Substance P and the neurokinin 1 receptor

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Chapter 6

Comparison of RIA and LC-MS/MS for the determination of Substance P in brain microdialysates of freely moving animals

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Abstract

Neuropeptides have gained interest for their role in numerous central nervous system processes in the last decade. Generally neuropeptides are analyzed using radio immune assay (RIA), which can handle complex matrices and have excellent sensitivity. A limitation of RIAs is their selectivity and the stability of the antibodies. Other detection methods are available but their sensitivity is generally too low for detection of neuropeptides in microdialysates. In this study we developed an HPLC method in combination with tandem mass spectrometry, sensitive enough to detect Substance P in brain microdialysates.

RIA and HPLC- MS/MS were compared as detection methods for Substance P. It appeared that concentrations of Substance P were approximately five-fold lower when LC-MS/MS was used than with a RIA. Local potassium stimulation in the dorsal raphe nucleus of rat induced a significant increase of the extracellular Substance P concentration. The increased concentration was detected by both analysis methods. In the striatum however, this effect was not observed.

Our results indicate that LC-MS/MS offers an excellent alternative for the conventional RIA analyses of Substance P in microdialysates. LC-MS/MS has at least comparable sensitivity but is arguably more selective. The significantly lower SP levels found with LC-MS/MS indicate that the RIA does not discriminate between Substance P and degradation fragments. In the worst case RIA is unable to discriminate between Substance P and related peptides from the neurokinin family. This may have major consequences for the interpretation of previously published studies using RIA.

6.1 Introduction

Substance P (SP) is abundantly present in the brain. SP belongs to the neurokinin family, which also consists of neurokinin A and B and hemokinin 1 (Hokfelt *et al.*, 2000; Duffy *et al.*, 2003).

Neurokinin receptors that have been classified thus far are the NK_1, NK_2 and NK_3 receptors. Whereas NK_1 and NK_3 receptors are distributed throughout the brain, NK_2 expression is limited to cortex, hippocampus, septum and thalamus of the brain (Saffroy *et al.*, 2003). Although all neurokinin peptides have some affinity for other NK receptors, SP predominantly binds to NK_1 receptors (Maggi, 1995; Regoli *et al.*, 1994).

Preclinical research on SP is complicated by species differences in NK_1 receptors distribution throughout the brain. Moreover, species variation of the NK_1 receptor (Sachais *et al.*, 1993; Beresford *et al.*, 1991; Rigby *et al.*, 2005), might result in divergent NK_1 agonist and antagonist affinities between various animal species. Finally, NK_1 receptor function also varies between species, which adds an extra layer of complexity when interpreting the results of preclinical research to the clinic (Beresford *et al.*, 1991).

Besides SP's role in the regulation of autonomic processes and pain physiology, several studies have indicated that the neuropeptide might be involved in stress response and affective behavior (Severini *et al.*, 2002; Kramer *et al.*, 1998). However, clinical studies on the relevance NK_1 antagonists for affective disorders have been far from unequivocal (Kramer *et al.*, 2004; Adell, 2004; Ebner & Singewald, 2006). Currently NK_1 antagonists are used in the clinic as anti-emetics to treat chemotherapy-induced emesis.

Neurotransmission of SP has been shown to occur via co-transmission, that is to say that SP is a neurotransmitter as well as neuromodulator (Otsuka & Yoshioka, 1993;Levita *et al.*, 2003). Both clinical and preclinical research indicates that SP is involved in multiple pathophysiological disorders of the central nervous system (CNS). Accordingly, it is of interest to study the processes involved in the release and control of extracellular SP (Rupniak & Kramer, 1999).

Many complications are encountered when attempting to measure extracellular levels of SP in brain. Total tissue content of SP will only yield limited information on actual release of SP as it represents both the intra- and extracellular compartment (Gamse *et al.*, 1981). To measure extracellular SP levels accurately in the brains of freely moving animals demands both an adequate sampling technique and a state of the art analysis. In theory the push pull technique is suitable for sampling of extracellular SP, however its damaging impact on the surrounding brain tissue cannot be ignored, which has limited its practical application considerably (Brodin *et al.*, 1983; Lindefors *et al.*, 1985a). The currently preferred method for sampling extracellular levels of SP is undoubtedly microdialysis (Ebner & Singewald, 2006),

but this technique also has limitations especially when it is used for monitoring neuropeptide levels.

