SEROTONERGIC SYSTEM AND ITS INTERACTION WITH NEUROINFLAMMATION

by

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Abstract

Serotonin (5-HT) is a neurotransmitter that is mainly expressed in brain where serves a wide array of physiological and behavioural functions. Literature described that some mediators of inflammation (i.e. cytokines) have been implicated in the modulation of monoaminergic response and this may be associated with pathophysiology of depression and in the responsiveness of antidepressant treatment in both humans and animals (Capuron and Miller, 2011). A hypothesis suggests that cytokines may affect the serotonergic system through p38 MAP kinase dependent mechanisms particularly at the serotonin transporters (Zhu et al., 2006) and $5HT_7$ receptors (Lieb et al., 2005; Mahe et al., 2005). The aim of this study was to show the interaction of Interleukin 1β (IL-1β) or p38 MAP kinase on serotonin transporter (SERT) and $5HT_7$ receptors in cells lines and native tissue, highlighting the biochemical mechanism of this system. The IL-1β and p38 MAP kinase activator, anisomycin, did not show any effect on 5-HT uptake and p38 MAPK activation in rodent native brain tissue, in human platelets and in cell lines in contrast to literature reports (Zhu et al., 2010). A different method was then used in which a release of cytokines was induced directly in the rat brain through an i.c.v. LPS treatment. Although pro-inflammatory cytokines involved in the change of animal mood, such as IL-1ß and TNFα, showed a significant increase in cortex and striatum, a modulation of SERT activity in term of K_m and V_{max} was not detected, confirming again that no interaction between cytokines, p38 MAP kinase and SERT function *in vitro* nor *in vivo* was evident. In contrast, this study revealed a positive interaction between $5HT_7$ receptors and p38 MAP kinase in glia cells. However, this pathway was not present in cortical neurons where $5HT_7$ receptors did not activate p38 MAP kinase but instead increased the AMPAR subunit, GluR1 and CREB phosphorylation. The effect on GluR1 was reversed by the specific $5HT_7$ antagonist, SB258719, and the PKA inhibitor, H89, confirming the specificity of response for $5HT_7$ receptors and the involvement of PKA in the mediation of GluR1 phosphorylation. In conclusion, this study displayed a lack of interaction between IL-1β and p38 MAP kinase on rat SERT while highlighting the effect of $5HT_7$ receptors on p38 MAP kinase, with different functions between glial and

neuronal cells. Noteworthy, this is the first report that showed a positive interaction between $5HT_7$ receptors and AMPA which stimulates new investigation into the role of $5HT_7$ receptors in neuronal plasticity.

Dedication

I would dedicate this thesis to all people who have contributed in the realization of this personal achievement.

In particular, thanks to my wife, Emanuela, for her constant support during these years.

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List of Abbreviations

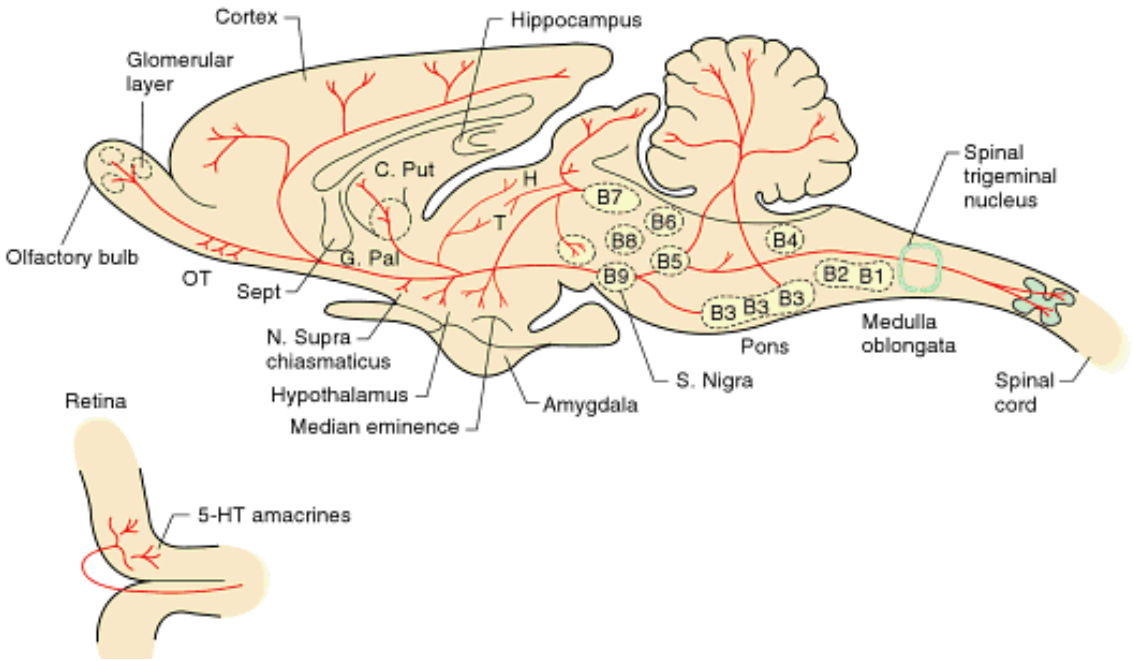
5HT: *serotonin*; DRN: *dorsal raphé nucleus*; MRN: *median raphé nucleus*; ACC: *anterior cingulate cortex*; SERT: *serotonin transporter;* MOA, MOB: *monoamine oxidases A and B;* MDD: *major depressive disorder; LSD: least significant difference;* LPS: *lipopolysaccharide; IL: interleukin;* IFNγ: *interferon gamma;* TNFα: *tumor necrosis factor alpha;* HPA: *hypothalamic-pituitary-adrenal*; 5-HIAA: *5-Hydroxyindoleacetic acid*; DA: *dopamine;* DOPAC: *dihydroxyphenylacetic acid;* NET: *noradrenalin transporter*; DAT: *dopamine transporter; AR: adenosine receptors*; H_3R: *histamine receptors*; BDNF: *brain-derived neurotrophic factor; PKG and PKC: phospho kinase G and C; SSRI: serotonin reuptake inhibitors*; NA: *noradrenalin; SCN: suprachiasmatic nucleus; cAMP: adenosine cyclic 3' 5'-monophosphate*; AC: *adenylate cyclase; LTP: long term potentiation;* sAHP: *slow afterhyperpolarization;* EPSPs: *excitatory postsynaptic potential;* IDO: *indoleamine 2,3-dioxygenease;* TRP: *tryptophan*; KYN: *kynurenine; FBS: foetal bovine serum;* PBS: *phosphate buffered saline; PRP: platelet rich plasma; ACF: artificial cerebrospinal fluid; DIV:* days *in vitro;* FSK: forskolin; AMPAR: *AMPA receptor; PTX: pertussis toxin;* PDE: *phosphodiesterase*

Chapter 1 Introduction

Section 1.1 Serotonergic system overview

Serotonin (5HT) is a neuromodulatory neurotransmitter expressed in brain where it serves a wide array of physiological and behavioral functions. 5HT is also present, at a high concentration, in platelets from where it is released as a result of blood coagulation; this leading to its initial discovery (Zucker *et al.*, 1953). The first histological localization of 5HT was performed by histofluorescence techniques, which revealed the preferential localization of serotonergic neurons in cell body groups along the midline of the brainsteam (Dahlström and Fuxe, 1964). Nine groups of 5HT containing cell bodies were designated, B1-B9 (see diagram below). Development of immunohistochemical techniques made more accurate the detection of serotonergic neurons which were classified in two groups: the rostral group, confined to the mesencephalon and rostral pons, with major projections to the forebrain, and the caudal group, extending from the caudal pons to the caudal portion of the medulla oblongata, with major projections to the caudal brainstem and to the spinal cord. The rostral group includes the dorsal raphé nucleus (DRN) and median raphé nucleus (MRN) and together provides the majority of 5HTergic innervation to forebrain areas including the cerebral cortex, hippocampus, limbic system. 5HTergic innervations were also observed in hypothalamus (Jacobs and Azmitia, 1992). 5HT is stored in vesicles and is released into the synaptic cleft following the stimulus. 5HT transmission is regulated through the 5HT

transporter (SERT, also known as SLC6A4), which transports 5HT from the extracellular to the intracellular space, tryptophane hydroxylase which is the enzyme that regulates 5HT synthesis and monoamine oxidases (MOA, MOB) which regulate the degradation of 5HT and to its metabolites. Once 5HT is released, the synaptic signal is translated into the cell through specific receptors that are classified in seven families, $5HT_{1-7}$ receptors, comprising a total of 14 structurally and pharmacologically distinct mammalian 5HT receptor subtypes. All these receptors regulate the complexity of serotonergic transmission and its involvement in a number of psychiatric conditions and behavioural traits.



Schematic drawing depicting the localization of serotonergic cell body groups in a parasagittal section of the rat central nervous system and their major projections. OT, olfactory tubercolum; Sept, septum; C. Put, nucleus caudate –putamen; G. Pal, globus pallidus; T, Thalamus; H, habenula. Diagram obtained from Basic Neurochemistry: Molecular, Cellular and Medical Aspects. 6th edition. Siegel GJ, Agranoff BW, Albers RW, et al., editors. Philadelphia: Lippincott-Raven; 1999

Section 1.2 Effect of neuroinflammation on the serotonergic system

Cytokines are messenger molecules with low molecular weight mainly involved in the regulation of the immune response. They are mostly produced by immune competent cells such as lymphocytes, macrophages, astrocytes and microglia and can be distinguished in two general groups: pro-inflammatory molecules that activate the immune response stimulating the defense system and anti-inflammatory molecules which counteract the action of pro-inflammatory mediators thus dampening the immune response. The primary role of cytokines is therefore the regulation of the inflammatory response system. However, many clinical and preclinical studies have revealed that mediators of inflammation are also active in the CNS since an abnormal regulation of cytokine secretion may be associated with a negative effect on brain function which is a causative factor of behavioural, neuroendocrine and neurochemical features of major depressive disorder (MDD)(Raison et al, 2006; Schiepers et al.,2005; Capuron and Miller, 2011). Several clinical studies have suggested that depressive disorders are associated with an abnormal increase of pro-inflammatory cytokines, while this level is often normalized with treatment response (Anisman et al.,2005; Dantzer et al., 2005; Lanquillon et al., 2000; Kulmatcycki and Jamali, 2006; Brien et al., 2006).

Study to assess whether cytokines may be used as biological biomarkers for depression showed that mRNA expression of four pro-inflammatory cytokines IL-1β, IL-6, IFNγ, TNFα were significantly elevated in 20 depressed patients with respect to 22 healthy controls (table 1).

Table1. Cytokines in depressed patients and healthy controls (Tsao et al., 2006).

	Total participants		
	Healthy control n=22	Patients (n=20)	P values (t-test)
IL-1β	0.14 ± 1.18	0.74 ± 0.95	0.0113
IL-6	0.04 ± 0.10	0.61 ± 0.70	0.0017
IFNγ	0.10 ±0.17	1.12 ±1.43	0.0047
TNFα	0.02 ±0.04	0.58 ±0.70	0.0020

All results were expressed as mean ± S.D. and compared between controls and patients at baseline by Student t-test

After a treatment with SSRI fluoxetine (20 mg daily for 3-month) which caused a reduction of 45% of the depression rate score in all subjects, only the IFNγ mRNA expression decreased significantly (Tsao et al., 2006).

In a different study, quantitative measurement of IL-6, IL-8, TNFα, was performed in blood sample collected from 15 normal volunteers and 28 patients with major depression. As results, serum TNFα, IL-8 increased significantly in depressive patients compared to normal control whether IL-6 showed no significant difference between the two groups (Table 2).

Table 2. Measurement of the inflammatory marker in normal controls and patients with depression (Mikova et al., 2001).

	Total participants		
	Healthy control n=15	Patients (n=28)	P values*
IL-8 (pg/ml)	60 ± 68	110 ± 147	0.03
IL-6 (pg/ml)	9.6 ± 11.3	15.6 ± 28.4	0.7
TNFα (pg/ml)	14.7 ± 8.0	33.4 ± 27.4	0.005

*All results were expressed as mean ± S.D. * Statistical analysis was performed using ANOVA, ANCOVA and Fisher's least significant difference (LSD).*

Literature also revealed as the pro-inflammatory cytokine, IL-12, may have a role in the psychopathology. Recent clinical study involved 30 patients with

MDD showed a significant increase of IL-12 in plasma of depressive patients compared to normal control (Lee and Kim, 2006). Twenty cytokines were simultaneously assessed in 49 individuals with MDD and 49 healthy subjects. Multiple proinflammatory and two antinflammatory cytokines were significantly elevated in the MDD sample. The appearance of depressive symptoms seems to be more robustly associated with the increase of plasma concentrations of IL-1β, IL-1α, IL-2, IL-6, IL-8, IFNy (Simon et al., 2007).

