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Title Page

SUBSTANCE P NEUROKININ 1 RECEPTOR ACTIVATION WITHIN THE DORSAL RAPHE NUCLEUS CONTROLS SEROTONIN RELEASE IN THE MOUSE FRONTAL CORTEX

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Running title page

Running title: Control of Cortical 5-HT Release by Substance P

Statistics of the manuscript:

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Figures: 4
References: 40/40
Abstract: 232/250
Introduction: 542/750
Results and Discussion: 2100

Nonstandard abbreviations: aCSF: artificial cerebrospinal fluid; AUC: area under the curve; DRN: dorsal raphe nucleus; FC: frontal cortex; NK: neurokinin; MNR: median raphe nucleus; SSRI: selective serotonin reuptake inhibitor; SP: substance P; 5-HT: serotonin; vH: ventral Hippocampus.

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Abstract

Preclinical studies suggest that Substance P (SP) neurokinin 1 (NK1) receptor antagonists are efficient in the treatment of anxiety and depression. This therapeutic activity could be mediated via stimulation of serotonin (5-HT) neurons located in the dorsal raphe nucleus (DRN), which receive important SP-NK1 receptor immunoreactive innervations. The present study examined the effects of intra-raphe injection of SP on extracellular 5-HT levels in the frontal cortex, ventral hippocampus and DRN by using intracerebral microdialysis in conscious mice. Intra-raphe SP injection dose-dependently decreased cortical 5-HT release while no effects were detected in the ventral hippocampus. Cortical effects were blocked by the selective NK1 receptor antagonist GR205171, and completely dampened in mice lacking NK1 receptors. Furthermore, genetic (in knockout 5-HT_{1A} -/- mice) or pharmacological inactivation of 5-HT_{1A} autoreceptors blocked cortical responses to SP. Contrasting with its cortical effects, intra-raphe SP injection increased 5-HT outflow in the DRN in wild-type mice, this effect being potentiated by a local perfusion of the selective 5-HT_{1A} antagonist, WAY100635. Finally, SP-induced changes in frontal cortex and DRN dialysate 5-HT levels were blocked by the DRN perfusion of the AMPA/kainate ionotropic receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX). These data support the hypothesis that SP-induced over-activation of 5-HT_{1A} autoreceptors within the DRN limits cortical 5-HT release. A better knowledge of the complex relationship between tachykininergic, serotonergic and glutamatergic systems within the DRN might help better understand the pathophysiology and subsequent treatment of depression.

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Introduction

Substance P (SP), a small peptide that belongs to the tachykinins family with neurokinin A (NKA) and neurokinin B (NKB) is widely distributed in the brain, specifically in limbic regions and brainstem nuclei such as the dorsal raphe nucleus (DRN) (Froger et al., 2001; Commons et al., 2002; Lacoste et al., 2006). In several species including rodents and humans, SP distribution overlaps with that of its high-affinity NK1 receptor (Ribeiro-da-Silva and Hokfelt, 2000). Recent clinical and preclinical studies have pointed out the potential therapeutic action of SP (neurokinin 1) receptor antagonists in major depressive disorders (Kramer et al., 1998; Chahl, 2006). Data obtained from NK1 receptor knockout mice have suggested that the antidepressant-like action of NK1 receptor inactivation may result, at least in part, from an increase in central 5-HT neurotransmission through functional desensitization of 5-HT_{1A} autoreceptors located in the DRN (Froger et al., 2001). Pharmacological arguments from wild-type mice chronically treated with a NK1 receptor antagonist also favour this hypothesis (Guiard et al., 2005). Interestingly, such a desensitization of somatodendritic 5-HT_{1A} autoreceptors resembles that induced by chronic treatment with selective serotonin reuptake inhibitors (SSRIs) (Blier and De Montigny, 1980; Hjorth et al., 2000). Thus, the enhancement of serotonergic neurotransmission would be a common element in the antidepressant-like activity of both SSRIs and NK1 receptor antagonists.

Given evidence that NK1 receptor antagonists stimulate the DRN-5-HT system, it may be postulated that endogenous SP limits 5-HT release at serotonergic nerve terminals. However, initial *in vitro* electrophysiological

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recordings suggested that SP excites DRN 5-HT neurons via glutamatergic afferents (Liu et al., 2002). These findings were consistent with intracerebral *in vivo* microdialysis experiments indicating that SP injection into the DRN in conscious rats produces a small, transient increase in hippocampal 5-HT release (by > 30% for 20 min compared to vehicle controls) (Gradin et al., 1992). The latter findings have been challenged recently by *in vivo* electrophysiological data, suggesting that the effects of SP depended on the location of the recording within the DRN, with excitation predominating in the dorsal part of the DRN and inhibition more ventrally (Valentino et al., 2003). Thus, whether 5-HT neurotransmission would be increased or decreased in projection brain regions of the DRN, would depend on the specific DRN sub-region that projects to the serotonergic nerve terminal area studied. Based on their findings, Valentino et al., (2003) have drawn an *in vivo* model of SP regulation of 5-HT neuronal activity. They propose that excitation of DRN 5-HT neurons synaptically linked with glutamate neurons expressing NK1 receptors, allows 5-HT release and subsequent activation of somatodendritic 5-HT_{1A} autoreceptors. However, this theory is limited by the fact that 5-HT releasing properties of SP have yet to be demonstrated. We therefore employed both genetic and pharmacological approaches to clarify the interactions between SP and 5-HT_{1A} autoreceptors. To better define the effects of proximal and distal intra-raphe SP injection on extracellular levels of 5-HT ([5-HT]ext), we performed intracerebral *in vivo* microdialysis studies in awake, freely moving mice with probes implanted either in a brain region containing numerous 5-HT nerve terminals (frontal cortex, ventral hippocampus) or in the vicinity of 5-HT cell bodies in the DRN.

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Material and Methods

Animals. Male C57BL/6 wild-type and NK1 receptor knock-out mice were derived from a stock of genotyped animals received from the animal facility of University College London, United-Kingdom. As well, male wild-type and 5-HT_{1A} receptor knock-out mice also raised on a C57BL/6 genetic background were bred in our animal care facility (Univ. Paris XI, France). All animals were matched for age (8-10 weeks old), weight (25-35g) and kept under standard housing conditions. Experiments were conducted in compliance with the guidelines for care of experimental animals (permissions #92-196 to AMG).

