Substance P and the neurokinin 1 receptor

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Chapter 4
DIVERGENT SUBSTANCE P-SEROTONIN INTERACTIONS IN PREFRONTAL CORTEX AND VENTRAL HIPPOCAMPUS OF THE GUINEA PIG

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Abstract

The neuropeptide substance P (SP) has gained interest as a target in the treatment of depression and anxiety. Several studies have reported on substance P-serotonin interactions in the brain. Studies in rats and mice have shown that inhibition of the NK_1 receptor, to which SP preferentially binds significantly increases the effect of selective serotonin uptake inhibitors (SSRIs) on serotonin levels in prefrontal cortex. This indicates that augmentation strategies with NK_1 antagonists might improve antidepressant treatment.

As the pharmacology of substance P is species dependant, here we studied whether the interplay between a NK_1 antagonist and an SSRI also occurs in the guinea pig. This is of interest because SP neuro-architecture in guinea pigs is more akin to humans than in rats and mice.

Whereas robust effects of the SSRI fluoxetine (10 mg/kg intra-peritoneal) were observed on serotonin levels in median prefrontal cortex, no augmentation was seen when fluoxetine was co-administered with the selective NK_1 antagonist GR 205171 (0.63 mg/kg sub-cutaneous. Interestingly, when the co-administration was studied in the ventral hippocampus, a pronounced augmentation of the effect of fluoxetine on serotonin levels was observed.

The present study supports the idea that profound differences exist in substance P pharmacology between guinea pigs and rodents. The observation that the SP-serotonin interaction in guinea pigs only occurs in the hippocampus, indicates that SSRI augmentation with an NK_1 antagonist may be more effective in depression with high co-morbid anxiety than with core symptoms of depression.

4.1 Introduction

Substance P (SP) is a member of the neurokinin family, which further consists of neurokinin A and B and the more recently discovered hemokinin I (Zhang *et al.*, 2000; Duffy *et al.*, 2003). Although the involvement of substance P in pain perception and emesis has been known for nearly half a century (Zubrzycka & Janecka, 2000), its relevance for psychiatric disorders has only been subject of investigation in the last decade (Chahl, 2006).

The increased interest in substance P as possible target to treat psychiatric disorders has led to a substantial increase of pharmacological studies on SP neurotransmission and its interaction with other neurotransmitter systems. However, when studying SP neurotransmission and pharmacology one is confronted with a number of problems.

First, the *in vivo* assessment of SP itself forms a major obstacle. Both microdialysis and bioanalysis of SP appeared to be far more complicated than with classical neurotransmitters such as monoamines (this thesis; Ebner & Singewald, 2006). Second, a limiting factor with pharmacological studies is the marked species variety with respect to neurokinin receptors. Three types of neurokinin receptors have thus far been identified (NK_1, NK_2 and NK_3). However, SP preferentially binds to NK_1 receptors. The amino acid sequences of these receptors differ between species (Sachais *et al.*, 1993), as a result the affinities of NK_1 antagonists and agonists vary considerably between species. Whereas the differences between human, guinea pigs and gerbils are not extreme, affinities in mice and rats severely deviate from those in humans (van der Hart *et al.*, 2002; Beresford *et al.*, 1991). This issue becomes even more relevant when compounds are being studied for drug development purposes. An additional complicating factor is that NK_1 receptor function might be different between species (Watling *et al.*, 1994).

Because of its possible relevance for the treatment of depression, several studies have investigated the interaction of SP with serotonin. Overall, an inhibitory effect of SP was observed on the serotonergic system and in particular on the firing rate of dorsal raphe nucleus (DRN) serotonergic neurones in rats (Haddjeri & Blier, 2000), offering a rational for using NK_1 antagonists in the treatment of depression. However, NK_1 receptors could not be demonstrated on the serotonergic cell bodies in rat, suggesting an indirect effect through glutamate and/or GABA. Alternatively, localization of NK_1 receptors on serotonergic terminals has been hypothesized (Adell, 2004; Conley *et al.*, 2002).