Passage of an analyte through the microdialysis membrane is typically expressed as percentage recovery. Earlier work has shown that multiple problems are encountered when applying microdialysis to sample neuropeptides (See chapter 6, this thesis). Ideally, recovery should instantaneously change when probes are transferred from an analyte free solution, to a solution containing the analytical standard. Chapter 6 it was demonstrates that the physico-chemical properties, the cut-off value of the membrane and the inclusion of bovine serum albumine (BSA) in the solution critically influence neuropeptide dynamics in the dialysate. These dynamics are arguably the most essential aspect of *in vivo* measurements. Yet, in the past very little attention was paid to the dynamics of SP in microdialysis experiments and one may therefore question how relevant previous studies were. Some other aspects of microdialysis make the analysis of neuropeptides in biomatrices difficult. Apart from non-specific binding of neuropeptides to the sampling system, levels of these compounds in brain dialysates are low and make ultrasensitive analysis methods mandatory. Typically, this has forced researchers to use antibody assays like RIA or ELISA when measuring peptides in dialysates.

Several issues are of crucial importance when antibody assays are being used to measure neuropeptide concentrations in dialysates.

1) Cross reactivity with other neuropeptides can be detrimental to the analysis. Neuropeptides are metabolized *in vivo* by peptidases. The resulting fragments (Michael-Titus *et al.*, 2002), may also have considerable affinity for the antibody, and compromise the analysis of the original compound (Nilsson *et al.*, 1998).

2) The bioanalytical matrix can influence high affinity binding of antibodies to the neuropeptides. For instance ionic strength, pH and temperature conditons can be crucial in obtaining optimal results (Midgley, Jr. *et al.*, 1969). As all these factors can influence the binding characteristics, a uniform matrix within a bioanalytical batch is desirable. Obviously, ionic strength will change with local potassium stimulation, which and may also influence binding to the antibody. In all, as a typical bioanalytical experiment is measured and compared to a calibration line in a single matrix, these effects are not compensated.

3) Another confounding factor could be the measures taken to reduce non-specific binding of the analyte to the materials used for sample collection. As discussed (above and in chapter 6), addition of BSA to the perfusion solutions reduces nonspecific binding to the sampling system. Although albumine is also used in the antibody assays its concentration should not exceed a certain level, which limits its use (Amino *et al.*, 1983).

4) Antibodies are derived from biological systems and their performance may therefore not always be consistent. Whereas common methodological protocols can be used, clear differences between the overall sensitivity of the antibodies can

be observed between batches. Likewise, complicating factors like cross reactivity and dependence on physical or chemical properties might also differ between batches.

Overall these issues make antibody assays a bioanalytical tool that is difficult to constantly control over extended periods of time when applied to *in vivo* samples.

Liquid chromatography with tandem masspectrometry (LC-MS/MS) is a technique that allows analysis of compounds by selection of ionized compounds through electrostatic fields, using their mass-charge ratio. Tandem masspectrometry (MS/MS) can be used in combination with liquid chromatography, thus allowing separation of a compound of interest from a complex matrix in conjunction with identification of mass and mass products. These features make the methodology very robust, selective and sensitive. Given the identification and chromatographic possibilities, this method is not prone to the issues described for antibody assays. Arguably, mass spectrometry measures ions and hence separation from ions during the chromatography is needed to prevent ionization suppression (Beaudry & Vachon, 2006b).

Several attempts have been made to quantify SP with LC-MS/MS (Rieux *et al.*, 2007; Beaudry & Vachon, 2006a), however, to our knowledge no studies have so far been published using this method to quantify SP in brain dialysates. The current study was set up to develop such a method and compare it to antibody assays.

6.2 Materials and methods

6.2.1 Animals, Surgery and experiment

Rats (280-350 g; Harlan, Horst, The Netherlands) were used for the experiments. They 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_2). Bupivacain / norepinephrine was used for local anesthesia. The animals were placed in a stereotaxic frame (Kopf instruments, USA), and I-shaped probes (PES Fresenius membrane, 3 mm exposed surface; BrainLink, The Netherlands) were inserted into striatum.

Coordinates for the tips of the probes was for the ventral hippocampus: posterior (AP) = +0.9 mm to bregma, lateral (L) = +1.1 mm to midline and ventral (V) = -6.0 mm to dura (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 artificial CSF, which contained 142mM NaCl, 3mM KCl, 1.2mM $CaCl_2$, 1mM $MgCl_2$ and 0.025% BSA which was freshly prepared daily. The flow rate was set at 1.5 µl/min.