Microdialysis procedure. Concentric dialysis probes were stereotaxically implanted under anaesthesia (chloral hydrate: 400 mg/kg, intraperitoneally i.p.) into the frontal cortex (FC), ventral hippocampus (vH) (active length: 1.5 mm) or dorsal raphe nucleus (DRN) (active length: 1.0 mm). Coordinates from Bregma (AP; L; V in mm) were in the FC: 1.6; 1.3; 1.6; vH: -2.8; 3.0; 4 and DRN: -4.5; 0; 3.5. Animals were allowed to recover from surgery overnight, and, were continuously perfused with artificial cerebrospinal fluid (aCSF, in mmol/L: NaCl 147, KCl 3.5, $CaCl_2$ 1.26, $MgCl_2$ 1.2, NaH_2PO_4 1.0; pH 7.4±0.2) the next day. Dialysate samples were collected every 15 min for the FC and vH (flow rate: 1.5 µl/min) and every 30 min for the DRN (flow rate: 0.5 µl/min). Extracellular 5-HT levels were measured using a High Performance Liquid Chromatography system [limit of sensitivity ≈ 1 fmol per sample (signal-to-noise ratio =2)]. In each experiment, after one hour of stabilization, four samples were collected to measure basal 5-HT values (means±SEM). The administration of pharmacological agents occurred at t=0 and subsequent

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fractions were collected. SP (5-200 ng) was directly infused into the DRN [(0.1 µL/min for 2 min via a microinjector (Harvard Appartus, France)], by means of a silica catheter glued to the microdialysis probe. Both WAY100635 (100 µM: Guilloux et al., 2006) or DNQX (10 µM: Tao et al., 1997) were injected via the probe after their dissolving in the aCSF. The exact probe locations in brains were determined according to Bert et al. (2004).

Drugs. SP was obtained from Neosystem (Strasbourg, France). GR205171 [(N-((2-Methoxy-5-(5-(trifluoromethyl)-1H-tetrazol-1-yl)phenyl)methyl)-2 phenyl -3-piperidinamine) was a gift from GlaxoSmithKline (Harlow, United Kingdom). WAY100635 [(N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl)-N-(2- pyridinyl) cyclohexanecarboxamide trihydrochloride)] and DNQX [6,7-dinitroquinoxaline-2,3-dione] were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

Data analysis and statistics. Baseline levels of 5-HT were calculated by averaging the levels measured in the first four samples collected before treatment. After administration of the treatment, the 5-HT content of individual dialysate samples was measured and expressed as a percentage of the baseline mean. . The summed effects of each treatment over the course were measured by determining the area under the curve (AUC; mean±SEM) values for the amount of 5-HT outflow during the 0-120 min period post-treatment. Comparisons of the effects of the different doses of SP on extracellular 5-HT levels were performed on AUC values by using a one-way ANOVA followed by a Fisher's PLSD *post hoc* test. The overall effects of "drug pre-treatment

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and treatment" as main factors was assessed by using a two-way ANOVA followed by Fisher's PLSD post hoc test when appropriate. Finally, A Student's t-test was used to compare two experimental groups, in particular the effects of SP *versus* vehicle in NK1 -/- and 5-HT_{1A} -/- mutant mice.

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Results and Discussion

Intra-raphe injection of substance P reduced cortical extracellular 5-HT levels.

In NK1 wild-type (+/+) control mice, the intra-raphe (i.r.) injection of SP (50 and 200 ng) dose-dependently reduced extracellular levels of 5-HT in the frontal cortex (Fig. 1A,B). These findings concur with *in vivo* electrophysiological data which found that the neuronal activity of nearly 80% of DRN 5-HT neurons in rats was inhibited by the local microinjection of SP (Valentino et al., 2003). Interestingly, we found that the i.r. injection of the highest dose of SP (200 ng) failed to alter extracellular levels of 5-HT in the ventral hippocampus when compared with the corresponding group of vehicle-treated wild-type mice (AUC values: -0.7 ± 6.2% *vs* 3.1 ± 5.1%, respectively; $P>0.05$). It appears therefore that SP regulates the DRN activity in a region-dependant manner as previously proposed. A corollary of these intriguing findings is that the efferents of the DRN may differentially drive the activity of 5-HT neurons projecting to different forebrain structures, depending on whether these cells are activated or inhibited by SP (Valentino et al., 2005). From our neurochemical data, it can be postulated that the subpopulation of DRN 5-HT neurons projecting to the FC is inhibited by SP. In contrast, the lack of effect of i.r. injection of SP on extracellular 5-HT levels in the ventral hippocampus suggests that the latter region is not innervated by the DRN 5-HT neurons. This is in agreement with previous studies, which provided both anatomical and pharmacological evidence (Molliver, 1987; Kreiss and Lucki, 1991). Another possible explanation would be that the ventral hippocampus receives both types of innervations (i.e., neurons excited and inhibited by SP),

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leading to a blunted response. Although this would be important to address in future investigations, it is important to emphasize that the precise injection of drugs in mice is technically difficult and we have to consider the possibility that SP diffused outside the DRN injection site. The median raphe nucleus (MRN), an adjacent area expressing NK1 receptors (Saffroy et al., 2003), is a region of interest as it sends serotonergic projections to the hippocampus. Nevertheless, in contrast to the present results, initial observations indicate that the injection of SP into the MRN produces an excitatory effect on 5-HT turnover and likely neurotransmission in the hippocampus (Forchetti et al., 1982) strongly suggesting that in our experimental conditions the diffusion of SP was restricted to the DRN.

Involvement of NK1 receptors in the cortical effect of intra-raphe injection of substance P.