The link between cytokines and depression was also supported by the use of cytokines in the treatment of some pathological conditions. IFNα, well known for its antiviral properties, is currently used in the treatment of diseases like hepatitis C, malignant melanoma and renal cell carcinoma. Administration of cytokines to non-depressed humans often induces a number of neuropsychiatric effects. Some of these effects can be directly associated with behavioural states of depressive patients such as cognitive impairment, decreased appetite, fatigue and loss of libido (Scheibel et al., 2004; Lieb et al., 2006; Capuron et al., 2002). These symptoms almost immediately disappear after the termination of cytokine treatment, suggesting again an important role of cytokines in mediating these effects.

When cytokines are administered to animals, a number of behaviours termed “sickness behaviour” are induced, mimicking some effects observed in humans after cytokine therapy and in MDD (Dunn et al., 2005; Yirmiya et al., 2006; Sudom et al., 2004). The most common features are hyperthermia, hypomotility, hypophagia, hyperalgesia, decreased interest in exploring the environment, decreased libido and increased time spent in sleeping. Rats treated with a

lipopolysaccharide (LPS) bacterial cell wall component which stimulates a hyper-activation of the immune system, revealed a dose-related reduction of saccharin consuming, suggesting that the activation of immune functions induces anhedonia in rats. LPS treatment induced other depressive-like symptoms, such as anorexia and reduced body weight, as well as reduction of social exploration (Yirmiya et al, 1996). The behavioural changes induced by LPS are indeed similar to those induced by a direct administration of cytokines (Sudom et al., 2004).

More evidences of a link between the effect on animal behaviours and the depressive status induced by cytokines have been highlighted by the effect of common antidepressant compounds on animals treated with LPS or cytokines. LPS effects were reversed by an i.p. chronic administration of DAT and NET inhibitor, imipramine (Yirmiya R., 1996). The anhedonic effect (diminution in perceived reward or pleasure from otherwise rewarding stimuli or responses) induced by a single administration of LPS in rats was also prevented by a chronic treatment with selective inhibitor of noradrenalin transporters, Desipramine. In contrast, treatment with selective SERT inhibitor, paroxetine and serotonin/noradrenalin re-uptake inhibitor, Venlafaxine failed to alter LPS-induced behaviours, underlining the complexity of the mechanisms that regulate antidepressant efficacy in attenuating LPS-induced behaviours. In the same study, desipramine reversed a rise of plasma TNFα secretion following LPS treatment, suggesting that desipramine protective effect may be due to its ability to inhibit the release of pro-inflammatory cytokines (Shen et al., 1999). The behavioural and physiological responses to a single administration of LPS in

rats have also been shown to be counteracted by chronic treatment with fluoxetine, which reversed the LPS-induced reduction of food consumption and body weight and also blocked the LPS-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis. (Yirmiya et al., 2001). Studies in rats have shown that a single i.p. administration of IL-1β decreased the consumption of "free" chow, which reflects anorexia, and also reduced responding for "earned sucrose rewards", which reflects anhedonia. In the same investigation, fluoxetine abolished the effects of IL-1β on "earned" rewards, but was inactive in the reduction of chow intake, suggesting that this antidepressant drug was more active in preventing IL-1β-induced anhedonia (Merali et al., 2003). Further effects of antidepressant drugs in cytokine-induced sickness behaviours in animal models were reviewed in Dunn et al., 2005.

Once cytokines enter the CNS, possible mechanisms that have been suggested to explain the alteration of neuronal functions is the modulation of monoamine concentrations especially 5HT with an effect that occurs in different brain regions and is dependent on the cytokines used. The focus of this PhD on the effect of cytokines on serotonergic neurotransmission is generated by the fact that this interaction has been largely supported by many *in vitro* and *in vivo* studies (see below). Moreover, some antidressant drugs such as fluoxetine and paroxetine which target SERT showed to reverse the depression symtoms and, in parallel, normalized the inflammatory response confirming the presence of an interaction between serotonergic system and cytokines (Kenis and Maes, 2002). Moreover, some cytokines can direct modulate the expression and/or catalytic activity of SERT reflecting a change of 5HT level at the synapses as

described in the section 1.3.1. *In vivo* microdialysis experiment in freely moving rats revealed that i.p. administration of IL-6 affected 5HT metabolism in the nucleus accumbens as shown by a significant rise of the principal 5HT metabolite, 5-Hydroxyindoleacetic acid (5-HIAA). Cytokine-treated rats showed also a decrease of dopamine (DA) concentration with a high concentration of the DA metabolite, dihydroxyphenylacetic acid (DOPAC) maintained over time (Song et al., 1999). Measurements of monoamine levels in mouse brain after repeated i.p IL-2 administrations showed a significant change on DA and 5HT metabolism with a reduction of DA and DOPAC in the caudate and substantia nigra, 5HT reduction in the medial prefrontal cortex and 5HT elevation in the hippocampus (La costa, 2000). In the nucleus accumbens, IL-1β did not affect DA concentrations but it significantly increased extracellular 5-HIAA accumulation, suggesting an increase of 5HT activity (Song et al., 1999). Peripheral IFNα chronic treatment caused changes in both DA and 5HT levels in many brain areas. However, the most considerable effect was a depletion of 5HT in frontal cortex, hippocampus, amygdala, thalamus, and hypothalamus, suggesting that these brain areas may be involved in IFN-α-induced rat sickness behavior (Sato et al., 2006).

In an *in vivo* microdialysis study, i.p. LPS treatment displayed a significant dose-dependent increase of extra-cellular 5HT levels and 5-HIAA in the rat hippocampus. This increase also correlated well with a decrease in active behavioural activity (locomotion, grooming, eating, drinking) and an increase in extracellular corticosterone levels. I.c.v. application of human IL-1β mimicked the effect of LPS on serotonergic neurotransmission, behavioural activity and

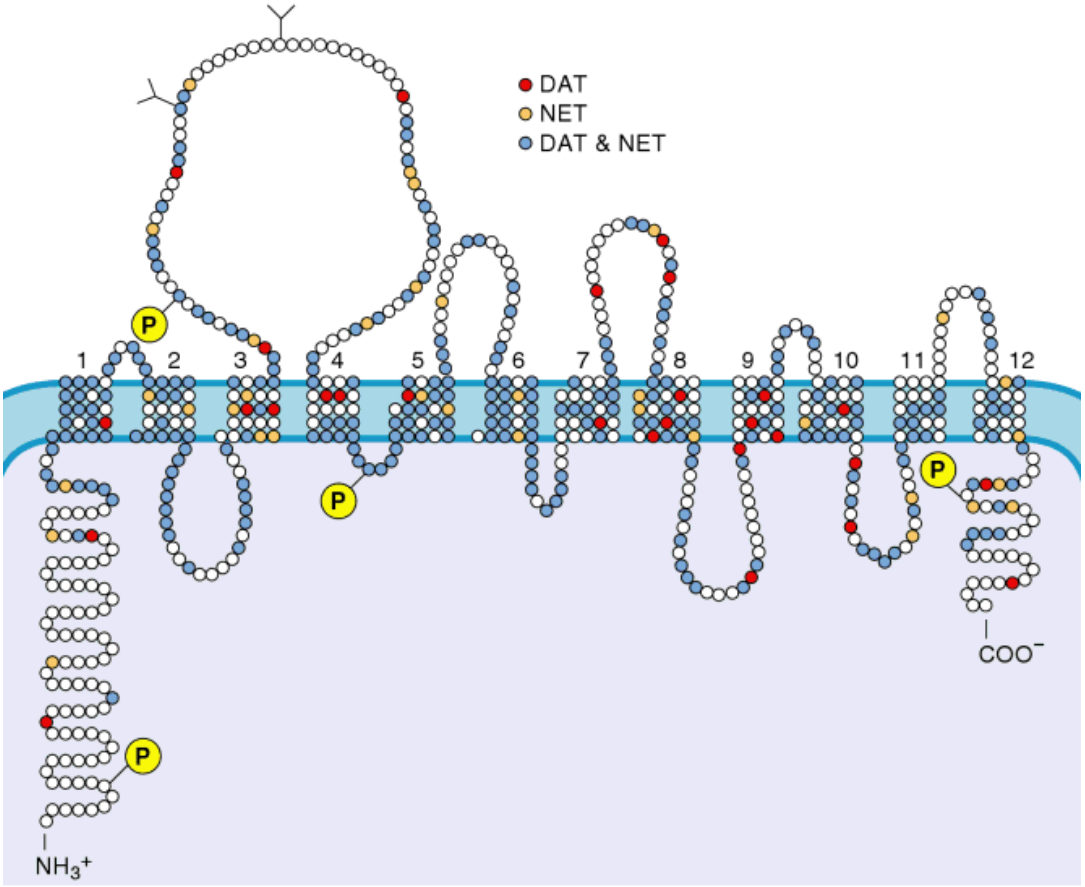
corticosterone levels. However, co-treatment with an IL-1 receptor antagonist reversed only the effect on extra-cellular 5HT concentration, suggesting that IL-1β may mediate LPS effect on hippocampal serotonergic transmission (Linthorst et al., 1995). All these studies therefore support a role for cytokines in the regulation of monoaminergic neurotransmission into the CNS, in particular in the change of serotonergic concentrations in brain.

Section 1.3 5HT transporter expression and structure

One of the most important targets involved in regulating levels of 5HT in the brain, is the 5HT transporter (SERT, also known as SLC6A4), which transports 5HT from the extracellular space and then regulates 5HT levels in the synapses. In the mature animal, it is located perisynaptically on presynaptic 5HT nerve terminals as well as on axons and 5HT cell bodies in the Raphe nucleus (Rudnick and Clark, 1993; Barker & Blakely, 1996; Blakely et al., 1998). The localization of 5HT transporter immunoreactivity in terminal endings and its concentration within varicosities are in good agreement with the high levels of 5HT and 5HT uptake found at these sites. SERT is predominantly expressed in caudate-putamen, cerebral cortex, amygdaloid complex, substantia nigra and Raphe nuclei, and low levels in the cochlear and vestibular nuclei and the cerebellum (Sur et al., 1997). Antisense oligonucleotide probes derived from the cloned human placental SERT cDNA (Ramamoorthy et al. 1993) reveal dense SERT hybridization signals overlying neurons, but not glia, of the dorsal and

median raphe nuclei and the serotonergic caudal linear nucleus in post-mortem human brain sections (Austin et al.1994).

SERTs are also expressed on non-neuronal cells including platelets, lymphoblasts, monocytes, enterochromaffin cells, endothelial cells and placental syncitiotrophoblasts (Daws and Gould, 2011). Human SERT (hSERT) is localized in the chromosome 17q11.2 (Ramamoorthy et al., 1993) and encodes a protein of 630 amino acids. The structure predicted for brain SERT is similar to the other monoamine transporters such as noradrenalin transporter (NET), and dopamine transporter (DAT) and belongs to the single gene SLC6 family. The molecular structure is a dimer of 60-70 KDa characterized by 12 hydrophobic transmembrane domains with cytoplasmic NH2 and COOH termini bearing greatest sequence identity to other gene family members in TMDs 1–2 and 5–8. It has been suggested that these conserved regions are involved in general transport functions while less conserved regions such as the COOH and NH_2 terminal are mainly involved in the pharmacological selectivity. Other studies where some regions of SERT were exchanged with NET did not show a loss of substrate or antagonism specificity suggesting that COOH and NH_2 were not important in target recognition (Zahniser et al., 2001). Transmembranes 3 and 4 are separated by a large, hydrophilic loop that bears two canonical sites for N-linked glycosylation (Ramamoorthy et al., 1993). SERT exists also in homo-multimeric complexes and contains potential phosphorylation sites for PKC, PKA and p38 MAP kinases as indicated in the diagram below (Ramamoorthy et al., 2010).



Putative structures of the rat 5HT transporter (SERT) showing homologous amino acids with the rat dopamine transporter (DAT), human norepinephrine (NET) or both. Possible phosphorylation (P) sites are shown, as are possible glycosylation sites on the large second extracellular loop.

Pre-synaptic receptors and intracellular signal pathways are linked with kinases regulating the activity of SERT in term of expression, functionality and transport in the plasma membranes. For example, activation of adenosine receptors (AR), $5HT_{1B}$, histamine receptors (H_3R) and BDNF/TrkB stimulates 5HT uptake (Launay et al., 1994; Zhu et al., 2004; Benmansour et al., 2008), whereas α2 adrenergic receptor stimulation reduces 5HT uptake (Ansah et al., 2003). The mechanism of the different efficiency of amine transport following SERT

phosphorylation is explained by a change of SERT recycling in the plasma membranes and its surface expression (Ramamoorthy et al., 2010).

Moreover, different human SERT variants associated with the risk of developing brain diseases showed an altered sensitivity to be activated by cGMP-dependent protein kinase (PKG) and p38 MAP kinase suggesting that an altered regulation of SERT by kinases may be associated with the risk of developing depression (Prasad et al.,2005). However, the degree to which kinases regulate SERT surface expression is controversial due to many discrepancies across different laboratories and unclear evidence supportive trafficking but also non-trafficking dependent mechanisms (Miller & Hoffman, 1994; Kilic et al., 2003).

