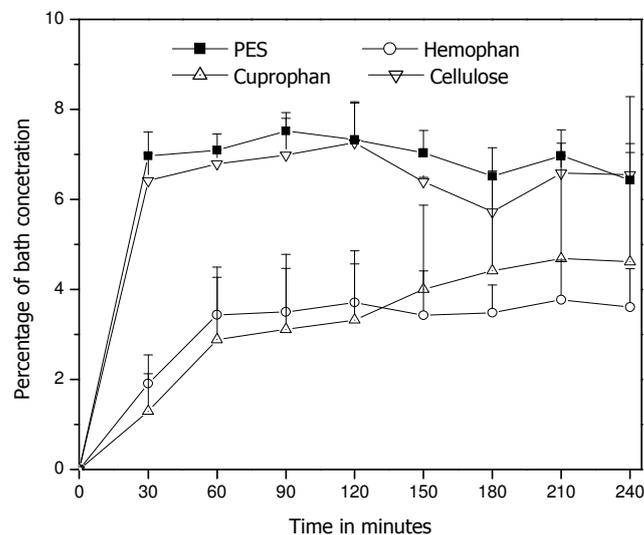
CRF concentrations were determined in the same way as the Substance P concentrations, except, standards and samples were pre-incubated with 100 µl of diluted CRF-antiserum (EURO-Diagnostica AB Malmö, Sweden) for 24 h at 4 °C. A cross reaction of CRF antiserum was 100% to CRF and less than 0.1% to other peptides. After addition of [<sup>125</sup>I] Tyr0-CRF (PerkinElmer Life sciences, Zaventem, Belgium), samples were further incubated for 24 h at 4 °C. Antibody-bound radioligand was separated from unbound radioligand, using a secondary antibody, as described for VP. The bound radioactivity was counted in a gammacounter (5000 Riastar, Packard, Illinois, USA). Control samples, either without standards or without antibodies, were incubated simultaneously to measure maximal tracer binding and unspecific binding, respectively. The detection limit of the assay was 0.8 fmol per sample.

#### **5.2.4 Data presentation**

Data are presented as percentage of batch concentration for the *in vitro* experiments. The data presented for the *in vivo* experiment are either in absolute output or as percentage of basal levels. For basal levels, three consecutive microdialysis samples with less than 50% variation were taken as baseline levels and set at 100%. Local infusion effects were expressed as percentages of basal level within the same subject. All data are expressed as mean ± SEM.

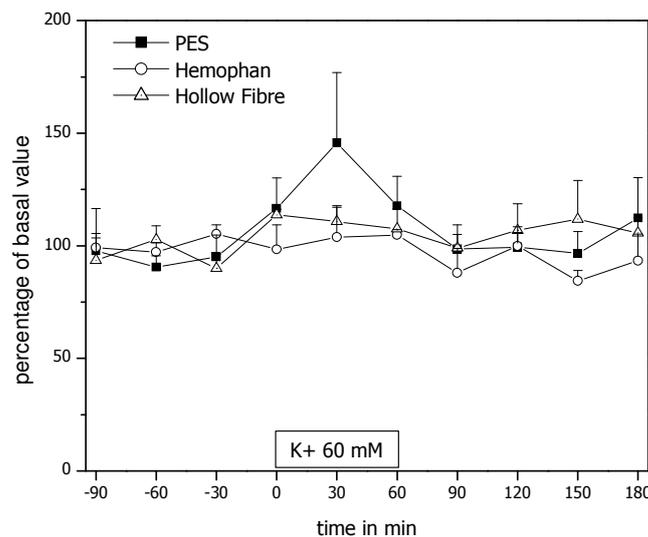
### 5.3 Results

To identify the membrane with the optimal recovery for neuropeptides, four different membranes were compared for the recovery of SP. These membranes varied in cut-off (6-20 kD), inner and outer diameter and physico-chemical properties. The custom made microdialysis probes were stabilized in an aCSF bath without any Substance P added and after a stabilizing period, the probe was transferred into a aCSF bath with a concentration of SP of  $10^{-9}$  M and samples were collected for 30 minute periods. Fig. 1 shows the dynamics of the different membranes used. It appeared that the dynamics of the hemophan and cuprophane probe were slower in establishing a steady flux compared to the PES and cellulose membrane. These latter membranes appeared to establish the equilibrium for the recovery of SP instantly. Furthermore, it appeared that both the cuprophane and hemophan membrane finally reached equilibrium the recovery was around 4% of the total bath concentration. Both PES and cellulose finally reached a recovery of 7.5% of the total bath concentration.