SP preferentially binds to and activates neurokinin 1 (NK1) receptors. However, it is well established that this neuropeptide may act as an agonist on NK2 and NK3 receptor sub-types, albeit with lower affinities (Maggi et al., 1993). Pharmacological and genetic experiments were thus conducted to assess whether the above inhibitory effects of SP on cortical 5-HT release were specifically mediated by NK1 receptors. In NK1 +/+ mice, the i.r. injection of SP (200 ng) did not modify cortical extracellular 5-HT levels when the potent and selective NK1 receptor antagonist GR205171 (30 mg/kg; i.p.) was given 30 min before (Fig. 1C,D). The dose of GR205171 was chosen on the basis of its capacity to block rat brain NK1 receptors *in vivo* (Rupniak et al., 2003). Similar results have been obtained using a genetic approach.

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Indeed, in contrast to NK1 +/+ mice, we showed that NK1 -/- mutant mice were insensitive to the i.r. injection of SP (200 ng) on cortical 5-HT release (Fig. 1E,F). It is noteworthy that neither the pharmacological nor the genetic inactivation of NK1 receptors altered basal extracellular levels of 5-HT in the FC of the mice (Table 1; Fig. 1D,F). These results concur with recent microdialysis experiments performed in awake mice (Zocchi et al., 2003; Guiard et al., 2004) suggesting a lack of tonic regulation of 5-HT transmission by SP. Interestingly, NK1 receptor antagonists have been reported to be efficient anxiolytic and antidepressant agents in several animal models (Chahl, 2006). As SP and 5-HT co-exist in a substantial part of the neuronal DRN population in human and rodent brains (Chan-Palay et al., 1978; Sergeyev et al.,1999), it is possible that 5-HT neurons may regulate the release of SP in stressful conditions (Ebner et al., 2004). Thus, the present results demonstrate that an increase in endogenous SP levels in the DRN (mimicked here by its local injection) specifically activates NK1 receptors, subsequently inhibiting cortical 5-HT neurotransmission. Additional work is now required to address how this particular effect of i.r. injection of SP affects depressive-like symptoms such as anhedonia or despair in various animal paradigms. Such studies could further support the hypothesis that the putative antidepressant activity of NK1 receptors antagonists is related, at least in part, to the blockade of SP neurotransmission within the DRN.

Indirect involvement of somatodendritic 5-HT$_{1A}$ autoreceptors in the cortical effects of intra-raphe injection of substance P.

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Given that SP was described as an excitatory neuropeptide, we raised the possibility that its neurochemical effects on cortical 5-HT release might indirectly recruit an inhibitory component in the DRN. Since somatodendritic 5-HT_{1A} autoreceptors play that role in the DRN (Hjorth et al., 2000), the i.r. injection of SP was evaluated in 5-HT_{1A} -/- mice. In these mutant mice, SP (200 ng) failed to modify extracellular levels of 5-HT in the FC (Fig. 2A,B). However, by using a genetic approach (e.g., constitutive disruption of 5-HT_{1A} receptors by homologous recombination of the gene), we cannot definitively state whether presynaptic (in the DRN) rather than postsynaptic (in the FC) 5-HT_{1A} receptors were involved in the inhibitory effects of SP on cortical 5-HT release. Indeed, evidence does exist that 5-HT_{1A} autoreceptors located in the prefrontal cortex are involved in a distal control of DRN 5-HT neuronal activity (Celada et al., 2001). In order to determine whether there is a preferential activation of presynaptic 5-HT_{1A} receptors in the SP response, the selective 5-HT_{1A} receptor antagonist WAY100635 was perfused for two hours into the DRN by reverse microdialysis in 5-HT_{1A} +/+ wild-type mice as previously described (Guilloux et al., 2006). In the FC of 5-HT_{1A} +/+ mice, pre-treatment with the selective 5-HT_{1A} receptor antagonist WAY100635 (100 µM, intra-raphe), but not with the vehicle, significantly blocked the effects of SP injection (200 ng) on extracellular levels of 5-HT (Fig. 2C,D). These results are consistent with the findings that both systemic and i.r injection of WAY100635 attenuated the predominantly inhibitory effects of SP on the DRN 5-HT neuronal activity (Valentino et al., 2003). In marked contrast to the effects observed in the FC, we showed here for the first time that the i.r. injection of SP (200 ng) increased extracellular levels of 5-HT in the DRN

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compared with the vehicle-treated group in 5-HT_{1A} +/+ mice, the latter effect being potentiated by WAY100635 (Fig. 2E,F). The opposite effects of SP on extracellular 5-HT outflow in the DRN and FC appear to be somewhat equivocal. Nevertheless, they allowed anticipating that SP-induced decreases in cortical 5-HT release resulted from an over-activation of inhibitory 5-HT_{1A} autoreceptors in the DRN. Previous studies in rats reported that relatively high extracellular 5-HT concentrations were required in the DRN to reduce forebrain 5-HT release through the activation of 5-HT_{1A} autoreceptors (Romero and Artigas, 1997; Tao and Auerbach, 2000). No direct evidence supporting these observations has been provided in mice. On the contrary, in a recent study, we reported that a two fold decrease in extracellular 5-HT outflow in the DRN was sufficient to trigger an equivalent increase in the FC in mice (Guiard et al., 2004). It therefore seems possible that activation of DRN 5-HT_{1A} autoreceptors in response to SP might contribute to the inhibition of cortical 5-HT release. Interestingly, despite evidence indicating a tonic inhibitory effect of DRN 5-HT_{1A} autoreceptors on 5-HT neuronal activity (Haddjeri et al., 2004), we observed that neither the pharmacological nor the genetic inactivation of 5-HT_{1A} autoreceptors altered the basal extracellular 5-HT levels in the frontal cortex and the DRN (Table 1, Fig. 2B,D). These findings are in agreement with initial microdialysis studies performed in mice at somatodendritic (Bortolozzi et al., 2004; Guilloux et al., 2006) and terminal levels (He et al., 2001; Knobelman et al., 2001a; Guilloux et al., 2006).

Role of excitatory amino acid receptors in the substance P-induced effect.