5HT transport operates by using the gradient of Na^+ and Cl^- ions as the driving force to accumulate 5HT inside the cells against gradient. Basically, the influx of 5HT is coupled with the influx of Na+ and Cl- ions and the efflux of K+ ion in each cycle. The rapid reuptake of 5HT in the synapses plays an important role in determining duration and intensity of 5HT transmission with post-synaptic receptors placed in limbic region mediating emotion and pre-synaptic receptors that exert inhibiting control of 5HT neurons. The interest for this neurotransmitter transporter is highlighted by the number of drugs targeting this protein that have relevant benefits in the treatment of psychiatry disorders such as depression and anxiety. For example, tricyclic antidepressants, a large class of anti-depressive compounds, are actually 5HT reuptake inhibitors (SSRI). In an attempt to understand the molecular mechanism of the positive effect of SSRI in depression, many publications highlighted that SSRIs increase the level

of 5HT by directing inhibition of transporter but also can alter SERT expression (White et al., 2005). Changes to the expression level of SERT in response to drugs could contribute to neuroadaptive changes associated with chronic antidepressant administration. However, the effects of antidepressants on the regulation of SERT expression remain largely unclear. It has been identified that a polymorphism in the promoter region of the human SERT gene resulted in a deletion of a 44-basepair repeat. Activity of the form with longer sequence (*l*) was three fold increased compared to the short form (*s*). Moreover PKC- and cAMP-activated transcription was significantly greater at the "*l"* SERT gene promoter *versus* the "s" promoter. This change reflects a different response to antidressant activity and response; for example patients with an "*l"* allele (either as "*l / l"* or "*l / s"*) exhibited an increase in the response to fluoxetine and placebo compared to subject with the "s" genotype (Lesch *et al.,* 1996).

Section 1.3.1 Effect of cytokines and their signal pathways on SERT

A mechanism proposed that may describe how cytokines alter 5HT levels in brain is by the modulation of 5HT transporter expression and then a change of re-uptake of 5HT at the synaptic cleft. Exposure of JAR human chotiocarcinoma cells to IL-1β for at least 16 hours at 37°C increased 5HT uptake two fold and this effect was mainly due to an increase of maximal velocity of uptake rate while effect on affinity was minimal. Further RTI-55 binding experiments confirmed an increase of density of SERT in the plasma membranes following treatment and Northern blot studies also showed an increase of 5HT transporter

mRNA in treated cells compared to control confirming an activation of SERT expression induced by IL-1β (Ramamoorty et al., 1995). The effect of other cytokines were revealed by additional kinetic analysis of SERT on JAR human chotiocarcinoma cells treated with 50ng/ml TNFα for at least 48 hours at 37°C which revealed a significant increase of the maximal velocity of uptake compared to non-treated cells whereas an effect on Km was absent suggesting a change of only SERT expression. The same publication reported no effect when cells were treated with increasing concentrations of IL-6 (0.1-100ng/ml; Mossner *et al.,* 1998). In contrast to previous reports, TNFα and INFγ decreased 5HT uptake when incubated for 72 hours with immortalized human intestinal epithelial cell line (Caco2 cells) due to a decrease in SERT expression (Foley et al., 2007). Treatment with INFα or γ for 3–6 h, but not for 30 min, increased the uptake activity of the 5HT transporter in human placental choriocarcinoma cells (BeWo cells) and this effect was accompanied by an increase of SERT mRNA. The same treatment increased the level of SERT mRNA in the midbrain and adrenal gland from mice suggesting that SERT espression is modulated also *in vivo*. Uptake rate in IFN-treated mice was not measured. The finding that treatment with interferons for a short time (30 min) did not alter 5HT uptake suggests that the uptake function of the 5HT transporter is not enhanced by short-term interferon-mediated signalling which involved a direct tyrosine phosphorylation of the 5HT transporter (Morikawa *et al.,* 1998). A different response in rat is suggested by an *in situ* hybridization study where a chronic i.p. administration of INFα (20000 up to 2000000 IU/kg for 14 days) did not produce any significant change in the 5HT transporter

mRNA levels in rat dorsal raphe nucleus (Sato T et al, 2006). The discrepancy observed in these findings may be due to a different effect of a chronic *vs* acute treatment with cytokines. Analysis of the effect of acute *vs* chronic IL-1β administration showed that despite the acute effect of cytokines being attenuated, the chronic IL-1β administrations showed an increase alteration of 5HT and NE utilization with an elevation of $5HT_{2C}$, $5HT_{1B}$ receptor expression (Anisman et al., 2008). Moreover, the levels of monoamines and associated metabolites after chronic and acute i.p. IFNα administration were measured in various regions of the rat brain using a high-performance liquid chromatography–electrochemical detection system. A depletion of 5HT in the frontal cortex, hippocampus, amygdala, thalamus and hypothalamus was observed in rats given a moderate-dose of IFNα for 14 days while acute IFNα did not change 5HT level (Sato et al., 2006). The importance of chronic exposure of cytokines on depression was confirmed by some particular situations characterized by a chronic low-grade inflammatory such as chronic stress, obesity and aging which appear as vulnerability factors for development neuropsychiatric diseases (Capuron et al., 2011).

The effect on SERT was not only due to the alteration of expression but also by a change of biophysical characteristics at the synapses. A paper revealed that IL-1β and tumor necrosis factor alpha (TNF-α) stimulated 5HT uptake in both the rat embryonic raphe cell line, RN46A, and in mouse synaptosomes. In RN46A cells, both cytokines stimulated 5HT uptake in a concentration- and time-dependent manner with a maximum effect at 100 ng/ml and after an incubation of 20 min with cells. Similarly, an increase of 5HT uptake was also

produced in mouse midbrain and striatal synaptosomes although the maximum effect occurred at lower concentrations (10 and 20 ng/ml for IL-1β and TNF-α respectively) and over a shorter incubation time (10 min). Interestingly, experiments on RN46A cell demonstrated that IL-1β and TNF-α treatments decreased K_m (uptake parameter which reflects the affinity of 5HT for transporter) whereas the B_{max} (uptake parameter which reflects expression of SERT in the membrane) was not changed by IL-1β and just partially affected by TNFα. Finally, in both RN46A cells and synaptosomes, the effects of these cytokines were completely (IL-1β) or partially (TNF-α) blocked by a selective p38 MAPK inhibitor, SB203580 suggesting that the regulation of neuronal SERT activity induced by cytokines may be mediated by p38 MAPK-linked pathways (Zhu et al., 2006). Interestingly, the role of p38 MAP kinase in the modulation of SERT had been already demonstrated in recombinant cells expressing human SERT and in RN46A cells. Measurement of 5HT uptake in these cells treated with the indirect unselective p38 MAP kinase activator, anisomycin (1μM), revealed an increased uptake rate due to a reduction in the Km. This study also revealed that anisomycin increased the 5HT uptake rate in human platelets (Zhu et al, 2005).

Literature has also reported that in CHO cells stably expressing human SERT, NET and DAT, activation of p38 MAP kinase increased 5HT and noradrenalin (NA) uptake but decreased DA uptake. This suggests that p38 MAP kinase mediates a catalytic activation or deactivation of all human monoamine transporters.

Taking together, these findings suggest that the regulation of neurotransmission by cytokines may depend on a direct change of the transporters functionality causing a different efficiency in the reuptake of monoamines, especially 5HT.

Section 1.4 $5HT_7$ receptor structure and function

$5HT_7$ receptors represent the most recently identified target within the big class of 5HT receptors (Barnes and Sharp et al., 1999). Structural studies showed that this receptor is a protein of about 445 amino acids and with the characteristic seven putative membrane spanning regions with a high degree of homology with other species. The human $5HT_7$ receptor gene is located on chromosome 10 (10q21-q24) (Gelernter et al., 1995), and contains several introns (Gellynck et al., 2008). The second intron corresponds to the carboxyl terminus of the $5HT_7$ receptor, from which at least four splice variants were reported. Human and rat tissue appear to express only three isoforms. In particular, rats express $5HT_{7A}$, $5HT_{7B}$, $5HT_{7C}$ because the exon responsible for the fourth isoform $5HT_{7D}$ is absent. Humans express $5HT_{7A}$, $5HT_{7B}$, $5HT_{7D}$ which are proteins of 445, 432 and 479 amino acids respectively (Gellynck et al., 2008). Investigation of the distribution of the different splice variants led to the observation that all variants were present in most tissues examined, although relative expression levels differ considerably. In man and rat, the $5HT_{7A}$ and $5HT_{7B}$ splice variants are the most abundant splice variants, whilst the rat $5HT_{7C}$ and human $5HT_{7D}$ splice variants represent 4% of the total brain $5HT_7$ mRNA (Vanhoenacker et al., 2000; Guthrie et al., 2005).

Many studies used *in situ* hybridization to determine where the $5HT_7$ receptor mRNA was expressed within the brain. These studies detected a high expression of $5HT_7$ receptor mRNA in mice and rats within regions of the thalamus, hypothalamus and hippocampus with generally lower levels in areas such as the cerebral cortex and amygdala (Neumaier et al., 2001). Immunocytochemistry analysis of $5HT_7$ receptor protein confirmed the distribution obtained with *in situ* hybridization, highlighting its expression in cell bodies and fibres of all CA fields (CA1, CA2, CA3) in the hippocampus and in the suprachiasmatic nucleus (SCN) of the hypothalamus (Muneoka et al., 2003). The distribution of $5HT_7$ receptors was also analyzed using the selective $5HT_7$ receptor antagonist radioligand [^{3}H]SB-269970 in various rodent (mouse, rat) and non-rodent (pig, marmoset) species (Thomas et. al., 2002) and human brain whole hemisphere autoradiography (Varnas K et. al., 2004). The results confirmed that $5HT_7$ receptors are most abundantly localized in the anterior thalamus and in the dentate gyrus. Other regions containing intermediate levels of $5HT_7$ receptors included the hypothalamus, anterior cingulate gyrus, hippocampus, amygdala and certain brainstem nuclei. A similar binding pattern, but with some notable difference, was found in rats, guinea-pigs and humans using [^{3}H]mesulergine as the radioligand (Hemedah et al., 1999). These data highlighted a relatively high binding density in the caudate-putamen in rats, guinea-pigs and human, although other studies did not report binding in this regions. In other regions, these findings confirmed the immunohistochemistry data.

Since the $5HT_7$ receptor is positively coupled with Gs protein, its activation stimulates an increase of intracellular adenylate cyclase (AC). This effect generally leads to important changes of cellular activity through an activation of various intracellular kinases (PKA and PKC) that trigger a series of down-stream signalling cascades and a consequent modulation of gene expression. Increase of cAMP induced by $5HT_7$ receptors was reported in various recombinant cell systems transfected with human and rat $5HT_7$ receptors (Mahe et al., 2004; Romero et al., 2006). Moreover, accumulation of cAMP induced by $5HT_7$ receptors was also discovered in many glioblastoma cell lines (Mahe et al., 2004), rat cultured astrocytes (Hirst et al., 1997) and guinea-pig hippocampal membranes (Thomas et al., 1999). Physiological functions of $5HT_7$ receptors in the central nervous system are various and depend on the area in which they are expressed. Overall, the expression of these receptors in the hypothalamus could be associated with the modulation of circadian rhythm, thermoregulation and endocrine regulation. The presence in thalamus, cortex and hippocampus might be important for sleep and mood regulation, and it has been suggested that thalamic $5HT_7$ receptors might be important in epilepsy. Finally, $5HT_7$ receptors in the hippocampus are of interest in synaptic plasticity which is important for learning and memory.

The role of 5HT in thermoregulation was well reported when Li et al (2001) showed that 8-OH-DPAT (non selective $5HT_{1A}$ and $5HT_7$ agonists) reduced body temperature in mice and this effect was preliminarily associated just to $5HT_{1A}$ receptors. The involvement of to $5HT_7$ receptors in the control of body temperature was assessed when further studies showed that selective $5HT_7$

receptor antagonists reversed the hypothermia induced by 5CT and 8-OH-DPAT (Hedlund et al.,2004). Recently, this finding was confirmed in mutant $5HT_7$ receptors knockout mice which were injected i.p. with the $5HT_7$ receptor agonist LP-211. This compounds seemed to dose-dependently reduce body temperature in $5HT_7$+/+ mice but not in $5HT_7$−/− mice confirming the capacity of $5HT_7$ receptor agonist to regulate body temperature *in vivo.*

Other investigations were focused to understand the role of $5HT_7$ receptors in the control of circadian rhythm. A first evidence of this modulation was provided in rat hippocampal slices where 8-OH-DPAT induced a phase shifts of the circadian rhythm of spontaneous neuronal activity within the SCN (Lovenberg et al., 1993). Further studies highlighted that this effect was reversed by unselective $5HT_7$ receptor antagonist, SB-269979 supporting the involvement of $5HT_7$ receptors (Sprouse et al., 2004). However, in one study, the selective $5HT_7$ receptor agonist AS19 administered in rats at one concentration failed to produce the effect observed with 8-OH-DPAT but the partial agonism of AS19 could make difficult the comparison (Cuesta et al., 2009). Moreover, studies availing of $5HT_7$ receptor knock-out mice and selective antagonists showed that $5HT_7$ is also involved in the photic regulation of retinohypothalamic input to the SCN (Gardani and Biello , 2008).