**Figure 1** *In vitro* recoveries of SP with different microdialysis membranes %  $\pm$  sem

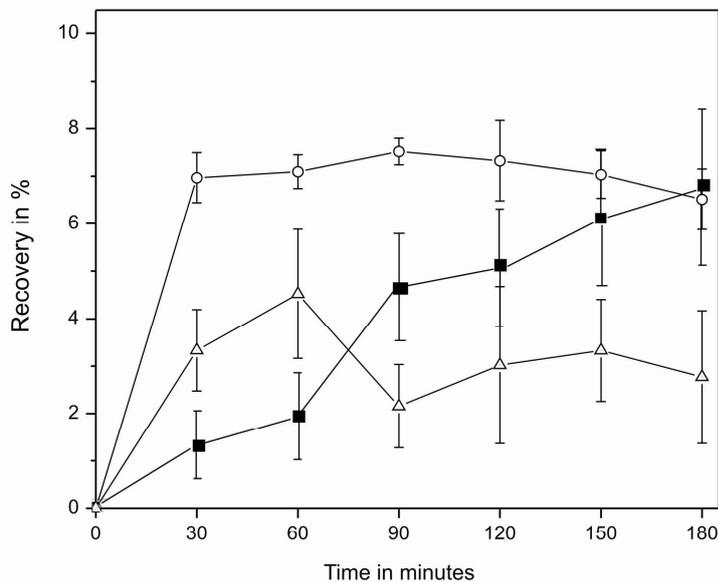
Fig. 2 shows the dynamics of Substance P levels *in vivo* in the ventral hippocampus of the rat. The absolute output of SP was comparable between membranes:  $1.35 \pm 0.19$ ;  $1.45 \pm 0.24$  and  $1.67 \pm 0.26$  fmol/sample for PES, hemophan and cellulose, respectively. The expected effect of a local increase of Substance P, in response to a high potassium concentration in the perfusion fluid, was not observed for the probes fitted with the hemophan and the hollow fiber membranes. PES was the only type of membrane with which the expected increase in Substance P was observed.



**Figure 2** *Effect of local potassium stimulation on extracellular Substance P levels in the ventral hippocampus of the rat*

Fig. 3 shows the *in vitro* recoveries of Substance P, vasopressin and CRF over a PES membrane. Prior to the *in vitro* recovery experiment, the optimal BSA concentration for the perfusion fluid was determined. For Substance P a BSA concentration of 0.025 % in the perfusion fluid was sufficient to prevent Substance P from sticking to the tubing and the probe. Further increasing the BSA concentration did not further increase the recovery of Substance P over the dialysis membrane (data not shown). For vasopressin this concentration was 0.2% BSA (data not shown) and for CRF a concentration of 0.5% was needed (Table 3). Substance P concentration in the perfusion fluid increases immediately after the

probe is placed in the perfusion fluid containing  $10^{-9}$  M Substance P. Vasopressin on the other hand shows a slow increase in recovery. After three hours of monitoring the recovery of vasopressin the experiment was discontinued; the concentration of the recovered of vasopressin was at that time still increasing. As with Substance P, the recovery of CRF stabilized instantly after the probe was placed in the beaker spiked with  $10^{-9}$  M CRF. The measured recovery for CRF was 3.15%, which was constant throughout the 3 hour monitoring period.



**Figure 3** *In vitro* recovery for Substance P (open circles), Vasopressin (closed squares) and CRF (open triangles) expressed in percentage of bath ( $10^{-9}$  M) concentration.

Table 4 shows the absolute measured concentration of Substance P in different brain areas using RIA in combination PES membrane probes. The data are in accordance with the reported distribution of Substance P throughout the brain. The Substance P concentration in striatum is higher than in the ventral hippocampus. The concentration measured in the dorsal raphe nucleus seems to be lower, but here the free dialyzable membrane surface is considerably smaller than the free dialyzable membrane used in the ventral hippocampus.