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The above findings, (i.e. SP-induced inhibition of cortical 5-HT release through an over-activation of DRN 5-HT_{1A} autoreceptors), may occur through a local elevation of endogenous 5-HT. According to Liu and Aghajanian (2002), such an elevation of 5-HT outflow occurring in the DRN could be attributable to a previous increase in glutamate transmission in the DRN. Consistent with this assumption are the observations that the DRN is endowed with a rich population of NK1 receptors especially dense on glutamate interneurons (Commons and Valentino, 2002, Liu et al., 2002, Commons et al., 2003). A last series of experiments was conducted to determine the putative involvement of glutamate in the neurochemical response to i.r. injection of SP. In our experimental conditions, we showed that the glutamate AMPA/Kainate receptor blocker DNQX alone produced a slight but significant decrease in cortical 5-HT release in NK1 +/+ mice. Despite its own effect, DNQX (10 µM, intra-raphe) significantly attenuated the SP-induced decrease in cortical 5-HT levels (Fig. 3A,B). This strongly suggests that the disinhibitory effect of the AMPA/Kainate receptor antagonist could be ascribed to a specific involvement of glutamate in the SP response. In line with this assumption, the increase in 5-HT outflow induced by i.r. injection of SP (200 ng) was also blocked by DNQX (10 µM, i.r.) in the DRN in NK1 +/+ mice (Fig. 3C,D), while DNQX alone had no effect on DRN extracellular 5-HT levels as demonstrated previously (Tao et al., 1997; Tao and Auerbach., 2003). Based on these results, it can be postulated that increased glutamate release in the DRN would occur after i.r. injection of SP. However, it is important to emphasize that the i.r. injection of glutamate receptor agonists enhances the release of 5-HT at both somatodendritic and

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nerve terminals levels (Tao and Auerbach, 2000), while in the present study the i.r. injection of SP produced opposite effects in the DRN and FC. Since SP-induced stimulation of glutamate transmission in the DRN is a short lasting effect (return to baseline ~10 minutes after injection: Liu et al., 2002), it is possible that we failed to detect any increases in 5-HT cortical release using intracerebral microdialysis. It is also possible that the elevated pool of extracellular 5-HT levels in the DRN of wild-type mice injected with SP did not solely originate from DRN 5-HT neuronal cell bodies or dendrites. Since the DRN receives serotonergic innervations from the other raphe nuclei (Tischler and Morin, 2003), the release of 5-HT may also result from the stimulation of AMPA/Kainate receptors on serotonergic afferents in the DRN (Figure 4). Alternative mechanisms, such as direct activation of NK1 receptors located on the 5-HT cell bodies, could also account for the inhibitory effect of SP on the serotonergic system. This hypothesis is supported by recent evidence showing that almost 30% of rats and mice DRN 5-HT neurons express NK1 receptors (Lacoste et al., 2006). Finally, NK1 receptors have been clearly identified on GABAergic neurons surrounding 5-HT cell bodies in the DRN and their activation might contribute, at least in part, towards SP-induced attenuation of cortical 5-HT neurotransmission.

In conclusion, our neurochemical data support the idea that increased brain SP levels, specifically in the DRN, may represent an important step in the pathophysiology of depression. In these conditions, the potential antidepressant effects of NK1 receptor antagonists (Chahl, 2006) could be related to their capacity to block or prevent the reported effect of SP on

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cortical 5-HT transmission. However, it is important to mention that despite the great enthusiasm raised by the first placebo-controlled trials, no antidepressant or modest efficacy of NK1 receptor antagonists were reported in subsequent clinical studies, leading several pharmaceutical companies to discontinue their research program in this field (Czeh et al., 2006; Holtzheimer and Nemeroff, 2006). Additional studies are needed to further characterize the real impact of tachykinins on the 5-HT system, as well as the pathophysiology and treatments of depression. In particular, based on the present data, it can be proposed that abnormal SP neurotransmission in the DRN is involved involved in the inadequate response to the selective serotonin reuptake inhibitors (e.g. long delay of action and/or resistance to the treatment) in some patients. Consequently, rather than being used as monotherapy, NK1 receptor antagonists could conceivably be prescribed as augmentation agents in combination with a traditional antidepressant (Ryckmans et al., 2002; Guiard et al., 2004). This should arouse our attention for future clinical investigations.

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Legends for Figures

Figure 1: **Effect of intra-raphe injection of substance P on 5-HT release in the frontal cortex mice.** (A, C, E) Data are means ± SEM of extracellular 5-HT levels expressed as percentages of basal values (arrows show the time of drug injection). (B, D, F) Data are area under the curve (AUC; means ± SEM) values calculated for the amount of 5-HT outflow. (B) One-way ANOVA on AUC values revealed a significant effect of treatment factor in the frontal cortex (FC) [$F_{(3,33)}$=6.1; p<0.01]. A pharmacological (D) and a genetic (F) inactivation of NK1 receptors were used to verify the selectivity of the SP response. (D) Two-way ANOVA on AUC values indicated a significant effect of pre-treatment (vehicle or GR205171: 30 mg/kg; i.p.) [$F_{(1, 31)}$ = 4.7 ; p<0.05], and treatment (vehicle or SP) [$F_{(1, 31)}$ = 14.1 ; p<0.001] factors, but no significant interaction between these two factors [$F_{(1,31)}$ = 2.9 ; p>0.05] in the FC of NK1+/+ mice. (F) A Student's t-test revealed no significant difference between the effect of vehicle and SP (200 ng) on extracellular 5-HT levels in the FC of NK1 -/- mice. *ns*: not statistically significant. **P<0.01 and ***P<0.001 significantly different from vehicle-treated group. ##P<0.01 001 significantly different from vehicle/SP (200 ng)-treated group. The number of determinations (n) and means ± SEM of baseline 5-HT levels expressed as fmol/sample for each experimental group in the FC were: vehicle (n=7; 9.7 ± 0.7), SP 5 (n=11; 7.9 ± 0.9), SP 50 (n=10; 10.6 ± 0.8) and SP 200 (n=11; 9.4 ± 0.6) (Fig 1A); vehicle/vehicle (n=7; 9.7 ± 0.7), GR205171/vehicle (n=11; 10.1 ± 2.1), vehicle/SP 200 (n=11; 8.6 ± 1.2) and GR205171/SP 200 (n=10; 8.9 ± 0.5) (Fig 1C); vehicle (n=7; 10.4 ± 0.7), SP 200 (n=7; 10.8 ± 1.2) (Fig

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1E). No significant differences were detected in baseline levels between experimental groups for individual experiments.