Studies highlighted expression of $5HT_7$ receptors in the hippocampus suggesting that $5HT_7$ receptors may play an important part in neuronal plasticity. Further studies were then performed to understand the physiological function of $5HT_7$ receptors in the control of neuronal firing and Long term potentiation (LTP) in hippocampus. Intracellular recording in area CA3 of

hippocampal slice showed that $5HT_7$ receptors reduced the amplitude of slow afterhyperpolarization (sAHP) in pyramidal neurons in a reversible, concentration-dependent manner. Mechanistically, this effect appeared to occur through an inhibition of Ca^{2+} activated K^+ channels (Bacon WL *et al.,* 2000). The reduction of sAHP induced by $5HT_7$ receptors was the mechanism that explained the increase of bursting frequency induced by 5CT in the same region (Gill at al., 2002). Moreover, the same study revealed that 5CT did not affect AMPA receptor mediated synaptic transmission as assessed by the effect of this $5HT_7$ receptor agonist on stimulus evoked field and intracellular recorded Excitatory PostSynaptic Potential (EPSPs).

In CA3 pyramidal slices the effect was similar to that reported in the CA1 region where 5CT activates $5HT_7$ receptors to increase neuronal excitability (Tokarski *el al.,* 2003). A role of $5HT_7$ receptors in neuronal plasticity was confirmed in mutant $5HT_7$ knock-out mice which showed a reduced ability to induce long-term potentiation (LTP) within the CA1 region of hippocampal slices (Roberts *et.al.,* 2004). In hippocampal cultured neurons, $5HT_7$ receptors activated the mitogen-activated kinases ERK1 and ERK2 which may be relevant for the effect on LTP (Errico et al., 2001).

The effect of $5HT_7$ receptor antagonists on neuronal physiology increased the interest to understand the role of $5HT_7$ receptors in brain. In particular, the interaction between antidepressants with $5HT_7$ receptors has forwarded a link with the control of mood. Several antidepressants induced c-fos expression in SCN and this effect was consistent with the expression of c-fos after the activation of $5HT_7$ receptors (Mullins et. al., 1999). Moreover chronic

antidepressant drug treatment led to a reduction of $5HT_7$ receptors binding sites in the brain (Mullins et. al., 1999). Activity in the forced swim test and the tail suspension test suggested a potential link between $5HT_7$ receptors and mood. Pharmacological blockade of the $5HT_7$ receptor or inactivation of the receptor gene led to an antidepressant-like behavioural profile, that is, reduced immobility (Hedlund et al. 2005; Guscott et al. 2005; Bonaventure et al. 2007; Wesolowska et al. 2006a, b, 2007). A synergistic interaction was also apparent between the $5HT_7$ receptor antagonist SB-269970 and antidepressants leading to reduced immobility in both the forced swim test and the tail suspension test (Bonaventure et al. 2007; Wesolowska et al. 2007). Thus, concurrent administration of citalopram, an antidepressant of the SSRI type, and SB-269970 has been shown to reduce immobility in the tail suspension test in C57BL/6 J mice (Bonaventure et al. 2007). This synergistic effect was also detected in the rat frontal cortex measuring the 5HT concentration after the exposure to citalopram and SB-269970. At a higher dose, SB-269970 alone reduced immobility in the mouse tail suspension test without any change of 5HT concentration in the rat frontal cortex (Hedlund et al. 2005; Bonaventure et al. 2007), However, the combination of a low dose of SB-269970 and a low dose of citalopram increased the level of 5HT in the frontal cortex (Bonaventure et al. 2007). A similar synergistic interaction between SB-269970 and citalopram has also been demonstrated in the forced swim test (Wesolowska et al. 2007). Other classes of antidepressants used in this study gave a similar positive interaction. Thus, when sub-threshold doses of imipramine, desipramine, and moclobemide were injected in the mouse in combination with SB-269970,

animals revealed reduced immobility in the forced swim test (Wesolowska et al. 2007). The interaction between SB-269970 and imipramine has also been shown in the forced swim test using Wistar rats in which the effect on immobility was accompanied with an increase in 5HT levels in the prefrontal cortex (Wesolowska and Kowalska 2008).

Another model to study mood disorders is the exposure to chronic unpredictable mild stress that generate a depression-like state in laboratory animals (Willner et al. 1992). In a recent study, rats exposed to such stress showed $5HT_7$ receptor mRNA upregulation in the hippocampus and hypothalamus, but not cortex (Li et al. 2009). The change in mRNA levels could be inhibited by treatment with fluoxetine and curcumin, an active ingredient in turmeric extracts (Li et al. 2009). Interestingly, a clinical study has shown that the $5HT_7$ receptor is relevant for the treatment of depression. Amisulpride is an atypical antipsychotic that is also a proven antidepressant (Smeraldi 1998). The antidepressant effect of amisulpride was firstly associated to somehow rely on its properties as a dopamine D2/D3 receptor antagonist, although the mechanism has never been satisfactorily explained. It has now been demonstrated that amisulpride has high affinity for $5HT_7$ receptors and that amisulpride reduces immobility in both the tail suspension test and the forced swim test in $5HT_7$ +/+ mice but not in $5HT_7$−/− mice (Abbas et al. 2009). These findings provide at least indirect evidence that the antidepressant effect of amisulpride is mediated by the $5HT_7$ receptor. It should also be noted that aripiprazole, another atypical antipsychotic that is successfully used to augment

the effect of traditional antidepressants (Berman et al. 2009), has high affinity for the $5HT_7$ receptor (Shapiro et al. 2003).

Section 1.4.1 $5HT_7$ receptors and neuroinflammation

Microglial cells and astrocytes represent the cellular sources of cytokines in the CNS. Microglia cells secrete Th1 cytokines (T helper type 1 cells) while astrocytes preferentially release Th2 cytokines (T helper type 2 cells). Since some psychiatric diseases seems to be associated with an Th1/Th2 imbalance with a shift to Th1, the equilibrium between microglial and astrocytes cytokine release may be an important feature in abnormal regulation of neuroinflammation leading to depression (Muller & Schwarz, 2007).

$5HT_7$ receptor expression functionally coupled with cAMP accumulation was confirmed in rat brain astrocytes (Hirst et al., 1997), in human microglia cells (Mahe *et al.,* 2005) and in human glioblastoma cell lines (U-373 MG, DBTRG-05MG, T98G, H4, U-138 MG, U-87 MG, CCF-STTG1, Hs 683) (Mahe et al., 2004). Moreover, 5HT has been shown to stimulate the expression and release of IL-6 in a human glioblastoma cell line U-373 MG and microglial MC-3 cell line (Lieb et al., 2005; Mahe et al., 2005). In human glioblastoma cell line U-373 MG, IL-6 release induced by 5HT was mediated by $5HT_7$ receptors. The time course experiment revealed two peaks, one after 4 hours and the second after 48 hours with a high level of IL-6 till 72 hours from stimulus. This data may suggest two transduction pathways are involved, one rapid and transient and other delayed but persistent. This effect was blocked in the presence of the

selective $5HT_7$ receptor antagonist, SB269970, while selective $5HT_{2A}$ and $5HT_3$ receptor antagonists were unable to reverse the 5HT induced IL-6 release. IL-6 release induced by 5HT was also blocked by p38 MAP kinase and PKC inhibitors (SB202190 and GF109203X, respectively) while p42/44 MAPK inhibitor PD98059 did not change the response suggesting that this effect is mainly mediated by p38 MAP kinase and PKC. Western blot analysis of cells lysates treated with 5HT showed that activation of p38 MAP kinase started at 5 min with a peak at 10 and 15 min and disappeared after 60 min, while activation of PKC stared at 2.5 min lasting untill 10min. In the attempt to further explore which PKC isoforms that were involved, a variety of PKC inhibitors were used. These data indicated that atypical PKCs (PKC δ and ε) were involved but not classical PKC (PKCα,β,γ). No consistent evidence for the involvement of transcription nuclear factor kappa B was reported. Within the human microglial MC-3 cell line, stimulation with the non-selective agonist of $5HT_7$ receptors, 5CT, caused an increase in IL-6 mRNA which was also abolished by the presence of the $5HT_7$ receptor antagonist SB269970 (Mahe et al., 2005). Considering that in this cell line, $5HT_7$ receptors increase cAMP increase and forskolin mimicked the effect of 5CT or 5HT on IL-6 expression, it has been suggested that cAMP intracellular signal pathway is involved.

Overall, these evidences suggest that $5HT_7$ receptors may play an important role in the regulation of cytokine release in the CNS. However, since four splice variants of $5HT_7$ have been identified, it is not yet known how different splice variants of the $5HT_7$ receptor may differentially modulate cytokine release.

Section 1.5 Alternative mechanisms of action of cytokine-induce depression.

As described in the previous section, clinical and preclinical studies suggested that the abnormal regulation of cytokines in the CNS may cause a change of neurotransmission which is strongly associated with the development of depression. Apart from the effect of cytokines on serotonergic system described above, alternative mechanisms seem relevant in the correlation between immune response with depression.

The main resource of cytokines in the brain are microglia and astrocyte which produce brain cytokines belonging to the Th1 (such as IFNγ, IL-1, TNFα) and Th2 (such as IL-4 and IL-10) class respectively. An inappropriate balance of microglia and astrocyte as found in some patients with depression (Johnston-Wilson et al., 2000; Miguel-Hidalgo et al., 2000; Rajkowska et al., 2007) could determine an abnormal regulation of these cytokines. Moreover, microglia and astroglia play a role through the secretion of neurotoxic substance and neurotrophic factors which influence the neuronal plasticity and neuronal toxicity confirming the importance of appropriate numbers of microglia/astrocyte in the CNS (Leah McNally et al., 2008)

In microglia, there is an induction of neuronal cytotoxicity *via* the cytokines-dependent stimulation of enzyme indoleamine 2,3-dioxygenease (IDO). IDO that converts the main precursor of serotonin, tryptophan (TRP), into kynurenine (KYN), has been demonstrated to be activated by cytokines, reducing TRP availability and consequently decreasing 5-HT concentrations in the brain. In parallel, a cytokine-induced augmentation of KYN produces an increase of the

NMDA receptor agonist quinolinic acid in the brain (Dantzer et al., 2010). A depletion of TRP along with an increase of quinolinic acid and consequently an altered glutamatergic neurotransmission are strongly associated with depression (Muller & Schwarz MJ, 2007).

Hyperactivity of the HPA axis induced by cytokines including increased production and release of CRH in response to stress, has been proposed as an alternative mechanism that associates inflammation with the developing of neuropsychiatric disorders (Raison et al., 2006). This hypothesis was confirmed in patients who developed depressive behaviours after INFα therapy and an exaggerated increase in adrenocorticotropic hormone and cortisol levels in response to INFα treatment (Capuron et al., 2003)

This hyperactivity of the HPA axis response may derive from the capacity of cytokines to alter GR expression and/or GR function (Pace et al., 2007, Miller et al., 1999 & 2001, Wang et al., 2004) which are very important to dampen the neuroendocrine and inflammatory responses induced by stress and pathogen exposure.

Another hypothesis, which links inflammation to neuroplasticity, is the regulation of the nerve growth factors like BDNF by cytokines. BDNF plays an important role in the control of neuronal differentiation, neuroplasticity and neuroprotection, therefore a loss of BDNF is increasingly being implicated in the pathophysiology of depression (Groves, 2007; Martinowich and Lu, 2007; Palomino et al., 2006). A production of BDNF was detected in immunecompetent cell lines and in inflammatory brain lesions, highlighting the possibility of a neuronal protective role of the immune system (Hohlfeld et al.,

2007). In the CNS, human cultured adult astrocytes stimulated with TNFα and IL-1ß showed two-fold increased mRNA expression of BDNF (Meeuwsen et al., 2003). Moreover, intra-hippocampal admistration of IL-1β and IL-1β receptor antagonist antibody (Amgen) into rat brain modulate the level of BDNF mRNA expression following learning and memory tasks and social isolation (Barrientos et al., 2003). Peripheral inflammation induced by LPS injection led to a 20% transient decrease of BDNF protein expression in mouse brain synaptosomes (Schnydring et al., 2007). In conclusion, these findings suggest that cytokines may regulate the level of BDNF within the CNS level that can ultimately alter synaptic plasticity and hence brain function though this hypothesis is young in its evolution (Raison et al., 2006).

Section 1.6 Aims

Overall, these studies suggest a correlation between immune activation and psychiatry symptoms but the number of mechanisms described indicates that the nature of interaction among cytokines, the brain function and the change of behavior is still not well identified. Understanding the mechanism that better described this interaction may help to create new prospects for developing new drugs with increased efficacy for the treatment of psychiatric diseases.