**Table 4** Basal concentration of SP measured in different brain areas of the rat

Brain area	Basal concentration (fmol/30 min sample $\pm$ sem)
vHipp (4 mm open)	1.29 $\pm$ 0.22
DRN (1.5 mm open)	0.76 $\pm$ 0.04
Striatum (3 mm open)	2.90 $\pm$ 0.16

Table 5 shows the basal output of vasopressin measured by microdialysis in 30 min samples in paraventricular nucleus of the hypothalamus (PVN) only. Vasopressin is not as widely spread throughout the brain as Substance P. Therefore vasopressin was not sampled in other brain areas.

**Table 5** Basal output of vasopressin in the para-ventricular nucleus of the hypothalamus.

Brain area	Basal concentration (fmol/30 min sample $\pm$ sem)
PVN (1.5 mm open)	0.085 $\pm$ 0.038

## 5.4 Discussion

Measuring neuropeptides by microdialysis remains a challenge. Clearly, the knowledge and experience obtained in 30 years of microdialysis research does not guarantee reliable measurements of neuropeptides.

Decades ago, Ungerstedt and co-workers tried to measure extracellular levels of dopamine in the brain using a dialysis fiber obtained from an artificial kidney to extract molecules from the extracellular space without inducing a net transport of fluid (Ungerstedt, 1991a). This method was rapidly adopted by several researchers and was successfully employed to monitor brain extracellular levels of monoamines and related compounds. By infusing compounds that decrease neuronal release of neurotransmitters, the method was shown to be useful for sampling neurotransmitters that were directly derived from neuronal release (Westerink & de Vries, 1989). After years of development, the method is now routinely used in research of CNS pharmacology and industrial drug development, especially for monitoring monoamines, like dopamine, serotonin and norepinephrine (Westerink, 2000b). However, in parallel to the routine application of the method, alternative applications of the method are currently also explored. As microdialysis monitors dynamic processes in time, any application should be validated to see whether it actually instantaneously reflects the exterior concentrations it samples. Even for monoamines like serotonin, it was shown that some membranes have delayed responses when suddenly exposed to changes in outer concentrations (Tao & Hjorth, 1992). As new applications tend to focus on monitoring compounds that are larger than monoamines (> 300 Da) but also significantly more non-polar, the dynamics of sampling becomes more complex.

**Membranes-materials:** Several issues are of importance when applying microdialysis to sample large non-polar compounds such as neuropeptides. The first important issues are the composition and structure of the membrane. Membranes typically consist of polymers that are produced by electrostatic polymerization. Different materials have been used throughout the years and most membranes are either cellulose derivatives (hollow fiber, cuprophane) or polyethersulfonic acid derivatives (PES, hemophane, HOSPAL). Whereas some membranes possess a charged surface (Hospal), which might add to overall recovery but might blunt dynamics, most are neutral in charge. Typically neutral membranes are best for sampling less polar compounds because a charged surface might repel non-polar compounds. However, the present results show that for sampling of Substance P, no pronounced difference is observed between the use of cellulose or polyether sulfone membranes. Another important issue is when sampling neuropeptide is the matrix in which the membrane is placed, as this can have a major influence on membrane passage and/or adsorption of the neuropeptides. The medium in which the *in vitro* recovery is measured can play a role, e.g. the protein content in which it is perfused (Tsuchida *et al.*, 2004). pH of

the solution can also have a major effect on the recovery, since it can affect the charge of the membrane and substrate and thus cause repulsion between the neuropeptide to be measured and the membrane, thereby decreasing recovery (Matsumoto *et al.*, 2003)

**Membranes-pores:** A feature that determines recovery by microdialysis probes to a large extent is the cut-off (pore size) of the membrane. Although the cut-off is a dynamic process and only defines a mean pore, it does not provide any indication as to how the pores are distributed, nor whether they are funnel shaped toward the inside or outside of the lumen, as is typically the case with the newer types of membranes. For Substance P, recovery was substantially lower when low cut-off membranes were used instead of high cut off membranes (see table 2 this chapter). This indicates that membranes with even larger pore membranes might be employed in sampling of neuropeptides. However, above 30 kD cut-off ultrafiltration becomes an increasingly significant factor. Therefore it is not recommendable to use this type of membranes, as it will influence the local physiology of the brain and thus the concentration of the analytes under investigation.