Figure 2: Effects of genetic and pharmacological inactivation of 5-HT$_{1A}$ receptors on substance P-induced changes in 5-HT outflow in the frontal cortex and dorsal raphe nucleus. (A, C, E) Data are means ± SEM of extracellular 5-HT levels expressed as percentages of basal values (arrows show the time of vehicle or SP injection while the grey line indicates the duration of intra-raphe perfusion of vehicle or WAY100635 through reverse dialysis). (B, D, F) Data are AUC (means ± SEM) values calculated for the amount of 5-HT outflow. (B) A Student's t-test revealed no significant difference between the effect of vehicle and SP (200 ng) on extracellular 5-HT levels in the FC of 5-HT$_{1A}$-/- mice. (D) A two-way ANOVA on AUC 5-HT ext values revealed a significant main effect of pre-treatment (vehicle or WAY100635: 100 µM) [$F_{(1,25)}$=3.8; p<0.05] and treatment (vehicle or SP) [$F_{(1,25)}$=15.6; p<0.001] factors, but no interaction between these two independent variables [$F_{(1,25)}$=1.2; p=0.27] in the FC of 5-HT$_{1A}$ +/+ mice. (F) A two-way ANOVA on AUC 5-HText values revealed a significant main effect of pre-treatment (vehicle or WAY100635) [$F_{(1,24)}$=5.5; p<0.05] and treatment (vehicle or SP) [$F_{(1,24)}$=35.5; p<0.001] factors, but no interaction between these two factors [$F_{(1,24)}$=2.7; p=0.1] in the DRN of 5-HT$_{1A}$ +/+ mice. Differences between groups of mice were determined by Fisher post hoc test. *ns*: not statistically significant. **P<0.01 compared to vehicle-treated mice, #P<0.05 compared to vehicle/SP treated wild-type mice. The number of determinations (n) and means ± SEM of baseline 5-HT levels expressed as fmol/sample for each experimental group in the FC were: vehicle (n=9; 9.8 ±

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0.7), SP 200 (n=9; 10.4 ± 0.7) (Fig 2A); vehicle/vehicle (n=7; 10.1 ± 0.9), WAY100635/vehicle (n=10; 10.2 ± 0.8), vehicle/SP 200 (n=11; 8.9 ± 1.4) and WAY100635/SP 200 (n=10; 10.9 ± 1.1) (Fig 2C); and in the DRN: vehicle/vehicle (n=5; 13.9 ± 2.1), WAY100635/vehicle (n=5; 16.1 ± 2.5), vehicle/SP 200 (n=8; 14.3 ± 1.7) and WAY100635/SP 200 (n=7; 12.5 ± 1.8) (Fig 2E). No significant differences were detected in baseline levels between experimental groups for individual experiments.

Figure 3: Effects of pharmacological inactivation of AMPA/Kainate receptors on substance P-induced changes outflow in the frontal cortex and dorsal raphe nucleus in wild-type mice. (A,C) Data are means ± SEM of extracellular 5-HT levels expressed as percentages of basal values (arrows show the time of drug injection while the grey line indicates the duration of intra-raphe perfusion of vehicle DNQX through reverse dialysis). (B,D) Data are AUC (means ± SEM) values calculated for the amount of 5-HT outflow. (B) An overall two-way ANOVA on AUC 5-HText values revealed no significant effect of pre-treatment factor (vehicle or DNQX: 10 μM) [$F_{(1,26)}$=0.0002; p=0.9], but a significant effect of treatment factor (vehicle or SP) [$F_{(1,26)}$=9.7; p<0.01] and a significant interaction between these two factors [$F_{(1,25)}$=12.1; p<0.01] in the FC of wild-type mice. (D) An overall two-way ANOVA on AUC 5-HText values revealed no significant effect of pre-treatment factor (vehicle or DNQX) [$F_{(1,20)}$=0.8; p=0.3], but a significant effect of treatment factor (vehicle or SP) [$F_{(1,20)}$=9.5; p<0.01] and a significant interaction between these two factors [$F_{(1,20)}$=4.4; p<0.05] in the DRN of wild-type mice. Differences between groups of mice were determined by Fisher post hoc test. *P<0.05, **P<0.01 and ***P<0.001 compared to vehicle-treated

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mice, #P<0.05 and ##P<0.01 compared to vehicle/SP treated mice. The number of determinations (n) and means ± SEM of baseline 5-HT levels espressed as fmol/sample for each experimental group in the FC were: vehicle/vehicle (n=7; 10.1 ± 0.9), DNQX/vehicle (n=6; 9.9 ± 1.1), vehicle/SP 200 (n=11; 9.4 ± 0.6) and DNQX/SP 200 (n=6; 8.7 ± 1.1) (Fig 3A); and in the DRN were: vehicle/vehicle (n=5; 13.9 ± 2.1), DNQX/vehicle (n=5; 15.1± 2.7), vehicle/SP 200 (n=8; 14.3 ± 1.7) and DNQX/SP 200 (n=6; 12.9 ± 2.1) (Fig 3C). No significant differences were detected in baseline levels between experimental groups for individual experiments.

Figure 4: Model of substance P regulation of 5-HT neurotransmission. In the DRN, SP activates NK1 receptors located on glutamatergic neurons (1) and produces glutamate release (black circles). (2) Since the DRN receives serotonergic innervations, the enhancement of glutamate release could stimulate 5-HT release (grey circles) through presynaptic glutamate AMPA/Kainate receptors on serotonergic afferents in the DRN [2a]. As well, 5-HT release could result from the activation of AMPA/Kainate receptor located on 5-HT cell bodies [2b]. (3) The excess of extracellular 5-HT levels in the DRN triggers a delayed inhibition of cortical 5-HT release via 5-HT$_{1A}$ autoreceptor over-activation.

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Table 1: Basal 5-HT levels across all wild-type and mutant mice.

	Basal values of 5-HT extracellular concentrations (in fmol/sample)	
	Frontal Cortex	**Dorsal Raphe Nucleus**
NK1 +/+ mice	9.3 ± 0.9 *(n=78)*	ND
NK1 -/- mice	10.6 ± 0.9 *(n=14)*	ND
5-HT1A +/+ mice	9.7 ± 0.8 *(n=68)*	13.7 ± 2.1 *(n=27)*
5-HT1A -/- mice	10.1 ± 0.7 *(n=18)*	ND

Data are means ± SEM of extracellular 5-HT levels expressed as fmol/20 µl in the FC and as fmol/10 µl in the DRN. The numbers of determinations are in parentheses. ND: not determined.