The mechanism stated in this thesis is a modulation of the 5HT transport as consequence of a direct modulation of SERT function induced by TNFα and IL-1β at the synapses level. This effect may have an important impact on 5HT post-synaptic signalling which links with cytokines release because the $5HT_7$

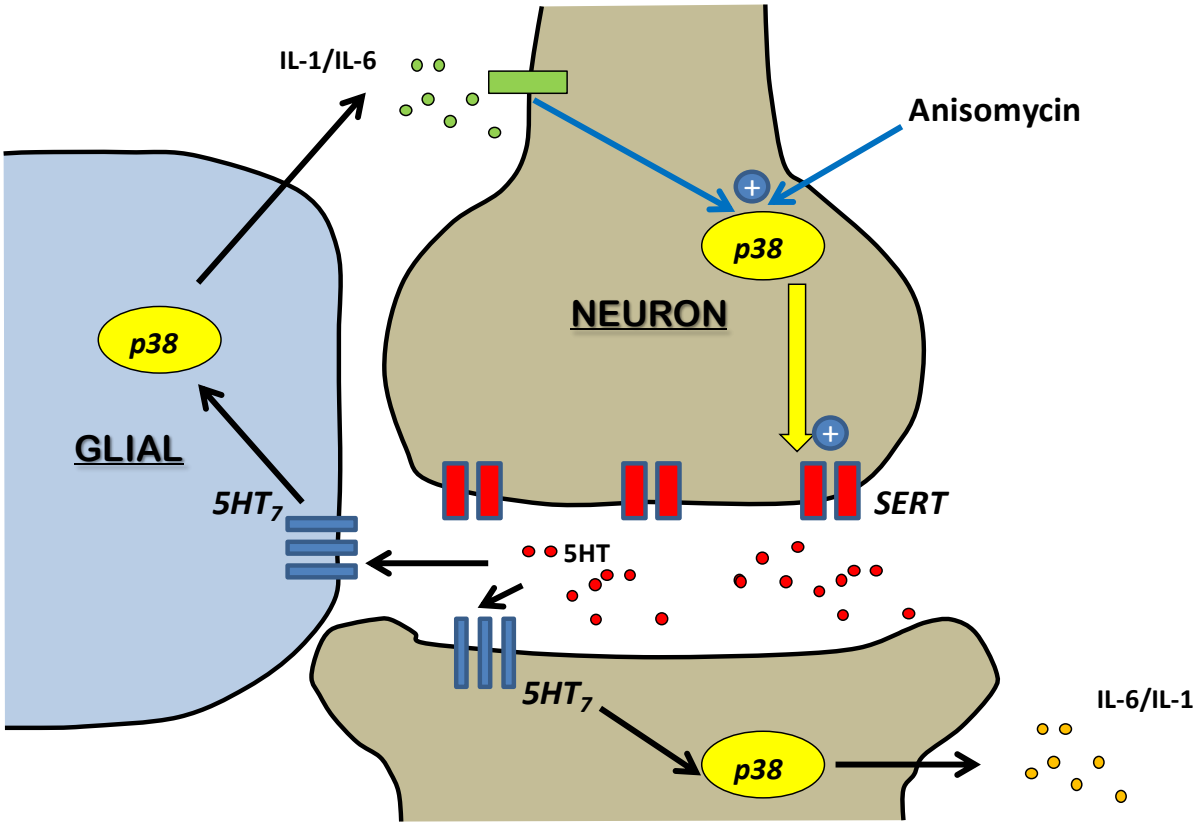
receptors expressed in glial cells potentiate the release of cytokines in the brain. It means that serotonin has a dual interaction with the cytokine pathway: on one hand increasing the 5HT transport, cytokines may induce critical changes of serotonin level at the synapses that may link to the development of depressive-like behaviors. On the other hand, serotonin may have a role in the release of cytokines in the brain and then an imbalance of Th1:Th2 types with consequently dysregulation of neuronal physiology.

All these processes involve a signalling cascade that have a common mediator, p38 MAP kinase, which is activated by numerous cytokines as well as by environmental and chemical stressors in many cases triggering inflammatory and apoptotic pathways (see figure below). .

This work was then focused on understanding the regulation of SERT induced by cytokines; in particular, studying if activation of p38 MAP kinase induced by IL-1β or anisomycin induced a significant change of rat SERT function and expression. The use of IL-1β and anisomycin was justified by the literature showing that IL-1β and anisomycin treatment induce a direct activation of SERT (Zhu et al., 2006) and the sickness behavior after LPS challenge is mainly mediated by IL-1β receptors *via* p38 MAP kinase (Zhu et al., 2010, Dunn et al., 2005). In order to correlate the proposed *in vitro* mechanism and the effect observed in animal model, this *in vitro* approach was followed by *ex vivo* studies in which SERT function was also measured in rats treated with LPS, a potent stimulator of inflammation. Moreover, additional studies were performed to characterize the expression and function of $5HT_7$ receptors in primary cultured

astrocytes and neurons and then explore the signalling cascade at downstream of $5HT_7$ receptor activation with regards the interaction with p38 MAP kinase.

The impact of this proposed study will be a clarification of the relationship between the serotonergic signal and cytokines pathway, in particular the interaction among cytokines, p38 MAP kinase, SERT and $5HT_7$ receptors in the context of immune response and psychiatry disorders. Understanding the mechanism of this interaction and which targets are more involved, will give the possibility to develop new antidepressant therapies based on the modulation of these pathways.



Hyphothesis of interaction between cytokines and serotonergic system.

Chapter 2 Materials and Methods

Section 2.1 Measurement of 5HT uptake in rat and mouse brain synaptosomes

The assay currently adopted to measure the SERT function is an [^{3}H]5HT uptake assay. This assay can be performed in both recombinant cells and in native tissue and give the measurement of 5HT uptake rate mediated by SERT. The native tissue has to be processed to obtain brain synaptosomes which are vesicles generated by the synaptic termination containing the targets present in the synaptic cleft. [^{3}H]5HT uptake was measured in brain synaptosomes prepared from Sprague Dawley rats (Charles river, 225-250g) housed in groups of 4 animals per cage and from C57BL/6N mice (Harlan, 12 weeks) housed in groups of 5 animals or in single animals per cage. The assay was performed using two different protocols. “Protocol 1” was based on those reported by Thomas *et al.,* (1987). “Protocol 2” was based on those reported by Zhu *et al.,* (2007).

Protocol 1

Brain tissue from single animal was dissected from different animalson ice and put into a tube containing 10ml cold 0.32M sucrose. The content of the tube was homogenised using 8 strokes of a Potters-Manheim homogeniser set at 50 rpm. The homogenate was centrifuged for 5 min at 1000xg at 4°C. Supernatant containing synaptosomes was collected, transferred into a new tube and diluted 1:2 (V/V) in 0.32M sucrose. 10μl of synaptosomes were stored at -20°C and

use for protein determination. 100μl of synaptosomes (30-40μg/well) were pre-warmed in a bath at the assay temperature of 30°C for 10 min. 350μl assay buffer (118mM NaCl, 4.9mM KCl, 1.5mM $CaCl_2$*$2H_2O$, 1.2mM $MgSO_4$*$7H_2O$, 25mM $NaHCO_3$, 1.2mM NaH_2PO_4, 0.06mM L-ascorbate, 10mM glucose, 0.01mM pargyline) with vehicle (0.1% DMSO in assay buffer) or compounds were then added to 100μl synaptosomes for 5-30 min. 5HT uptake was initiated by adding 50μl of [^{3}H]5HT (10-20 nM final concentration) and after 10 min the reaction was stopped and synaptosomes separated by rapid filtration through GF/B Whatman paper using Brandel harvester. Filter was washed there times with ice-cold assay buffer. After immersing filters in scintillation liquid for 6 hours, radioactivity was evaluated by scintillation counting. Counts were normalized per min and per mg of protein detected in the frozen samples.

Protocol 2

The brain tissue from single animal was dissected out on ice as above and homogenized in 10 volumes of cold 0.32M sucrose using 8 strokes of a Potters-Manheim homogeniser set at 400 rpm. The homogenate was centrifuged for 10 min at 800xg at 4°C. The supernatant was transferred into a new tube and centrifuged at 10000xg for 15 min at 4°C. The pellet with synaptosomes was resuspended in 40 volumes of assay buffer containing 130mM NaCl, 1.3 mM KCl, 2.2mM $CaCl_2$*$2H_2O$, 1.2mM $MgSO_4$*$7H_2O$, 1.2mM KH_2PO_4, 0.1mM L-Ascorbate, 10mM Glucose, 0.1mM Pargyline. 10μl of synaptosomes were stored at -20°C and use for protein determination. 100μl of synaptosomes (30-40μg/well) were pre-warmed in a bath at the assay temperature of 37°C for 10

min and then incubated with vehicle (0.1% DMSO in assay buffer) or compounds which were p38 MAP kinase activator, anisomycin (0.2-5μM), p38 MAP kinase inhibitor, SB203580 (0.2-5μM) or IL-1β (10-200ng/ml) for 5-30 min in assay buffer. 5HT uptake was initiated by adding 50μl of [^{3}H]5HT (10-20nM final concentration) and after 5 min at 37°C, the reaction was stopped by rapid filtration as described in the protocol 1.

Total counts obtained by filters were corrected for non specific signal obtained by parallel samples incubated with the selective SERT inhibitor Fluoxetine (10μM, Tocris). Analysis of protein concentration was performed by Bradford standard method.

Section 2.2 Measurement of p38 MAP kinase phosphorylation in rat and mouse brain synaptosomes

100μl of striatum or cortex synaptosomes were added to 400μl assay buffer (protocol 2) containing drugs and incubated in a bath at 37°C for 10-30 minutes. Samples were not standardized for protein. Synaptosomes were then centrifuged at 10000g for 20 min at 4°C, and pellet was re-suspended in 100μl 1X Lysis buffer containing 20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM Na_2EDTA, 1mM EGTA, 1% Triton, 2.5mM $Na_4P_2O_7$, 1mM β-Glycerophosphate, 1mM Na_3VO_4, 1μg/ml Leupeptine. Samples were then kept on ice for 20 min, centrifuged 13000g for 20 minutes and than the supernatant was transferred in a new tube. 10μl was stored at -20°C and use for protein determination and the left solutionwas finally analysed in duplicate with phospho-p38 MAP Kinase

ELISA kit (Cell Signalling, Cat#7946) according to the instructions provided.Briefly, sample was diluted 1:1 with Sample Diluent (supplied in each PathScan® Sandwich ELISA Kit). 100µl of each diluted cell lysate was added to the appropriate well and incubate over night at 4°C. Plate was then washed four times with 1X Wash Buffer (200µl each time for each well) and incubates with Detection antibodies for 1 hour at 37°C. Each well was washed as described before and 100µl HRP-link secondary antibodies was the added for 30 min at 37°C. Washes were repeated as before and 100µl of TMB substrate was added each well for 10 min at 37°C. Reaction was stopped by addition of 100µl Stop solution and adsorbance at 430nm was detected using spectrophotometer (SpectraMax, Molecular Devices).

Section 2.3 Maintenance of HeLa, RBL2H3 and hSERT-LLCPK

Hela cells (HeLa) and Rat basophilic leukemia cells, RBL2H3, have become readily accessible commercially and were received frozen in dry-ice from ECACC. Epithelial cell line from pig kidney (LLC-PK) expressing human SERT (hSERT-LLCPK) was kindly provided by GSK starting at passage 10. Cells were cultured in 75 cm^2 tissue culture flask at 37°C in an atmosphere with 5% CO_2, 100% relative humidity. All cells were grown in Dulbecco's Modified Eagles Medium (DMEM, Gibco 41966-029) with 10% (v/v) foetal bovine serum (FBS) supplemented with 100 IU/ml penicillin and 100µg/ml streptomycin (HeLa and RBL-2H3) or 400µg/ml Hygromycin-B (LLCPK). To maintain healthy proliferation, the cells were routinely passaged every two days At approximately

100% confluency, media was aspirated off the cell monolayer and the cells washed in 5ml Phosphate-Buffered Saline (PBS; Sigma). Cells were then dissociated from a flask with 2 ml 0.05% Trypsin/EDTA solution (Gibco) for 2-5 mins. The reaction was quenched with media containing 10% serum and spit in new flasks. All cell culture was completed in a microbiological safety cabinet, preventing contamination with bacteria, fungi and viruses. Before using cell in the assay, cells were cultured for at least two weeks in normal conditions (at least 4 passages) and then Hela cells and RBL2H3 were used between passage 4 and 8 and hSERT-LLCPK were used between passage 12 and 15.

Section 2.4 Measurement of 5HT uptake in recombinant cell lines

Cells were placed in 96-well plate one day before the experiment at the concentration of 20000 cell/well. After 24 hours, medium was aspirated and replaced with 100µl assay buffer (10mM HEPES, 130mM NaCl, 1.3mM KCl, 2.2mM $CaCl_2*2H_2O$, 1.2mM $MgSO_4*7H_2O$, 1.2mM KH_2PO_4, 0.1mM L-Ascorbate, 10mM Glucose, 0.1mM Pargyline) containing vehicle (0.1% DMSO in assay buffer) or compounds for 5-30 min at 37°C. 5HT uptake was initiated by adding 100µl of [^{3}H]5HT (100 nM final concentration) and after 10 min at 37°C, buffer was aspirated and cells were washed there times with ice-cold assay buffer. Cells were solubilised in 50µl Microscint 20 (PerkinElmer, cat# 6013621) and radioactivity was counted with TopCount plate scintillation counter (PerkinElmer).