**Non-specific binding:** Any bioanalysis of non-polar compounds is compromised by non-specific binding of the compound to the sampling system. Although microdialysis probes are constructed of fused silica, and peek tubings are used for connections in order to minimize these effects, non-specific binding remains an issue. To this end several approaches have been proposed to circumvent these events. Some authors use cyclodextrins to chelate the compounds under study, preventing them from attaching to the surface of sampling/analytical systems (Caille *et al.*, 2007; Walker *et al.*, 1999). Although very functional in its approach, given the low molecular weight of cyclodextrins (mw ~ 1.1 kDa) compared to the cut-off of the membranes, inclusion of these compounds to the dialysis fluid might lead to infusion into the extracellular fluid. The cyclodextrins can then interact with multiple systems in the extracellular space, making this approach undesirable. Another approach to reduce non-specific binding, which was used in this study, is the inclusion of albumine in the dialysis fluid. As the molecular weight of BSA is much higher than the cut-off of the membrane (mw ~ 66 kDa), no risk of contamination of the extracellular space is present. Indeed, in the present study we observed that inclusion of BSA significantly elevated recoveries for CRF (see table 3 this chapter). The necessity to increase the BSA concentration for vasopressin and CRF supports the idea that microdialysis has to be optimized for individual neuropeptides to guarantee reliable quantification. In the case of vasopressin and CRF their polarity is the most likely cause non-specific binding. Therefore, it is not likely that recoveries can be improved by changing the type of membrane, but that a solution must be found in which a compound is used that prevents the non-specific binding.

***In vivo* sampling of Substance P:** While *in vivo* sampling of Substance P has already been described in 1983 in the caudate putamen of the rat (Brodin *et al.*,

1983), membrane properties have to our knowledge never been studied. Initial *in vitro* recovery studies showed pronounced differences for the hollow fiber and PES membranes vs. the hemophane membrane. However, no such differences were observed when the membranes were applied *in vivo* in the ventral hippocampus. All types of probes yielded about 1.5 fmol Substance P per 30 min of microdialysate sample. However, when using potassium to evoke a response, only in the case of the PES membrane was an increase in Substance P levels of about 45% (Fig. 3). Using cellulose, the increase observed upon potassium stimulation was much less pronounced reaching no more than 20%. It is not clear whether this occurs by reduction of Substance P entering the membrane or restriction of potassium diffusion from the probe, and should be further elucidated; for instance by looking at the effect of potassium on monoaminergic neurotransmitters. Microdialysis in multiple areas of the brain showed that Substance P levels are highest in striatum (2.9 fmol/sample), while lower levels were observed in the ventral hippocampus (1.29 fmol/sample) and DRN (0.75 fmol/sample). Previously various concentrations of Substance P in different brain areas, ranging from 0.1 fmol per 30 min sample in striatum up to 8 fmol per 30 min sample in the amygdala. It is clear however, that the reported concentrations are connected with the type of antibody used.

**In vivo sampling of CRF and vasopressin:** CRF and vasopressin could be extracted using the PES membrane. A complicating factor however, is the blunted dynamics when the membranes are exposed to sudden changes of the external concentration of the neuropeptide. It took a considerable time before equilibrium was reached, this lag-time will skew any pharmacology measured *in vivo*. Even with increased levels of albumine (up to 0.5%), the delayed response to concentration changes could not be reduced. However, as shown in Table 5, it was possible to measure vasopressin concentration in the PVN.

### Conclusions

The current study evaluated the possibility of applying microdialysis for monitoring brain levels of neuropeptides. Although multiple studies have been published (Ebner & Singewald, 2006; Wotjak *et al.*, 2008), none of them have systematically investigated the characteristics and dynamic behavior of the applied microdialysis probe, to better understand the phenomena underlying the observed signal. It can be concluded that, Substance P can be reliably monitored using a PES membrane with a 20 kDa cut-off. However, applying this membrane to more non-polar or larger neuropeptides such as vasopressin or CRF was less successful. Finally, it was shown that the physico-chemical properties of membrane affect the recovery and recovery rate of the studied neuropeptide, this may in turn lead to incorrect conclusions about their concentrations.

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