Microdialysis samples were collected for 30 min periods into mini-vials already containing 65 µl of RIA buffer for those analyzed in a radio immuno assay. Alternatively, samples were collected without additive, when analyzed on the LC/MS/MS. Microdialysis samples were collected by an automated fraction collector (CMA 142) 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.

6.2.2 Analysis

6.2.2.1 RIA

In each assay, standard curves were prepared for SP by using known amounts of synthetic SP (SIGMA, St Louis, Missouri, USA) $1*10^{-9}$M - $5*10^{-14}$ prepared in aCSF RIA buffer (at a 45:65 ratio). Standards and samples were pre-incubated with 100 µl of diluted SP-antiserum (Euro-Diagnostica, AB Malmö, Sweden) for 48 h at 4 °C. Cross reaction of SP antiserum was 100% to SP and less than 0.1% to other peptides. After addition of [^{125}I]Bolton-Hunter-SP 8,000 cpm in 100 µl (Amersham Biosciences, Buckinghamshire, UK), all 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). Separate 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 1 fmol per sample.

6.2.2.2 LC/MS/MS

To 45 µl standards and samples, 5 µl of internal standard (SP-5*C^{13}-1*N^{15}) was added and the sample was injected onto a C18 BDS Hypersil 10 x 2.1 mm guard column with a particle size of 5 µm. The initial mobile phase condition consisted of acetonitrile (0.1% formic acid) and ultra-purified water (0.1% formic acid) at a ratio of 5:95. From 0 to 3 min, the ratio was maintained at 5:95. From 3 to 8 a gradient was a plied to go stepwise to a ratio of 80:20. At 8.5 min the mobile phase composition was reverted to 5:95 and the column was allowed to equilibrate for 3.5 min for a total runtime of 12 min. From 0 min to 5.5 min and from 8 to 12 min the flow was diverted directly to waste, to prevent salt and protein overload on the mass-spectrometer. The flow rate was fixed at 0.25 ml/min.

The detection was performed using an API 4000 MS/MS system consisting of a API 4000 MS/MS detector and a Turbo Ion Spray interface (both from Applied Biosystems, the Netherlands). The acquisitions were performed in positive ionization mode, with ion spray voltage set at 5.3 kV with a probe temperature of 450 °C. The instrument was operated in multiple-reaction-monitoring (MRM) mode for detection of SP (precursor 450.0 amu, product 600.5 amu) and SP-5*C13-1*N15: (precursor: 452.0 amu, product: 603.5 amu).

6.2.3 Data presentation

Data are presented 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. Data was statistically analyzed by one or two way ANOVA; $p < 0.05$ was considered significant.

6.3 Results:

6.3.1 Analysis method

Fig. 1A shows the variation in SP-5*C^{13}-1*N^{15} levels measured by RIA overnight and fig. 1B shows a typical chromatogram of Substance P and its internal standard SP-5*C^{13}-1*N^{15}.

A B

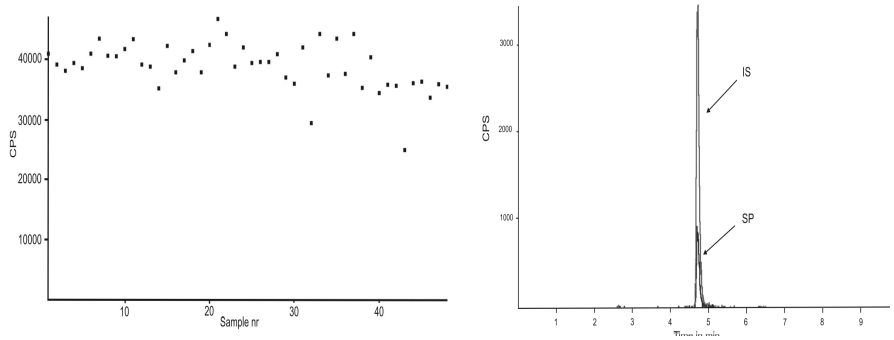

Figure 1 *Internals standard of SP tested for stability overnight (1A) and a typical chromatogram of SP and internal standard (IS), measured by LC-MS/MS (1B)*

Detection limit of SP in the RIA was around 1 fmol/45 µl. Levels of SP varied between 1 fmol and 13 fmol per sample. A typical calibration curve of SP by RIA is shown in Fig. 2A For the LC/MS/MS detection with a stable isotope of SP as internal standard levels of SP varied between 0.23 fmol and 4.6 fmol/sample. Analysis of Substance P with LC-MS/MS was linear over three decades as shown in Fig. 2B.