Section 2.5 Measurement of 5HT uptake in human blood platelets

Human blood was collected in K3-EDTA tubes and centrifuged at 200xg for 10 min at room temperature. The supernatant, that corresponds to platelet rich plasma (PRP) was collected and platelets were counted by flow cytometry and diluted in Krebs assay buffer (130mM NaCl, 1.3mM KCl, 2.2mM $CaCl_2*2H_2O$, 1.2mM $MgSO_4*7H_2O$, 1.2mM KH_2PO_4, 0.1mM L-Ascorbate, 10mM Glucose, 0.1m Pargyline) up to 20 million platelets /ml. 100µl assay buffer containing $2x10^6$ platelets were pre-warmed in a bath at 37°C for 10 min. 100µl assay buffer with vehicle (0.1% DMSO) or anisomycin was then added for 5-30 min. 5HT uptake was initiated by adding 50 nM [^{3}H]5HT and after 10 min, the reaction was stopped by rapid filtration through GF/B. Whatman paper washing three times with ice-cold assay buffer. Radioactivity was counted as described above.

Section 2.6 Measurement of p38 MAP kinase phosphorylation in recombinant cell lines

Cells were placed in 6-well plates one day before the experiment at the concentration of $1x10^6$ cell/well. On the day of experiment, cells were treated with vehicle (DMSO final 0.1%) or compounds for 5-20 minutes in medium and then lysed with 100µl 1X Lysis buffer described above. In some experiment, cells were pre-treated with p38 MAPK inhibitor, SB203580 for 30 min before the

stimulation with drugs. All samples were then kept on ice for 20 min, centrifuged at 13000g for 20 minutes and finally analysed with phospho-p38 MAP Kinase ELISA kit (Cell Signalling) according to the instructions provided.

Section 2.7 LPS treatment and sample preparation for the ex vivo experiment

A intracerebroventricular cannula was implanted in Sprague Dawley rat (Charles river, 225-250g) to allow injection of LPS in the brain. After 1 week of recovery, rats were i.c.v. treated with vehicle (artificial cerebrospinal fluid, ACF, containing 125mM NaCl, 2.5mM KCl, 2.5mM $MgCl_2$, 1.7mM $CaCl_2$, 2mM $Na_2HPO_4*7H_2O$) or LPS from Escherichia coli serotype 0111:B4 (20µg/dose in 5µl ACF). After 2 hours, rat brains (four animals/group) were removed and cut into parasagittal halves. One half was dissected to prepare synaptosomes from cortex and striatum as described in the section 2.1 (protocol 1)Pre-frontal cortex, hippocampus and striatum were dissected from the other half, quickly frozen on dry ice and stored at -80°C to measure cytokine levels by multiplex technology (see section 2.7.1).

Section 2.7.1 Assessment of cytokine level in the brain tissue by multiplex technology

IL-1β, IL-6, IL-10, TNFα and IFNγ, levels were measured in cerebral homogenate with a Luminex instrument, using a multiplex suspension array technology. Basically, specific primary antibodies recognizing a cytokine are

conjugated with beads emitting different fluorescence. Once added to the samples, analyte and primary antibodies form immunocomplexes that are recognized as well by secondary antibodies (Detection antibody) which are coniugated with another fluorescent dye. The quantification was performed by the Luminex instruments, which identifies the type of beads and the fluorescence intensity of Detection antibody. Concentrations were obtained from fluorescence values by comparison with a standard curve. The assay was carried out using Bioplex instruments (Biorad). Kit and reagents were purchased from Millipore (MILLIPLEX MAP Rat Cytokine/Chemokine Panel, cat N° RCYTO-80K). Brain tissues were placed in a tube and weighed. 5µl lysis buffer (Saniquin) for mg of tissue was added to the tube andthen samples were homogenized with minipestles, (Blu PP pestle Cod. Z359947 Sigma, Cordless motor Cod. Z359971 Sigma), incubated for 10 min on ice and centrifuged at 13200 RPM at 4°C for 2 minutes. Supernatant was diluted 1:3 in assay buffer and 50µl/well were loaded on the kit plate in duplicate. The assay was performed following the instructions provided. Protein concentrations of samples were not detected and then cytokines concentration was not standarized to protein. Cytokines measured were IFNγ, TNFα, IL-1α, IL-1β, IL-6 and IL-10.

Section 2.8 Rat astrocytes preparation

Rat cortical astrocytes were obtained from twelve 2-3 day old Sprague-Dawley pups. Rat brains were dissected out and placed in Petri dish with cold HBSSH

buffer (pH 7.3) containing 5mM potassium chloride (KCl), 0.44mM potassium phosphate monobasic anhydrous (KH_2PO_4 an.), 137mM sodium chloride (NaCl), 0.34mM sodium phosphate dibasic anhydrous (Na_2HPO_4 an), 4.2mM sodium bicarbonate ($NaHCO_3$), 0.1% D-Glucose, 10mM Hepes, 100 U/ml penicillin and 100µg/ml Streptomycin. Under stereo microscope, peel off the meninges and transfer cortex to a tube containing cold HBSSH buffer. Cortices were minced with needle-sharp tweezers and transferred into three 50ml Falcon tubes containing 30ml of enzymatic dissociation solution (0.1% trypsin and 0.02% DNase I type IV, D5025, Sigma). The tube was incubated for 20 min at 37°C. After a centrifugation at 228g for 5 min, the pellet was filtrated through a cell strainer filter of 70µM (BD Falcon) into a new 50ml tube. Cells were centrifuged again and resuspended in medium 15% FBS (500ml of DMEM, 75ml of FBS, 5ml of Pen/Strep, 2.2ml of Glucose).

Cells were then placed in Poly-D-lysine (30000-70000) coated T175 flask (Nunc) for one night at 37°C, 5% CO_2. Medium was changed with new medium 15% FBS at 2 days *in vitro* (DIV) and 10%FBS at 5 DIV. Rat astrocytes were detached with 0.05% trypsin plus 10mM EDTA, centrifuged and resuspended in freezing medium (DMEM, 15% FBS, 10% DMSO). Purity of culture was not detected. Aliquots were then stored at -80°C. Rat astyrocytes used in the assay were placed in 96 or 6-well plate at the density of 700-800 cells/mm^2 and cultured for 12-14 DIV.

Section 2.8.1. Rat neuronal cultures preparation

Cortical and hippocampal neuronal cultures were obtained from embryos at day 18/19 of gestation (E18/19) of Sprague-Dawley rats. Rat brains were dissected out and hippocampi were quickly isolated at 4°C in HBSS pH 7.3 buffer containing 10mM Hepes, 100U/ml penicillin and 100µg/ml Streptomycin (HBSS buffer). Hippocampal and cortical cells were treated with HBSS buffer added with Trypsin 0.1% at 37°C for 10 min. In the last 5 min of incubation, cortical cells required an addition of 166µg/ml of DNAse I which facilates the cell dissociation. After a single wash with HBSSH buffer containing 10% FBS and two additional washes with HBSSH buffer, cells were mechanically dissociated by triturating with Pasteur pipets. Cells were than placed in Poly-L-lysine (P9404, MW >30000 kDa) coated 12- or 96-well plates at the density of 700-800 cells/mm^2 in serum-free Neurobasal medium supplemented with B27 supplement, 500µM Glutamine, 100U/ml penicillin and 100µg/ml Streptomycin. In the hippocampal neurons, medium was supplemented with 12.5µM glutamate (Sigma, St. Louis, MO, USA). Cells were grown at 37°C, 5% CO_2 and half the volume of the medium was changed after 4 days with complete medium without glutamate. In all assays, neuronal primary cultures were used after 8-10 days in culture. All reagents were purchased from Gibco/Invitrogen (Paisley, UK) except those in which supplier were indicated.

Section 2.8.2. Purity of cultured neuronal preparations

Purity of cultured neuronal cells was assessed by immunoflorescence. Cells were grown on 12-well plate in coverslips at the density of 500 cell/mm^2 under normal cultured conditions (see above).Onthe day of analysis the cells were processed at room temperature as described below. Cultured medium was aspirated, washed once in a small volume of PBS buffer and then incubated for fixation in PBS containing 4% paraformaldehyde and 4% sucrose for 20 min. Cells were then washed for 5 min in PBS and then incubated in PBS with 5% FBS (quenching solution) for 30 min for blocking non-specific binding sites. In order to allow the permeabilization of antibodies, cells were incubated with PBS containing 0.1% Triton X-100 and 5% FBS for 10 min and then washed for 5 min with quenching solution. Cells were incubated with astrocyte primary antibodies anti-GFAP (Sigma G9269, 1:1000, from rabbit) or neuron-specific nuclear protein primary antibodies anti-NeuN (Chemicon, MAB377, 1:200, from mouse) diluted in quenching solution for 1h, Unbound antibodies were eliminate by three washes of 5 min and then secondary antibodies anti-rabbit AlexaFluor 594 (Molecular Probes/Invitrogen, 1:1000, from donkey) or anti-mouse AlexaFluor 488 (Molecular Probes/Invitrogen, 1:1000, from goat) was diluted in quenching solution and added for 45 min. After two washes with quenching solution and one with distilled water, coverslips were mounted with one drop of VECTASHIELD® Mounting Medium with DAPI (H-1200, Vector Laboratories,UK) and closed with nail polish. In order to quantify the purity of culture, 5 fields per coverslip are acquired using LEICA microscope (IM 50

program) and 20x objective. For each field, 3 pictures (blue, green and red channels) are acquired with the following parameter settings:

DAPI (blu channel): exposure time 56.4 msec, gain/offset threshold: 26/1.17/6
NeuN (green channel): exposure time 11 sec, gain/offset threshold: 22/1.10/9
GFAP (red channel): exposure time: 1.1 sec, gain/offset threshold: 45/0.58/9

The number of cells positive for each of the three markers was manually counted from the acquired pictures.

Section 2.9. RNA extraction and quantification

Total mRNA from primary neurons and astrocyte was extracted using RNeasy mini kit from Qiagen (Qiagen Inc, Valencia, CA, USA). Briefly, cells were grown in 12-well plate in normal conditions. Medium was aspirated and cells were washed two times with ice-cold PBS. 350µl RLT buffer was added to cells for the lysis. 350µl of 70% ethanol was added to cell lysate which was then putted in a RNeasy spin column. Samples were centrifuged for 15sec at 10000xg. 700µl Buffer RW1 was added to the RNeasy spin column and centrifuged for 15sec at 10000xg to wash the spin column. The RNeasy spin column was washed two other times with Buffer RPE using the same procedure described previously and finally mRNA was eluated with 50µl Rnase-free water. Genomic DNA contaminants were removed from each samples using an RNAse-free Dnase I supplied by Applied Biosystem (Foster City, CA, USA). Briefly, samples

were incubated with RNAse-free Dnase I for 30min at 37°C. Samples were then centrifuged at 16000xg for 5 min and the pellet was resuspended in 50µl Rnase-free water. Purified RNA was quantified using an Agilent 2100 Bioanalyzer following the Agilent RNA 6000 Nano kit protocol (Agilent Technologies Inc., Santa Clara, CA, USA). Briefly, the gel was prepared by mixing 65µl filtered gel with 1µl of dye and the tube was centrifuged at 13000Xg for 10 min at room temperature.9.0 µl of gel-dye mix was added to the RNA 6000 Nano chip. 5 µl of RNA 6000 Nano marker was loaded in all sample wells following by 1µl of sample in each well. The chip was now ready to quantify the mRNA by the Agilent 2100 bioanalyzer within 5 min.

Section 2.10 Reverse Transcribed PCR

Complementary DNA (cDNA) was generated by reverse transcription of 100ng of total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystem). The reaction consisted of 1X RT buffer, 2.5mM $MgCl_2$, 1mM dNTPs, 2.5µM random hexamers, 0.4U/µl RNase Inhibitor, 1.25U/µl MultiScribe RT. The mixture was incubated consecutively at 25°C for 10 min, 48°C for 30 min and finally 95°C for 5 min. PCR was carried out in a reaction volume of 30µl using HotStarTaq(R) PCR from Qiagen using this protocol: 10ng cDNA, 1X PCR buffer, 200µm dNTP's, 300nM Primers, 1 unit Taq in Dnase-free water. After an initial incubation of 15 min at 95°C, 40 cycles of amplification were performed consisting of 30 sec denaturation at 95°C, 30 sec annealing at 60°C

and 40 sec extension at 72°C. The reaction was terminated with a final extension of 10 run min at 72°C. Primer pairs were reported in Table 3.