In line with the inhibitory action of SP on serotonergic activity an interesting observation was made by Guiard *et al.* (2004). Whereas NK_1 antagonists were devoid of pronounced effects on brain serotonin levels when administered systemically alone, a clear augmentation was observed when a NK_1 antagonist was

co-administered with a SSRI (Guiard *et al.*, 2004). Augmentation with NK_1 antagonists has the potential to improve antidepressant therapy and this has certainly stimulated research into SP-serotonin interactions. However, most of this research has been performed with rats and mice, and one may question whether these results can be safely translated to humans. Given the different NK_1 antagonist affinities and divergent NK_1 receptor functions between species, it is important to study how universal this augmentation really is (Sergeyev *et al.*, 1999; Commons & Valentino, 2002; Beresford *et al.*, 1991). Accordingly, we investigated co-administration of the SSRI fluoxetine with the selective NK_1 antagonist GR 205171 in guinea pigs. Because NK_1 receptor function may differ between brain regions we studied the interaction in both prefrontal cortex and ventral hippocampus in freely moving guinea pigs.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Male Dunkin Hartley guinea pigs (350-500g; Harlan, Horst, the Netherlands) were used for the experiments. Guinea Pigs were housed in pairs in plastic cages (35 x 55 x 20 cm) and had access to food and water *ad libitum* untill surgery. After surgery the animals were housed individually in plastic cages (32 x 49 x 30 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.

4.2.2 Surgery

Guinea Pigs were anesthetized using isoflurane (2%, 800 ml/min O_2). Lidocain (10 % w/v) was used for local anesthesia. Each animal was placed into a stereotaxic frame (Kopf instruments, USA) and I-shaped probes with hospal membrane (6 kD, 4 mm exposed surface, Brainlink, Groningen, the Netherlands) were inserted into the prefrontal cortex (PFC) and ventral hippocampus (vHip). Coordinates according to the guinea pig brain atlas of Luparello (Karger, Switzerland, 1967) were: anterior to intra-aural + 15 mm, lateral to midline + 1 mm and ventral to dura – 4.5 mm for PFC and anterior to intra-aural + 4.9 mm, lateral to midline + 6.5 mm, and ventral to dura – 9.0 mm for vHip. The probe was then fixed to the skull with dental cement and a screw. Flunixin (1 mg/kg s.c.) was administered as peri/post-operative analgesic.

4.2.3 Experiments

Experiments were carried out 24-48 hours after surgery. On the day of the experiment, the probes were connected with flexible PEEK tubing to microperfusion pumps (CMA 102, Sweden). The dialysis probes were perfused with a Ringer solution containing 147 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgCl_2$ at a flow rate of 1.5 µl/min. Microdialysis samples were collected at 30 min intervals into mini-vials already containing 45 µl 0.02 M formic acid. Samples were collected by an automated fraction collector (CMA 142, Sweden), and stored at -80 °C until further analysis. After completion of the experiments the guinea pigs were sacrificed. The brains were removed and stored in paraformaldehyde solution (4% w/v). The location of each probe was verified histologically, according to Luparello in coronal sections of the brain (Luparello, Karger, Basel, Switzerland, 1967).

4.2.4 Drug administration

Fluoxetine and GR 205171 (kindly donated by Servier, France) were dissolved in ultra purified water. Fluoxetine injections were given intraperitonealy at a dose of 10 mg/kg and GR 205171 injections were given intraperitonealy at a dose of 0.63 mg/kg. GR 205171 was given at t = 0 minutes and fluoxetine was given at t=30 min. Volume of injection was 1 ml/kg. Injection times are indicated by arrows in the figures.

4.2.5 Analysis

Concentrations of serotonin, norepinephrine and dopamine were determined in the same sample, by HPLC and electrochemical detection. Samples were split into two aliquots; one was used for the simultaneous analysis of norepinephrine and dopamine and the second aliquot was used for the analysis of serotonin.

4.2.5.1 Norepinephrine and dopamine

Separation:

Aliquots (20 µl) were injected onto the HPLC column (Reversed Phase, particle size 3 µm, C18, Thermo BDS Hypersil column, 150 x 2.1 mm, Keystone Scientific, USA) by a refrigerated microsampler system, consisting of a syringe pump (Gilson, model 402, France), a multi-column injector (Gilson, model 233 XL, France), and a temperature regulator (Gilson, model 832, France). Chromatographic separation was performed using a mobile phase that consisted of a sodium acetate buffer (4.1 g/l) with methanol (2.5% v/v), disodium EDTA (150 mg/l), octyl sulphonic acid (150 mg/l), and Tetramethylammonium (150 mg/l) and adjusted with glacial acetic acid to pH = 4.1 (isocratic). The mobile phase was run through the system at a flow rate of 0.35 ml/min by an HPLC pump (Shimadzu, model LC-10AD vp, Japan).