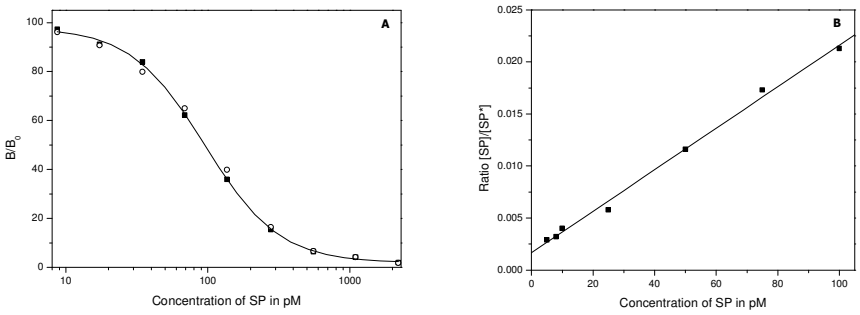

Figure 2 *Examples of typical calibration curves for SP measured by RIA (A) and with LC-MS/MS (B)*

It appears that the LC-MS/MS measurements give more reproducible calibration curves of SP than the RIA analysis. The detection limit of SP measured with RIA ranged from 1 to 3 fmol/sample, depending on the antibody batch that was used. For the LC-MS/MS analysis the capacity of the guard column was found to be the critical factor that determined the limit of detection.

6.3.2 Result of analysis method *in vivo*

Fig. 3 shows the basal striatum and DRN concentrations of SP measured by RIA or LC-MS/MS. It appears that the basal level of SP in the striatum measured by RIA is significantly higher than that measured with LC-MS/MS. A comparable effect was observed in the basal levels of SP in the DRN as well. The difference observed in the striatum is approximately seven-fold and in the DRN threefold. Both in striatum and DRN the difference was statistically significant according by one way ANOVA ($p < 0.001$).

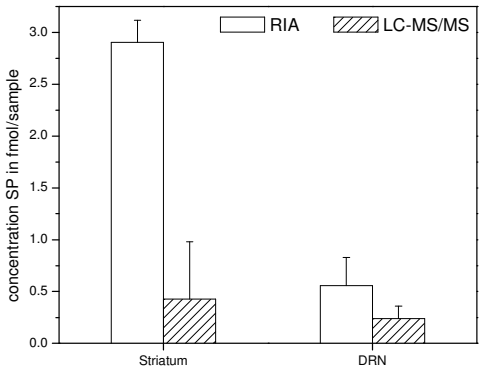

Figure 3 *Basal output of Substance P in the striatum and DRN measured with either RIA or LC-MS/MS. *** indicates p < 0.001*

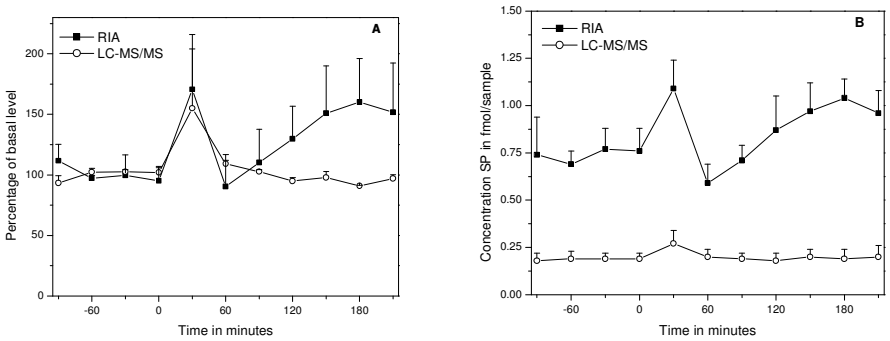

Figure 4 *Effect of local potassium infusion into the dorsal raphe nucleus. Panels A and B show the relative and absolute data, respectively. * indicates p < 0.05*

By reversed microdialysis it is possible to locally infuse compounds or to change the ionic balance in the target area. An increased extracellular potassium or calcium concentration leads to increased exocytosis of the neurotransmitter, which in turn increases the extracellular neurotransmitter concentration. Local stimulation of potassium in the dorsal raphe nucleus of rats showed an increase in extracellular SP levels (Fig. 4). The relative increase in extracellular levels of SP is

comparable between the analysis methods. A significant time effect at t = 30 minutes is seen for the local potassium stimulation (two way ANOVA, using Student Newman Keuls post-hoc test: $F_{7,42} = 2.309$, $p < 0.05$) on extracellular SP concentration, although this effect could not be attributed to a specific analysis method.