Table 3. Primers for $5HT_7$, $5HT_6$ and $5HT_4$ receptors

Gene	Sequence
$5HT_7$	Forward: ATCTTCGGCCACTTCTTCTGCAACG Reverse: CAGCACAAACTCGGATCTCTCGGG
$5HT_6$	Forward: CCATCTGCTTCACCTACTGC Reverse: TCTGAATCTGAGTTTGGCGG
$5HT_4$	Forward: TTGGCTGCTTTGGTCTCTGTCCGC Reverse: TGCAAGGCTGGAACAACATCGGC

PCR products were separated electrophoretically with 1.5% agarose gels. Agarose 1.5% in 1X TAE buffer (Tris 40mM, 1mM Glacial acetic acid, 0.1% EDTA pH 8) was heated in microwave until fully dissolved. After a brief cooling, a 1X SYBR safe DNA gel stain (Invitrogen) was added that binds to the DNA and allows visualization under UV illumination. Samples were mixed with a 6X DNA loading buffer composed by Bromofenol Blue 0.25% w/v, Xylene cyanol FF 0.25% w/v and Ficoll (Type 400) 15% and ran alongside DNA molecular weight markers (100bp ladder, Invitrogen) for 1 hour at 100V.

Section 2.11 cAMP assay

Intracellular cAMP accumulation was measured in neurons using cAMP Hi Range kit supplied by Cisbio (Bagnols-sur-Cèze, France). This assay is based on two fluorophores, a donor (Europium cryptate) and an acceptor (d2).

Excitation of the donor by an energy source (e.g. flash lamp or fluorometer laser) triggers an energy transfer to the acceptor if they are within a given proximity to each other. The acceptor in turn emits light at its given wavelength. This phenomenon is called FRET (Fluorescence Resonance Energy Transfer). In this specific case, the assay is a competitive immunoassay using cryptate-labeled anti-cAMP and d2-labeled cAMP. When cAMP is present in solution, it displaces the d2-labeled cAMP determining a decrease of emit light. A standard curve with cAMP solutions of known concentrations is run in parallel. The protocol described by suppliers was adapted to neurons cultured in adhesions. Neurons cultured in 96 well plates were incubated with vehicle (0.1% DMSO) or antagonists for 15 min at 37°C in medium before an addition of 5HT or forskolin (FSK) in the presence of 500µM 3,7-Dihydro-1-methyl-3-(2-methylpropyl)-1H-purine-2,6-d ione (IBMX). After 30 min, medium was fully replaced with 100µl lysis buffer containing cryptate-labelled anti-cAMP and d2-labelled cAMP. After 3 hours, europium cryptate fluorescence and time-resolved fluorescence emitted from acceptor were measured 400µs after excitation at 340 nm at both 615 and 665 nm using a Victor V fluorimeter (Perkin Elmer). Results were calculated from the 665nm / 620nm ratio and expressed in Delta F% following this equation:

$$\text{DeltaF\%} = \frac{\text{Sample ratio} - \text{Ratio neg}}{\text{Ratio neg}}$$

Where “sample ratio” was the 665nm/620nm ratio of each sample and “ratio neg” was the 665nm/620nm ratio calculated in wells where d2-labeled cAMP was not added. Sample cAMP concentrations were calculated from a standard curve.

Section 2.12 Analysis of expression, phosphorylation and transport of expressed protein

Section 2.12.1 Treatment and sample preparation

Cultured neurons and astrocytes grown in 12-well or 6 well plates were treated with different compounds in medium at 37°C for the indicated time points. After a brief wash with ice-cold phosphate buffered saline (PBS, Invitrogen), cells were scraped into 100µl/well (12 well) or 200µl/well (6 well) Lysis Buffer containing 10mM Tris (pH 7.4), 100mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 20mM $Na_4P_2O_7$, 2mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% Sodium dodecyl sulfate (SDS), 0.5% deoxycholate, 1mM phenylmethanesulfonyl fluoride (PMSF), one tablet of complete mini protease inhibitors (Roche, Indianapolis, IN, USA) and 1% phosphatase inhibitors I and II (Sigma). Cells lysates were transferred to tubes and kept at 4 °C for 30 min. Lysates were then centrifuged for 10 min at 13,000 x *g*, supernatants were transferred to new tubes and stored at -80°C. Samples were analysed by western blot to quantify total and phosphorylated protein expression (see section 2.12.4 and 2.12.5.).

Section 2.12.2. Cell surface protein isolation

Protein present at the cell surface can be purified using EZ-link sulfo-NHS-SS-Biotin, a thiol-cleavable amine-reactive biotinylation reagent. Basically, this procedure uses a cell-impermeable, cleavable biotinylation reagent (Sulfo-NHS-SS-Biotin) to label exposed primary amines of proteins on the surface of intact cells. Treated cells are then harvested, lysed and the labeled surface proteins are affinity-purified using the capacity of biotin to bind streptavidin and avidin with high specificity.

In this biotinylation procedure, cultured neurons grown in 6-well plates were transferred onto a pre-chilled platform and all subsequent steps were conducted at 4ºC. Cells were washed with ice-cold PBS and then incubated for 20min with 1mg/ml biotin (EZ-Link Sulfo-NHS-SS-Biotin; Pierce, Rockford, IL, USA) dissolved in PBS. After three rinses in ice-cold 50mM glycine in PBS to quench biotin, cells were rinsed in PBS and then scraped in 200µl/petri dish of homogenization buffer (0.1% SDS, 0.5% NP40, 0.5% sodium deoxicolate, 150mM NaCl, complete mini protease inhibitors, phosphatase inhibitors cocktail I and II in PBS, pH 7.4). Homogenates were centrifuged at 10,000 x *g* for 20min to pellet the insoluble fraction. The supernatant (total extract) was analyzed for total protein content with a Micro BCA kit (Pierce). For purification of the biotinylated surface protein fraction, 50µg of total proteins were incubated with 80µl of 50% slurry Neutravidin resin (Pierce) for 2h at 4ºC, by using spin columns with top and bottom caps. After washings with homogenization buffer (1min centrifugation of spin columns at 1,000 x *g* at 4ºC for three times),

biotinylated surface proteins were eluted by application of 100µl of elution buffer (Invitrogen NuPage LDS sample buffer 4x diluted to 1x with H_2O and added with 50mM ditiothreitol as reducing agent) to the resin, heating at 95ºC for 5 min, and centrifugation for 2min at 1,000 x g. Eluted fractions represented the purified biotinylated surface proteins (surface fraction) and were ready for gel loading (see section 2.12.4 and 2.12.5.).

Section 2.12.3. Protein sample quantification

Section 2.12.3.1. Bradford standard method

The protein concentration of samples was determined using Bio-rad reagent (Bio-Rad, UK) thet was based on an assay first described by Bradford in 1976 (Bradford 1976, Anal Biochem. May 7;72:248-54. Bio-rad reagent was diluted 1:5 with ddH20, and filtered through chromatography paper (Whatman, UK) to remove particulate matter. Bovine serum albumin (BSA) was diluted to 0, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml with H_2O for use as protein standards. Filtered Bio-rad reagent (200µl) and 4µl of each sample were added to well of 96-well plate. The plate was mixed and incubated for 10 minutes at room temperature in a dark place. Coomassie® Brilliant Blue G-250, included within the reagent, shifts absorbance from 465 nm to 595 nm in the presence of protein. Absorbance was measured at 595 nm in a spectrophotometer (Spectramax 400) and BSA standards used to calculate a protein concentration/absorbance curve. Protein

concentration of each sample was calculated from the BSA standard curve, from the absorbance reading at 595 nm.

Section 2.12.3.2. Micro BCA

An alternative assay was used to determine protein concentration when protein concentration was very low or alternatively when small volumes were available. It was based on the micro BCA kit (Pierce). Micro BCA working reagent was prepared by mixing 25 parts of Micro BCA reagent MA, 24 parts Reagent MB with 1 part of Reagent MC. Bovine serum albumin (BSA) was diluted to 0, 0.7, 1.5, 3, 6, 12, 25 and 50µg/ml with H_2O for use as protein standards. Micro BCA Reagent (150µl) and 150µl of each sample were added to a well of a 96-well plate. The plate was mixed and incubated for 2 hours at 37°C in a dark place. Bicinchoninic acid included within the reagent has a strong absorbance at 562 nm in the presence of protein. Absorbance was measured then at 562 nm in a spectrophotometer and BSA standards used to calculate a protein concentration/absorbance curve as above.

Section 2.12.4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein were separated according to the molecular weight using dodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The equipment used to run this assay was a XCell SureLock™ Mini-Cell (Invitrogen)

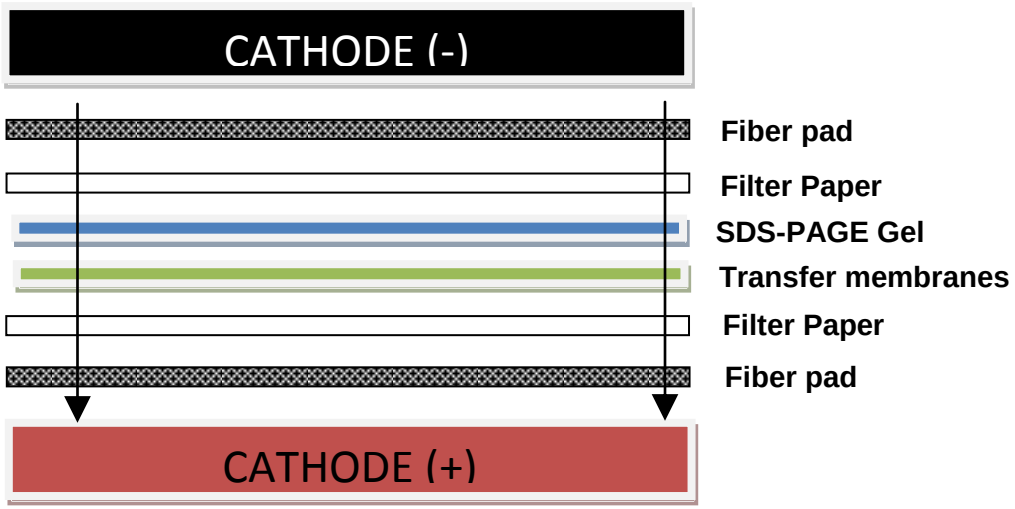
which allows SDS-PAGE to run in pre-cast mini-gels. The gels usedwere 1.5 mm 4-12% Novex Bis-Tris Pre-Cast Mini Gels (Invitrogen).

Equal amounts of cell lysates (~6-10µg protein) prepared in an equal volume of 4X NuPAGE® LDS Sample Buffer and 10X NuPAGE® Sample Reducing Agent, were boiled for approximately 10 minutes, and centrifuged briefly to remove particulates prior to loading; 25-30µls for 10 well combs, 15-20µls for 15 well combs. Prestained protein molecular weight markers (Novex® Sharp Pre-stained Protein Standard) were loaded (10µl) to visualise migration, and identify approximate the molecular weight of protein bands. Electrophoresis was performed at 50V for 1 hour per gel.

Section 2.12.5. Western blot

To detect specific proteins resolved in polyacrylamide gels, polyclonal or monoclonal antibodies were used to label the antigenic region of the target protein. After separation, proteins were transferred to polyvinylidene difluoride membranes (Amersham plc., Buckinghamshire, UK) using a Mini Trans-Blot module (Bio-rad). Polyvinylidene fluoride (PVDF) was used during this study which can retain approximately 170µg protein/cm^2 (Millipore). Pre-soaked fiber pads and correctly sized Whatman paper were stacked ensuring air bubbles did not form. One fiber pad, and one papers were layered, before polyacrylamide gel (soaked briefly in transfer buffer) was placed on the stack, followed by the PVDF membranes and one fiber pad and one Whatman papers. Proteins

transferred towards the anode; an optimal transfer required 40-50V for approximately 2 hour (see diagram below).



Transfer Buffer pH 8.3	
Tris	25mM
Glycine	192mM
Methanol	20%

Table 4. Constituents of Western blot transfer buffer (stored at room temperature).

Before incubation with primary antibody, PVDF membrane was placed in blocking buffer, for approximately 1 hour at room temperature to reduce non-specific background binding.

10x Tris-Buffered Saline (TBS) pH 7.6	
Tris	0.5 M
NaCl	2.8 M
KCl	27 mM

Table 5. Constituents of Western blot wash buffer. N.B. 1 ml of Tween 20 was added to the 1 litre of 1x TBS to make TBS-T. 5% low-fat dry milk was added to TBS-T to make blocking buffer (stored at room temperature).

Blocking buffer was Tris Buffered Saline (Table 5) added with 5% low fat skimmed milk and 0.1% Tween-20. Membranes were subsequently processed with primary antibodies rabbit anti-GluR1 Ser845P (Sigma A4477; 1:1000), rabbit anti-GluR1 (Millipore #04-855; 1:1000), rabbit anti-pan Cadherine (Abcam, Ab6528, dilution 1:1000) and mouse anti-GAPDH (SC-32233 from Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:10000). Membranes were washed three times in 0.1% Tween-20 in TBS (10 min every wash) and reincubated with the peroxidase-conjugated secondary antibodies goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP for 1 hour (both from Santa Cruz; 1:5000). The chemiluminescence reaction was developed with ECL Plus (Amersham) and detected with Luminescent Image Analyzer LAS-4000mini (Fujifilm, Tokyo, Japan). Optical densities were determined by densitometric

analysis with Quantity One software (Bio-Rad Laboratories Inc., Hercules, California, USA).