Electrochemical detection:

Norepinephrine and dopamine were detected electrochemically using a potentiostate (Antec Leyden, model Intro, the Netherlands) fitted with a glassy carbon electrode set at +500 mV vs. Ag/AgCl (Antec Leyden, the Netherlands).

Data were analyzed by Chromatography Data System (Shimadzu, class-vp, Japan) software. Concentrations of norepinephrine and dopamine were quantified by the external standard method.

4.2.5.2 Serotonin

Chromatography:

Aliquots (20 µl) were injected onto the HPLC column (Reversed-phase, particle size 3 µm, C18 ODS Hypersil column, 100 x 2.0 mm, Phenomenex, USA) by a refrigerated microsampler system, consisting of a syringe pump (Gilson, model 402, France), a multi-column injector (Gilson, model 233 XL, France), and a temperature regulator (Gilson, model 832, France). Chromatographic separation was performed using a mobile phase consisting of a sodium acetate (4.1 g/l) with methanol (4.5% v/v), disodium EDTA (500 mg/l), heptane sulphonic acid (50 mg/l), and triethylamine (30 µl/l) and adjusted with glacial acetic acid to pH = 4.74 (isocratic). Mobile phase was run through the system at a flow rate of 0.4 ml/min by an HPLC pump (Shimadzu, model LC-10AD vp, Japan).

Electrochemical detection:

Serotonin was detected electrochemically at +500 mV, using the same method as described for norepinephrine and dopamine.

4.2.6 Statistical evaluation

Four consecutive microdialysis samples with less than 50 % variation were taken as baseline levels and set at 100%. Drug effects were expressed as percentages of basal level (means ± SEM) within the same subject. Statistical analysis was performed using Sigmastat for Windows (SPSS Corporation). Treatment or dose effects were statistically evaluated using two-way ANOVA for repeated measures, followed by Student Newman Keuls post-hoc test. The level of statistical significance level was set at $p<0.05$.

4.3 Results

4.3.1 Prefrontal cortex

Extracellular serotonin in the prefrontal cortex was monitored throughout the entire experiment. All groups received two i.p. injections. At t=0 min the guinea pigs received either vehicle or the NK_1 antagonist GR 205171 (0.63 mg/kg) and at t = 30 either vehicle or fluoxetine (10 mg/kg). The effects on extracellular serotonin in the prefrontal cortex are shown in Fig. 1.

No significant difference between the different treatment groups, not considering the time effect, could be observed ($F_{(2,12)}$=3.725; p=0.055). Yet, a significant interaction between the groups and time was observed ($F_{(16,75)}$=4.151; p<0.001). Within the vehicle and fluoxetine treated group an increase of extracellular serotonin was observed at t=40 minutes, 210 minutes after administration of fluoxetine. If GR 205171 is administered 30 minutes prior to fluoxetine, the rise of extracellular serotonin compared to baseline has an earlier onset, at t=150 minutes. This effect is also seen when both groups are compared to the vehicle treated group. A significant difference at any given time point between the GR 205171 and fluoxetine treated animals and those treated with fluoxetine alone could not be observed.