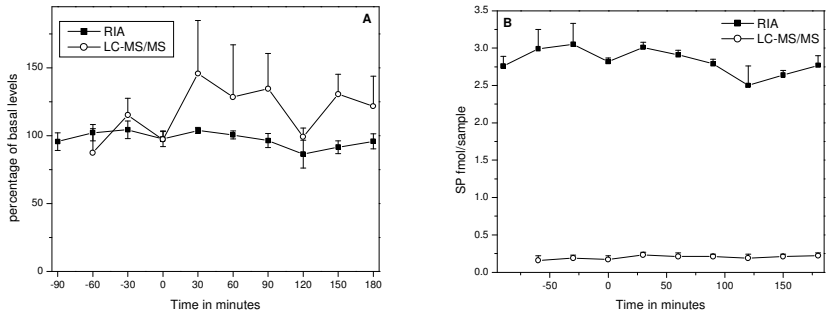

Figure 5 *shows the effect of a local infusion of potassium in the striatum. Panels A and B show the relative and absolute data, respectively. * indicates p < 0.05*

Local potassium stimulation in the striatum of the rat does not show any effect on the extracellular concentration of SP levels (Fig. 5), but when the same experiment was repeated and measured with LC-MS/MS a minor increase to 150% of basal values is observed. This effect was however not found to be statistically significant in a two way ANOVA ($F_{6,30} = 1.078$, $p = 0.398$).

6.3.3 *In vivo* experiments

As shown above, local stimulation for 60 minutes with 60 mM potassium resulted in increased extracellular SP up to 150% of basal levels in the dorsal raphe nucleus. Similarly we tried the effect of increased calcium concentration in the DRN. However, this did not appear to affect extracellular SP comcentrations, neither did the omission of calcium in the perfusion fluid (Fig. 6).

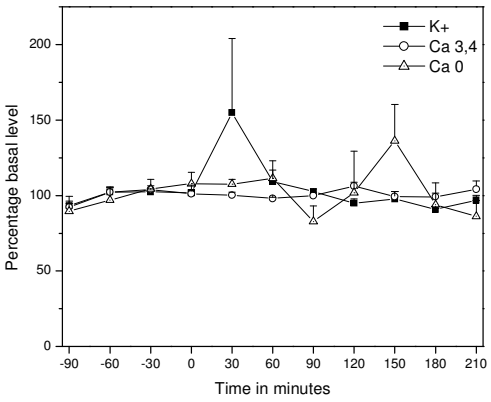

Figure 6 *Effects of different local infusions into the DRN on extracellular level of Substance P measured by LC-MS/MS.*

Local infusion in the striatum of a high potassium concentration in the perfusion medium resulted in an increase extracellular SP to 150% of basal level. Local infusion of high calcium or TTX did not alter extracellular SP concentrations (two way ANOVA for repeated measurements: $F_{14,84}$ =1.539, p = 0.115) (Fig. 7).

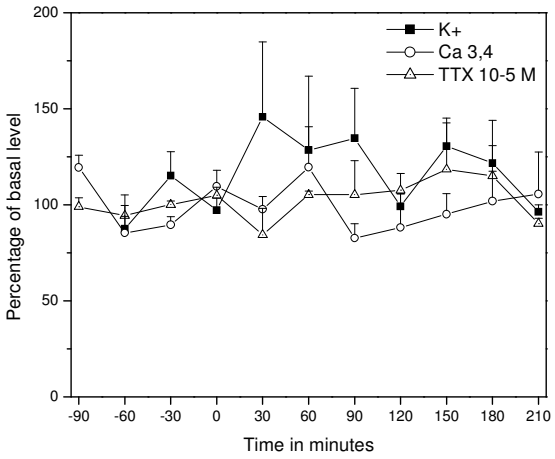

Figure 7 *Effect of different infusions into the striatum on extracellular SP measured by LC- MS/MS*

6.4 Discussion

Microdialysis is a well established technique that is routinely employed to monitor neurotransmitter release in the brains of freely moving animals. Conventionally the method is used to monitor small polar neurotransmitters like monoamines (norepinephrine, dopamine and serotonin), aminoacids (GABA, glutamate) and acetylcholine (Westerink, 2000). In the previous chapter we showed that a thorough study of membrane characteristics is necessary, to obtain optimal conditions for performing microdialysis to study neuropeptides. Here we show that optimization of the analytical conditions for studying neuropeptides is equally important.