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FIGURE 1

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FRONTAL CORTEX
NK1 +/+ mice

A

SP or
vehicle

vehicle
SP 5
SP 50
SP 200

Extracellular 5-HT levels
(% of basal values)
150
100
50
0
0 15 30 45 60 75 90 105 120
Time (min)

B

vehicle
SP
5 50 200 ng
AUC for cortical 5-HT
(% of basal values)
20
0
-20
-40
**
***

C

GR205171 or
vehicle

vehicle / vehicle
vehicle / SP 200
GR 205171 / SP 200
GR 205171 / vehicle

Extracellular 5-HT levels
(% of basal values)
150
100
50
0
SP or vehicle
0 15 30 45 60 75 90 105 120
Time (min)

D

vehicle
SP 200 ng
vehicle GR205171 vehicle GR205171
AUC for cortical 5-HT
(% of basal values)
20
0
-20
-40
***
##

FRONTAL CORTEX
NK1 -/- mice

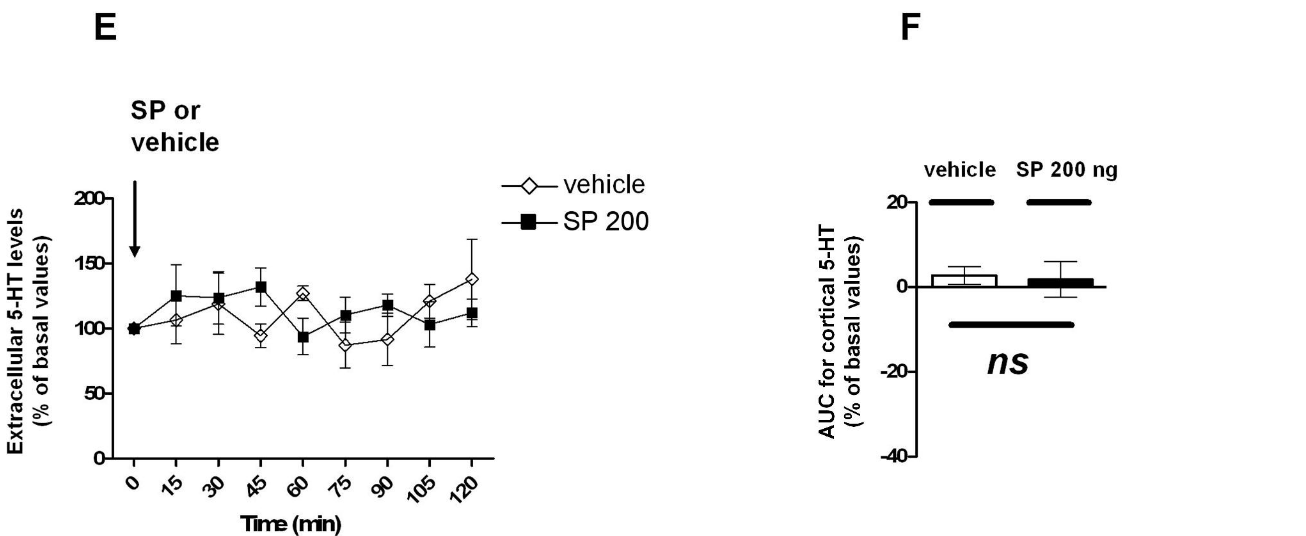

FIGURE 2

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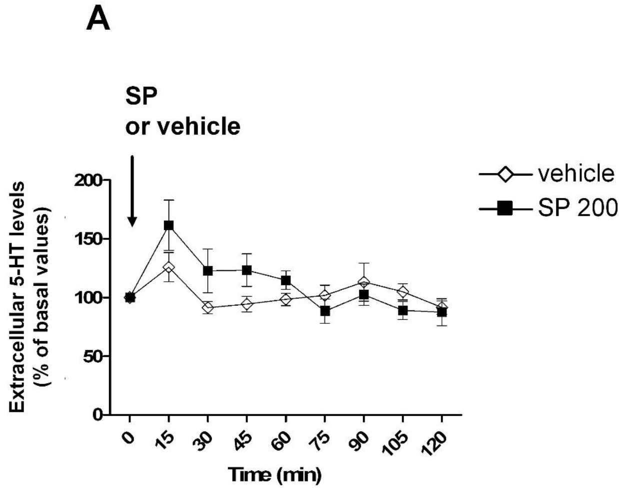
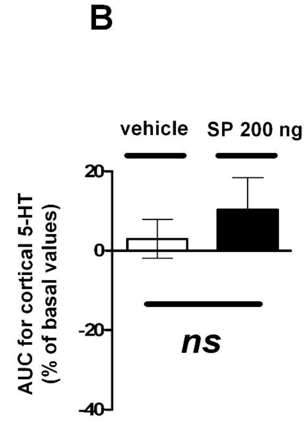
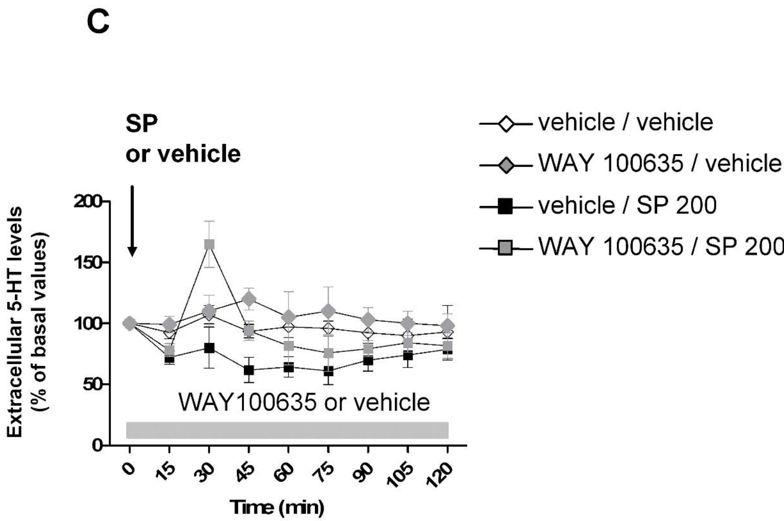
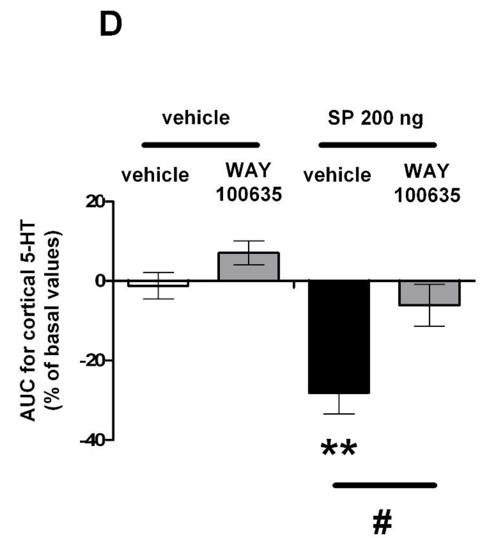
FRONTAL CORTEX
5-HT1A -/- mice