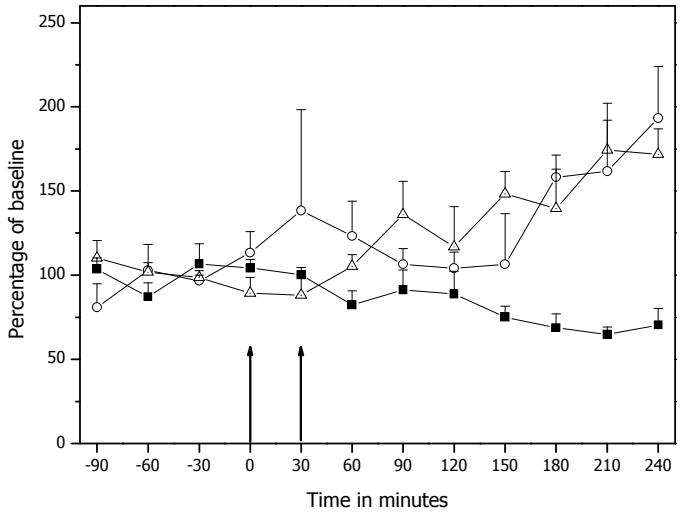

Figure 1 *The effects of vehicle (n=6, closed squares), fluoxetine 10 mg/kg (n=6, open circles) or a combination of GR 205171 0.63 mg/kg and fluoxetine 10 mg/kg (n=6, open triangles) on extracellular levels of 5-HT in the prefrontal cortex.*

In Fig. 2 the effects on extracellular norepinephrine is shown. No significant treatment effect on the extracellular levels of norepinephrine in the prefrontal cortex was found ($F_{(2,12)}$=3.650; p=0.055). Yet, an interaction between the different treatments and time was found ($F_{(16,102)}$= 4.656; p<0.001). Treatment with vehicle and fluoxetine was significantly different from baseline, prior to vehicle injection, from t=30 min to t=240 min. An additional effect of fluoxetine is seen at t=210 min to t=240 min (compared to t=30 min, prior to fluoxetine injection). The combination of GR 205171 and fluoxetine did not show a significant increase compared to baseline or the additional fluoxetine injection. The animals treated with fluoxetine alone compared to the animals treated with both GR 205171 and fluoxetine showed a significant higher increase in extracellular norepinephrine levels at t=180 to t=240 min. The animals treated with fluoxetine alone showed a significant increase of extracellular norepinephrine compared to the animals which were treated with vehicle at t=90 min and from t=180 min to t=240 min.

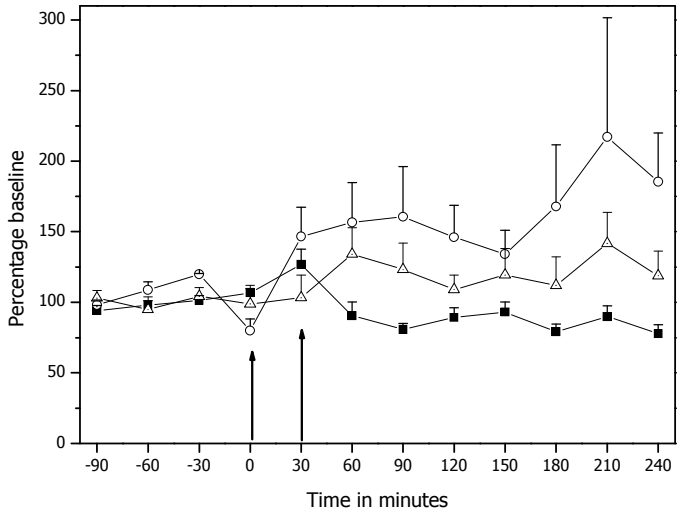

Figure 2 *The effects of vehicle (n=6, closed squares), fluoxetine 10 mg/kg (n=6, open circles) or a combination of GR 205171 0.63 mg/kg and fluoxetine 10 mg/kg (n=6, open triangles) on extracellular levels of NE in the prefrontal cortex.*

Extracellular dopamine levels in the prefrontal cortex of guinea pigs were monitored after injection of either vehicle or GR 205171, followed after 30 min by an injection of vehicle or fluoxetine. Fluoxetine alone or combined with GR 205171, had no effect on the levels of extracellular dopamine ($F_{(2,9)}$=0.863;p=0.454), nor was there any interaction effect observed between treatment and time ($F_{(16,61)}$=0.324;p=0.992).