Although antibody assays of SP in dialysates have been applied abundantly (Lindefors *et al.*, 1985b; Ebner *et al.*, 2004), no studies have described the quantification of SP in microdialysates using LC-MS/MS. This is primarily due to the complications that arise when studying microdialysates. For instance, Ringer used for microdialysis contains large amounts of salts and BSA, which interfere / foul the mass spectrometer. To prevent this, a a guard column was was successfully combinined with the LC step to remove BSA and desalt the solution.

Interestingly, it was observed that quantification of neuropeptide levels in dialysates using antibody assays give of up to 5 times higher levels of SP then quantification by mass spectrometry. This means that ~80% of the signal that is measured using the current antibody method would result from partial degradation products from SP or from other abundant neuropetides. Although not all published studies used the same antibody as the present study, observed levels where close to those found in the present antibody assays. In addition, when levels would have been as low as quantified with MS, RIA sensitivity would not have been sufficient. This observation is in line with several previous studies in which antibody assays were compared to masspectrometry (Nilsson *et al.*, 1998). For neuropeptide Y, somatostatin and galanin similar observations have been reported, indicating that data of antibody assays should be interpreted with care (Nilsson *et al.*, 2001). The major part of the signal measured for SP is apparently derived from degradation products of SP or from other tachykinins and their degradation products, rather than the SP itself. In this respect it is of interest that also the degradation products have been found to be functional *in vivo* (Khan *et al.*, 1995; Khan *et al.*, 1996).

In the DRN the relative effect of infusion of potassium is consistent between RIA and LC-MS/MS analyses. Both result in a rise of extracellular SP to about 150 % of basal values. The absolute values however, are 3-fold lower in the samples analyzed by LC-MS/MS. This indicates that the degradation products and other peptides additionally measured with RIA, show the same response to potassium stimulation as SP alone. In the striatum a small increase of 50% was observed in

the samples, measured by LC-MS/MS, but not in the samples measured by RIA. Other studies have reported a rise for potassium stimulation in different brain areas measured by RIA (for an overview see (Ebner & Singewald, 2006)).

No effect was observed when either the sodium channel blocker TTX or elevated calcium was infused into the DRN or striatum. As both RIA and MS analysis showed no significant effects, these data lead to the conclusion that SP is not modified by impulse dependent mechanisms or calcium dependent mechanisms. Since no effect was observed in the samples analyzed with RIA, the concentration of the degradation products of SP and other peptides are not TTX and calcium dependant either. This might be explained by the fact that the calcium dependent release of neuropeptides is regulated by the L-type calcium-channels and not as for e.g. norepinephrine, the N-type calcium channel (Hirning *et al.*, 1988;Perney *et al.*, 1986). These L- type channels are more sensitive to intracellular calcium, which appears not to be not affected by the extracellular changes of calcium (Perney *et al.*, 1986; Holz *et al.*, 1988). This is in contrast to *in vitro* results, where clear release of SP from slices was observed in response to increased extracellular calcium (Huang & Neher, 1996). SP appears to be insensitive to the effect of local TTX infusion. This is probably because neuropeptides often not only have neurotransmitter like transmission, but also a paracrine like transmission within the brain (Wotjak *et al.*, 2008). This concept has been postulated on the basis of anatomical studies, showing that some SP terminals are not in close proximity of the preferred NK_1 receptor (Liu *et al.*, 1994; Vruwink *et al.*, 2001). This could explain why the local TTX induced decrease of SP transmission is not observed, since paracrinic SP replenishes the local concentration at faster rate than the local exocytosis. Paracrinic transmission is also observed for serotonin and serotonin decreases after local application of TTX (Bunin & Wightman, 1998). Serotonin is however degraded faster than SP, which could explain the observed differences.

Overall the present study clearly shows that microdialysis of neuropeptides is complicated by multiple factors. Whereas antibody assays measure SP and its degradations products, the pharmacological response to potassium stimulation is similar in this study. SP levels as quantified by microdialysis appear to be not related to neuronal impulse flow or to be extracellular calcium dependent. The extent to which the parallel release of SP and its degradation products holds for additional pharmacological studies is unknown. In conclusion, mass spectrometry appears to be a more precise analysis technique than RIA and should be applied to ensure identity of the compound under study.

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