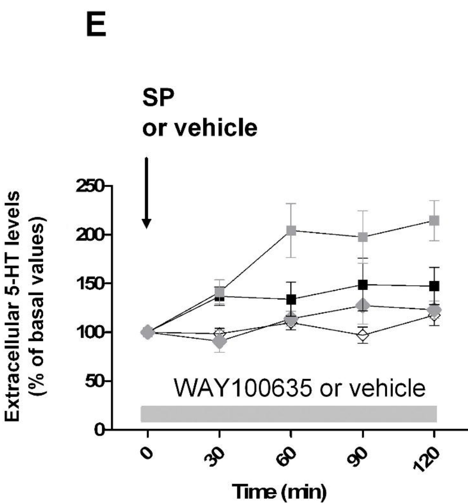


FRONTAL CORTEX
5-HT1A +/+ mice





DORSAL RAPHE NUCLEUS
5-HT1A +/+ mice



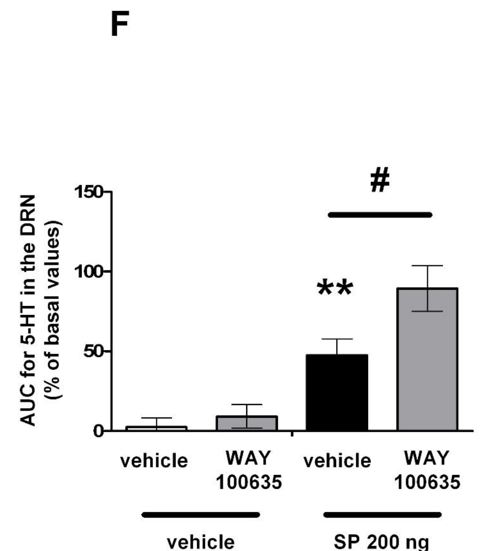


FIGURE 3

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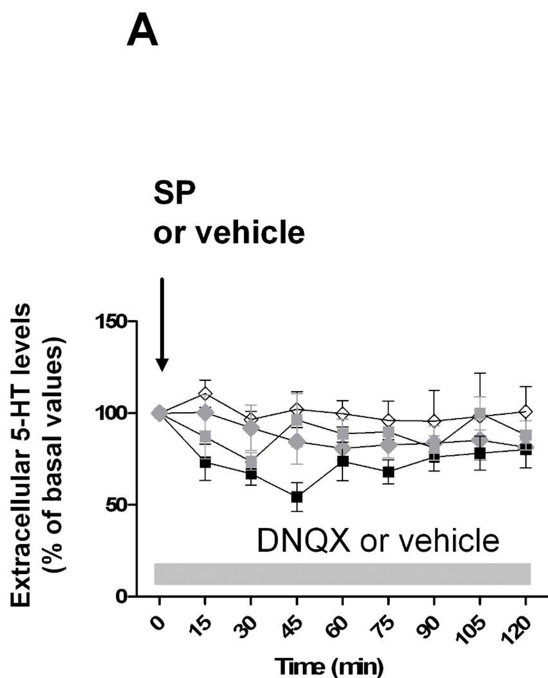
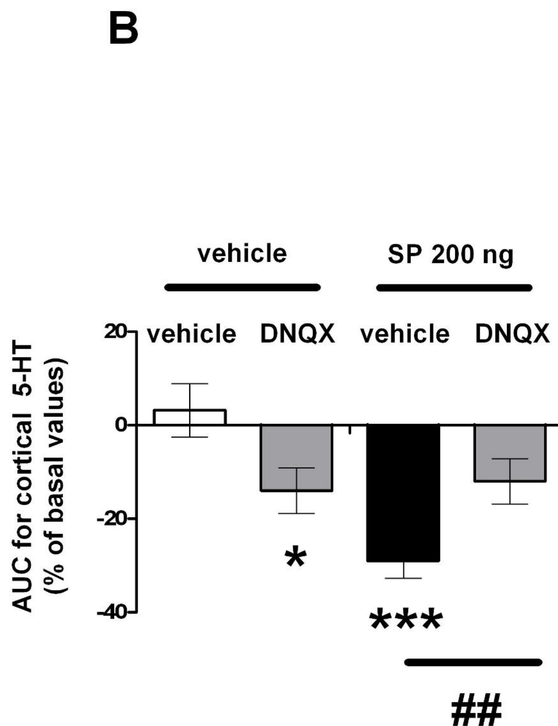
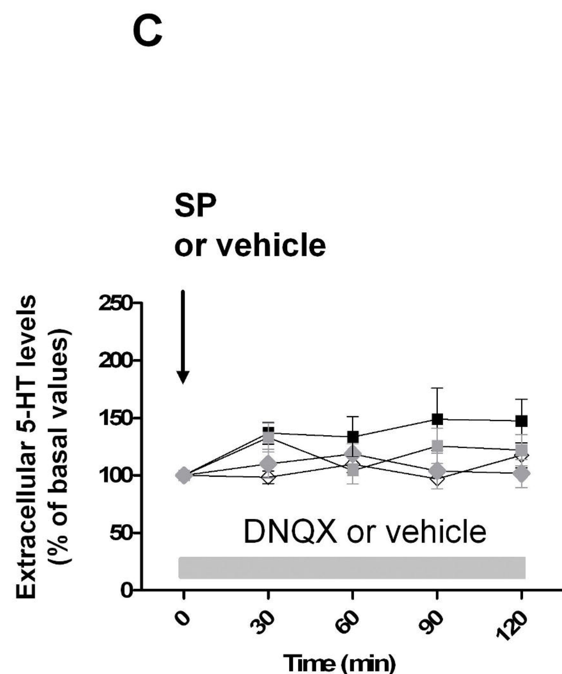
FRONTAL CORTEX