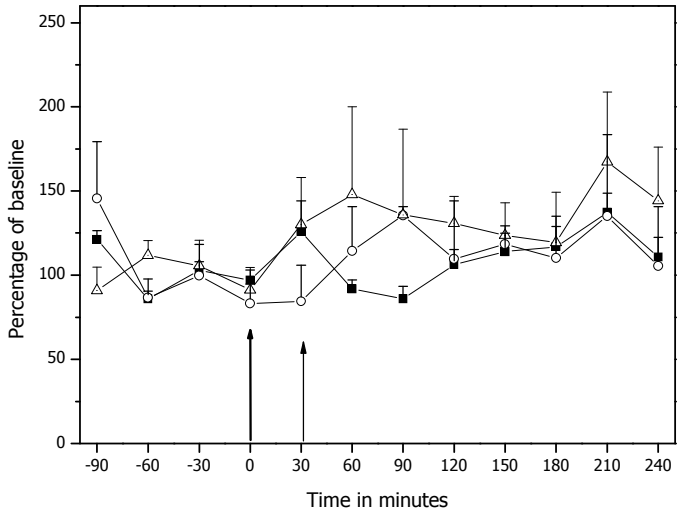

Figure 3 *The effects of vehicle (n=3, closed squares), fluoxetine 10 mg/kg (n=6, open circles) or a combination of GR 205171 0.63 mg/kg and fluoxetine 10 mg/kg (n=6, open triangles) on extracellular levels of DA in the prefrontal cortex.*

4.3.2 Ventral Hippocampus

Fig 4 shows the effect on extracellular levels of serotonin in the ventral hippocampus. Extracellular serotonin levels increased after administration of fluoxetine alone or combined with GR 205171 ($F_{(2,13)}$=6.973; p<0.01). However the time course differed between the treatment groups ($F_{(16,104)}$=6.011; p<0.001). Compared to baseline a significant increase was observed when the animals with treated with fluoxetine alone (t=210 minutes). This increase was present until the end of the experiment. For the treatment group, which received both GR 205171 and fluoxetine, the onset of the increase started at t=90. Apparently, the NK_1 antagonist enhanced the effect of fluoxetine in the ventral hippocampus on the extracellular level of 5-HT.

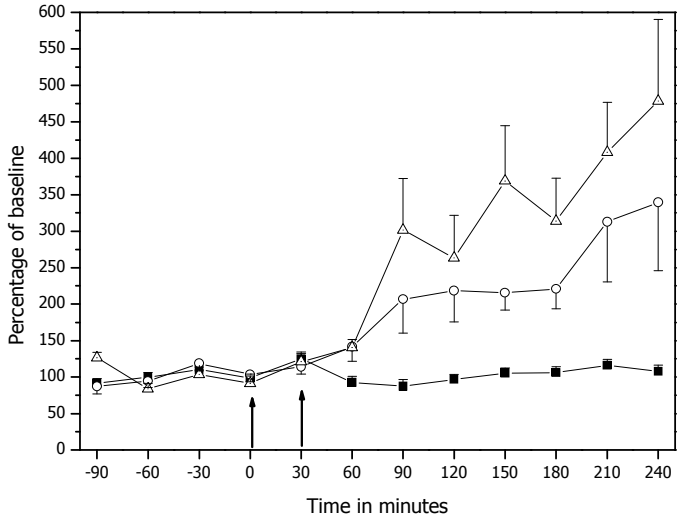

Figure 4 *The effects of vehicle (n=6, closed squares), fluoxetine 10 mg/kg (n=5, open circles) or a combination of GR 205171 0.63 mg/kg and fluoxetine 10 mg/kg (n=5, open triangles) on extracellular levels of 5-HT in the ventral hippocampus.*

Extracellular norepinephrine levels in the ventral hippocampus of guinea pigs were monitored for 240 minutes after injection of either vehicle or GR 205171 and 210 minutes after injection of vehicle or fluoxetine. The treatment combinations used in this study; vehicle alone, fluoxetine alone or fluoxetine combined with GR 205171, did show an effect on the levels of extracellular norepinephrine over time ($F_{(8,111)}$=2.482;p<0.05), but this effect could not be attributed to a specific treatment ($F_{(16,111)}$=1.661;p=0.065).

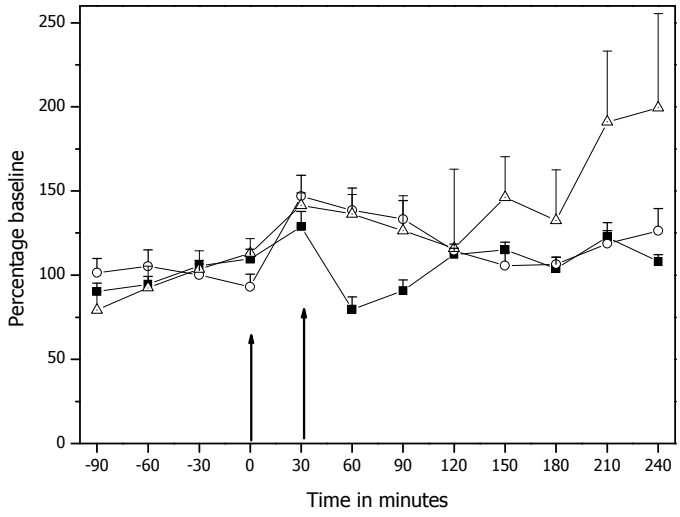

Figure 5 *The effects of vehicle (n=6, closed squares), fluoxetine 10 mg/kg (n=5, open circles) or a combination of GR 205171 0.63 mg/kg and fluoxetine 10 mg/kg (n=6, open triangles) on extracellular levels of NE in the ventral hippocampus.*

Extracellular dopamine levels in the ventral hippocampus of the guinea pigs were monitored for 240 min after injection of either vehicle or GR 205171 and 210 min after injection of vehicle or fluoxetine within the same animal. All experimental conditions; vehicle alone, fluoxetine alone or fluoxetine combined with GR 205171, showed no difference in effect on the levels of extracellular dopamine ($F_{(2,12)}$=0.567;p=0.582), nor was any interaction effect between treatment and time was observed ($F_{(16,82)}$=0.973;p=0.493).

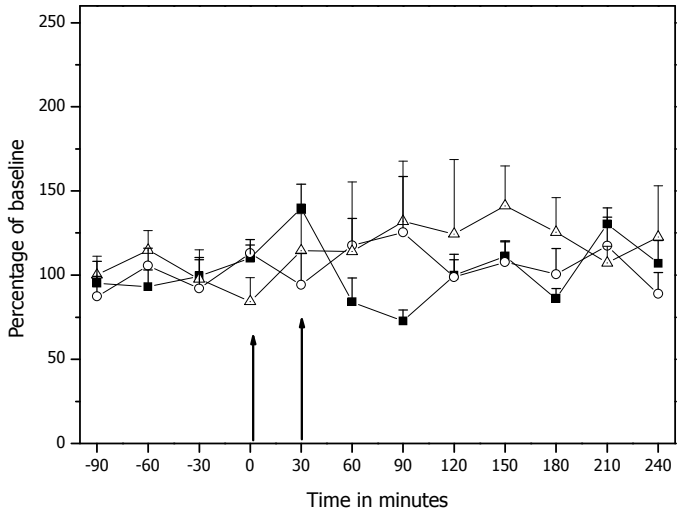

Figure 6 *The effects of vehicle (n=6, closed squares), fluoxetine 10 mg/kg (n=4, open circles) or a combination of GR 205171 0.63 mg/kg and fluoxetine 10 mg/kg (n=6, open triangles) on extracellular levels of DA in the ventral hippocampus.*

4.4 Discussion and conclusions

Given the sometimes marked species differences in receptor function and pharmacology it is not without risk to translate results from preclinical experiments to the human situation (DeGraba & Pettigrew, 2000; Bolton, 2007). The present study illustrates this by showing that NK_1 receptor pharmacology in guinea pigs markedly deviates from that previously reported for rodents. With targets that are very species dependant, such as NK_1 receptors, confirmation of the hypothesized mechanisms of action in multiple species is warranted, in particular when such data concern compounds that are used as a lead for clinical studies.