DORSAL RAPHE NUCLEUS



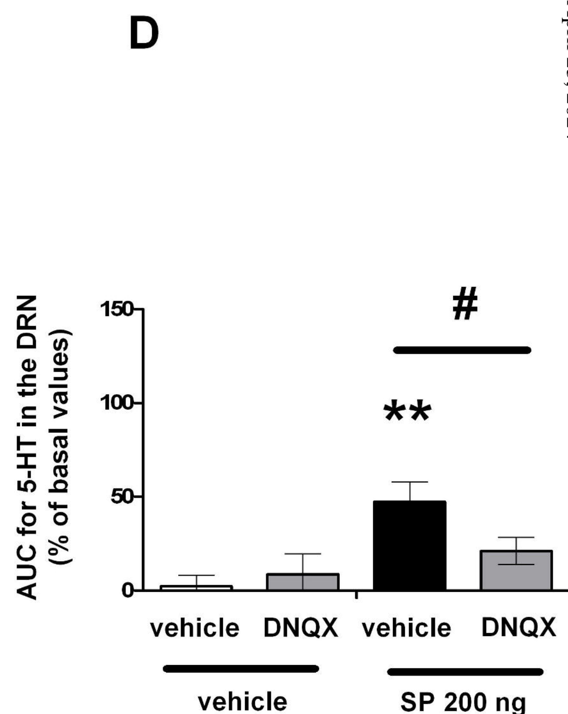


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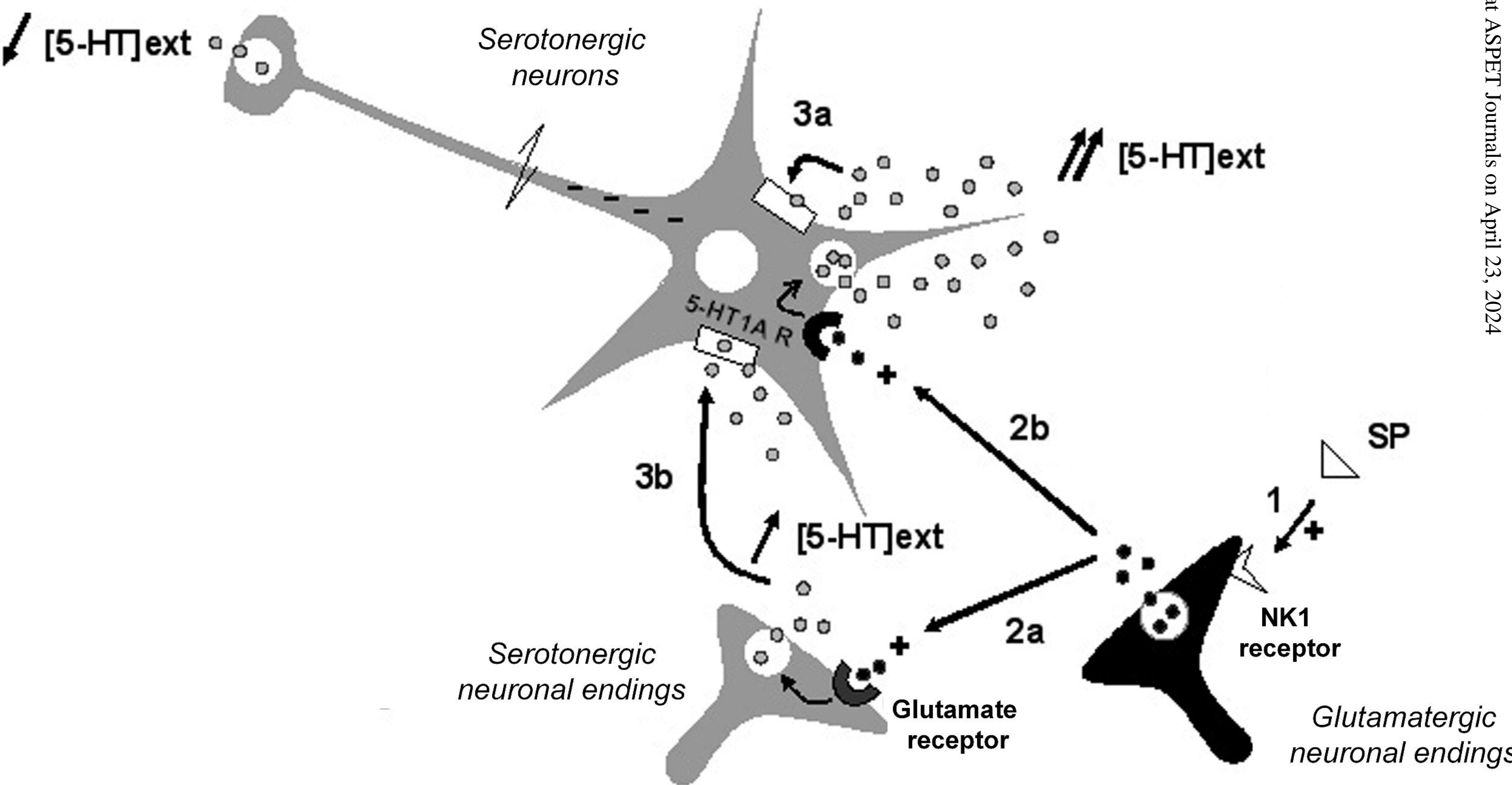
Figure 4



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