Treatment of depression is generally focused on the elevation of extracellular monoamine levels (Elhwuegi, 2004; Trudeau, 2004). As a non monoaminergic target, the NK_1 receptor to which substance P preferentially binds would have been a welcome addition to the existing neuronal targets in the treatment of depression and anxiety (Rupniak, 2002;Adell, 2004). However, the low efficacy of NK_1 antagonists in clinical trials came as an unpleasant surprise (Keller *et al.*, 2006). Yet, the irrefutable role SP in stress and anxiety (Ebner & Singewald, 2006) and the observation that an NK_1 antagonist augmented the effect of an SSRI on extracellular serotonin levels has kept interest in the neuropeptide alive (Guiard *et al.*, 2004). In this latter study a selective NK_1 antagonist had no effect in mice when given alone, but it significantly augmented the effect of a SSRI on extracellular serotonin levels in the frontal cortex. NK_1 receptors are mainly found in the dorsomedial part of the DRN. In rodents they are mainly located on non-serotonergic cells (Valentino *et al.*, 2003), but in humans NK_1 receptors are found on the serotonergic cell body (Sergeyev *et al.*, 1999). Moreover, substance P neurons are in contact with serotonergic and GABA-ergic neurons in the DRN in rats (Magoul *et al.*, 1986). Finally, local application of substance P into the dorsal part of the DRN increased the serotonergic firing rate in rats, whereas a decrease was observed when the neuropeptide was applied into the ventral part. Interestingly, systemic administration of NK_1 antagonists also increased DRN serotonergic firing rate indicating that NK_1 receptors are involved in the autoregulation of serotonergic activity in rats (Haddjeri & Blier, 2001). Accordingly, the augmentation by NK_1 antagonists of SSRI evoked increases in extracellular serotonin levels might relate to an attenuation of autoregulatory processes at the level of cell firing in the raphe nuclei. It is conceivable that, that this augmentation will result in improved efficacy and a reduced onset of action of the antidepressant.

The fact that the potency of NK_1 antagonists varies between species has been known for decades (Beresford *et al.*, 1991). Additionally, a different functionality of NK receptors among species has been equally well described (Rigby *et al.*, 2005; Sachais *et al.*, 1993). However, whereas behavioral work focused on animals that are more relevant for human pharmacology such as guinea pigs and gerbils, most

biochemical studies were performed in mice and rats (Malkesman *et al.*, 2007; Dableh *et al.*, 2005; Santarelli *et al.*, 2001; Vendruscolo *et al.*, 2003). This is surprising given the well known and pronounced species differences in both substance P pharmacology and neuroarchitecture. For instance, in mice a clear augmentation of cortical serotonin levels was observed when fluoxetine was administered in conjunction with a NK_1 antagonist (Guiard *et al.*, 2004), while our data in guinea pigs showed no such interaction in the median prefrontal cortex. In contrast, microdialysis in ventral hippocampus showed a significant augmentation of extracellular serotonin levels when fluoxetine and a NK_1 antagonist were combined. This result can be attributed to a different neuro-architecture of cortex and hippocampus in guinea pigs, where the median prefrontal cortex is exclusively innervated by the DRN. However, the ventral hippocampus receives innervations from both the dorsal and the median raphe nucleus (McQuade & Sharp, 1997). Thus, the observed differences may relate to a varying role of NK_1 receptors in the controlling of dorsal versus median raphe nucleus firing, in the different species. However, this is no more than speculative as in contrast to 5-HT_{1A} receptors relevant literature comparing the expression and function of NK_1 receptors between species does not exist (Price *et al.*, 1996).

NK_1 receptors are abundantly present in striatum and locus coereleus, and a local interaction with dopaminergic and noradrenergic systems can therefore be expected (Mantyh *et al.*, 1984). In the prefrontal cortex, an area with relatively low levels of substance P, fluoxetine alone, or in combination with GR 205171 did not exert any effect on dopamine levels. Norepinephrine, on the other hand, was increased following fluoxetine administration, but GR 205171 appeared to counteract this effect. This might be attributed to an excitatory effect of substance P on locus coereleus neurons that is counteracted by the combination of fluoxetine and GR 205171 (Blier *et al.*, 2004; Maubach *et al.*, 2002). However, in the ventral hippocampus, extracellular norepinephrine and dopamine was not influenced by fluoxetine or the combination of SSRI and NK_1 antagonist, suggesting regional differences. This absence of response is probably related to species variety in neuroanatomy and pharmacology of NK_1 receptors, as in rat NK_1 antagonist showed an effect on dopamine, which could not be shown in gerbils (Renoldi & Invernizzi, 2006).

In conclusion, the present study shows that an NK_1 receptor antagonist augments the fluoxetine induced increase of 5HT levels in ventral hippocampus of the guinea pig. A significant augmentation was not observed in medial frontal cortex, which is in contrast with a previous study in mice. The marked species difference in NK_1 receptor pharmacology indicates that an extensive study on the functionality, distribution and pharmacology of NK_1 receptors between species is needed before any further conclusions can be drawn as to how these results translate to the human situation.

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