Section 2.12.6. Measurement of levels of total CREB, phospho CREB[133] and phospho p38 MAP kinase using ELISA kit

Neurons cultured in 12 well plates were treated with antagonist or 0.1% DMSO for 15 min at 37°C in medium before the addition of agonist or forskolin. After stimulation, medium was aspirated and cells were scraped in to a lysis buffer containing 10mM Tris (pH 7.4), 100mM NaCl, 1mM EDTA, 1 mM EGTA, 1mM NaF, 20mM $Na_4P_2O_7$, 2mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1mM PMSF, protease inhibitors (1 table in 10ml, ROCHE) and 1% phosphatase inhibitors I and II (Sigma). Cells lysates were transferred in tubes and kept at 4 °C for 30 min. Lysates were then centrifuged for 10 min at 13000 g, supernatants were transferred into new tubes and stored at -80°C. ELISA kits were used to quantify total CREB and phospho CREB at serine 133 were supplied by Invitrogen (Cat#KHO0241), while those used for phospho p38 MAP kinase at tyrosine 180/182 were from Cell Signaling. Briefly, samples containing CREB [pS133] or CREB protein were diluted with Standard Diluent Buffer 1:10. Plates were covered and incubated 2 hours at room temperature. The liquid in each well was then aspirated following by four washings. After the addition of 100 µL of CREB [pS133] Detection Antibody solution into each well, the plate was incubated for 1 hour at room temperature. Liquid was aspirated and wells were washed as before. 100 µL Anti-Rabbit IgG HRP Working Solution was added to each well and incubated for 30 min at room temperature.

Liquid was aspirated and wells were washed as before. 100 µL of Stabilized Chromogen was added to each well and the liquid in the wells turned blue. After an incubation of 30 minutes at room temperature, in the dark, 100 µL of Stop Solution was added and absorbance of each well was read at 450 nm. Samples containing phospho p38 MAP kinase, were diluted with Sample Diluent Buffer 1:10 and processed ad described in the section 2.2.

Section 2.13. FLIPR/$[Ca^{2+}]_i$ assay

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurements were made using the FLIPR TETRA fluorescent imaging plate reader (Molecular Devices, Sunnyvale, CA). On the assay day, cultures were washed two times with Physiological Salt Solution (PSS; composition 145mM NaCl, 1g/L glucose, 5mM KCl, 2 mM $CaCl_2$, 20mM HEPES) supplemented with 2.5mM probenecid (Sigma-Aldrich). Then, cells were added with the cytoplasmic calcium indicator, Fluo-4 (Invitrogen) in the acetoxylmethyl ester form (2µM) in PSS containing 5mM probenecid and 0.04% Pluronic F-127 (Sigma-Aldrich). After 40 min at 37 °C, cells were washed 3 times as above and treated with compounds at 37 °C for different time points. When $[Ca^{2+}]_i$ responses were measured, plates were transferred to the FLIPRTETRA equipped with a cooled CCD camera with excitation at 470–495nM and emission at 515–575nM. Camera gain and intensity were adjusted for each plate to yield a minimum of 300 relative light units (RLU) baseline fluorescence. To assess the effect of 5HT and FSK receptor antagonists on the AMPA-triggered $[Ca^{2+}]_i$ response, cultures were exposed to different

concentrations of 5HT and FSK for 10 min, then exposed to increasing concentrations of AMPA for 120s. Prior to addition of stimulators, 5 fluorescence readings were taken to measure baseline. $[Ca^{2+}]_i$ responses were quantified as area under the curve of the time-response data over baseline.

Section 2.14. Compounds

Details of agonists and antagonists used across these assays were reported in the table below:

Compunds	Supplier*	Catalog number	Specificity	Reference
5HT	Sigma-Aldrich	H9523	Selective 5HT receptors agonist	Mahe et al, 2004
SB258719	Tocris,	2726	Selective 5-HT_7 antagonist	Thomas et al., 1999
GR113808	Tocris	1322	Potent and selective 5-HT_4 receptor antagonist	Mikami et al., 2008
SB399885	Tocris	3189	Potent and selective 5-HT_6 receptor antagonist pA_2= 7.85±0.04	Hirst et al., 2007
AS19	Tocris	1968	Potent 5-HT_7 receptor agonist, IC_{50} = 9±1 nM	Brenchat et al., 2009
Anisomycin	Tocris	1290	Protein synthesis inhibitor (blocks translation). Potent activator of stress-activated protein kinases (JNK/SAPK) and p38 MAP kinase	Xiong et al., 2006
SB203580	Tocris	1402	Selective inhibitor of p38 MAP kinase	Kumar et al., 1999
Rolipram	Tocris	0905	Selective PDE4 inhibitor Selective inhibitor of cAMP phosphodiesterase PDE4, IC_{50} = 2.0 µM	Teixeira et al., 1997
Papaverine	Sigma-Aldrich	P3510	Unselective PDE10 inhibitor	Nihsi et al., 2008
H89	Tocris	2910	Protein kinase A inhibitor that also inhibits several other kinases (IC50 values are 80, 120, 135, 270, 2600 and 2800 nM for S6K1, MSK1, PKA, ROCKII, PKBα and MAPKAP-K1b).	Chijiwa et al., 1989

*Tocris= Tocris Bioscience, Ellisville, MO, USA, Sigma Aldrich= St. Luise, MO, USA

Section 2.15. Data Analysis

Statistical analysis of the data was performed using STATISTICA 8 (Statsoft, Inc., Tulsa, OK, USA). The model used to analyse uptake, ELISA and densitometry data was an ANOVA test analysis of variance in which the different treatment groups were compared. These data were expressed as percentage or folds respect to the control. As post hoc analysis, a Dunnett's test was performed. In addition, when two groups needed to be compared, planned comparison of Least Squares means (LSD method) was performed. Transformation of average into square root was deemed necessary in order to improve compliance with normality assumption required for the mixed model. P-values lower than 0.05 are considered statistically significant.

Drug concentration-response curves from cAMP and FLIPR assay were fitted to a four parameter logistic equation using GraphPad Prism 5 (GraphPad Software Inc, San Diego, CA). Agonist potency was expressed as pEC_{50} (-log EC_{50}). The inhibition of antagonists was expressed as the pIC_{50} (–logIC_{50}), where IC_{50} represents the concentration of drug inhibiting 50% of agonist maximal response. pK_B values for antagonists were determined using the equation: pK_B =(-log([antagonist]/(concentration ratio-1)) where concentration ratio = ratio of the agonist EC_{50} in the presence and in the absence of antagonist.

Chapter 3 Results

Section 3.1 Effect of interleukin 1ß (IL-1ß) and p38 MAP kinase activator, anisomycin, on SERT function in rat cortex synaptosomes

The assay adopted to measure if the pro-inflammatory cytokine IL-1ß could modulate the functionality of SERT in rat native tissue was an *in vitro* [^{3}H]5HT uptake assay. This was performed in cerebral cortex synaptosomes due to the relatively high expression of SERT in this brain region, which improves the specific to non-specific signal. A basic pharmacological characterization of SERT was performed by measuring the uptake rate after incubation of increasing concentrations of 5HT. Cerebral cortex synaptosomes were prepared as described in protocol 1 (section 2.1), according to Thomas *et al.,* (1987). The saturation curve fitted to a one-site hyperbola, giving K_m and V_{max} values of 37.2 ±6.0 nM (n=3) and 0.68 ± 0.11 fmol/µg/min (n=3), respectively (Figure 1).

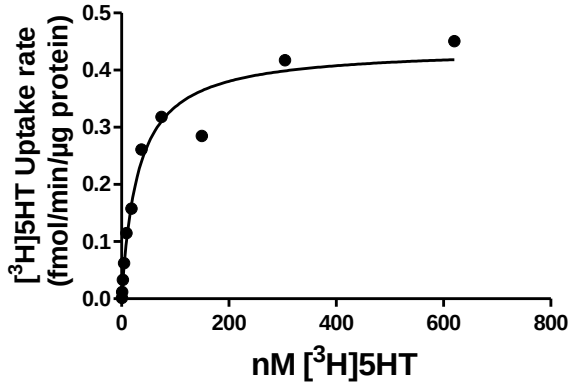


Figure 1. Representative saturation 5HT uptake curve on rat cerebral cortex synaptosomes. *The curve represents the specific [^{3}H]5HT uptake measured in a single animal in triplicate. 5HT was incubated for 10 min before stopping reaction by rapid filtration Non specific uptake was identified by the addition of citalopram (10 µM). Experiment was replicated three times.*

In order to verify if IL-1ß could modulate SERT function, 10 or 200 ng/ml IL-1ß were incubated with rat cerebral synaptosomes over incubation times of 10-30 minutes and then [^{3}H]5HT uptake rate was measured (figure 2).

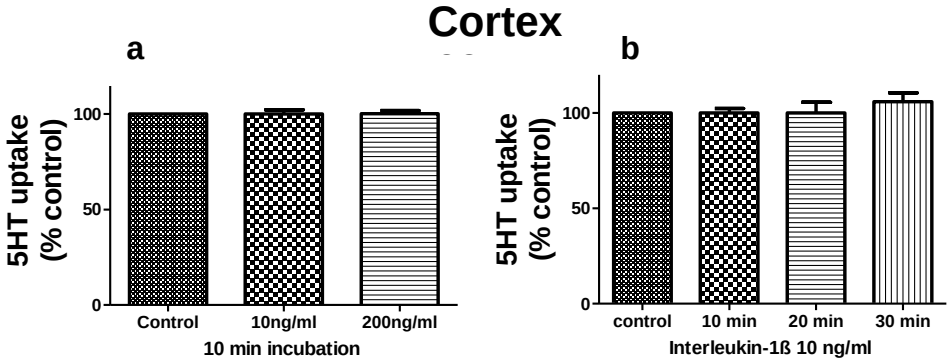


Figure 2. ***5HT uptake on rat cortex synaptosomes in the presence of IL-1ß.*** *(a) increasing concentrations of IL-1β (10, 200 ng/ml) incubated for 10 min. (b) 10 ng/ml IL-1β incubation at different end points (10, 20, 30 min). Values are expressed as the mean of three independent experiments ± SEM (n=3; one-way ANOVA).*

Data analysis showed that IL-1ß did not produce any effect on 5HT uptake, suggesting that IL-1ß was not involved in the regulation of SERT function under these assay conditions. Since the activation of SERT seems to be mediated by p38 MAP kinase (Zhu et al., 2005), which is also a main mediator of cytokine responses (Roux and Blenis, 2004), a further study was performed using a direct activator of p38 MAP kinase, anisomycin; increasing concentrations of anisomycin in a range that showed an activation of 5HT uptake in previous

study (Zhu et al., 2004), were incubated for 5, 10, 20 minutes and impact upon [^{3}H]5HT uptake was assessed.

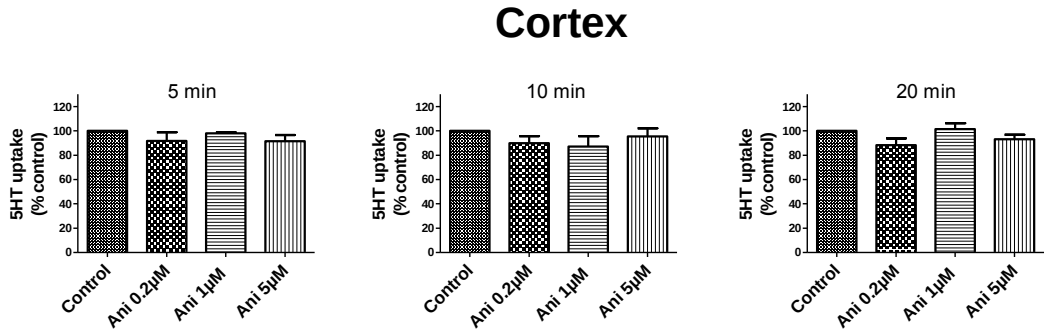


Figure 3. ***5HT uptake on rat cortex synaptosomes in the presence of anisomycin incubated for different time periods.*** *0.2, 1, 5 µM anisomycin was incubated for 5, 10, 20 min with synaptosomes before measuring 5HT uptake. Values are expressed as the mean± SEM of three experiments (n=3; one-way ANOVA).*

Graphs reported in Figure 3 showed that anisomycin did not produce any significant effect at all conditions tested. This finding was not in agreement with literature in which an activation of p38 MAP kinase induced by IL-1ß showed an increase of 5HT uptake in mouse synaptosomes (Zhu *et. al.* 2006).

In an attempt to produce comparable data with those reported in the literature where SERT activation was observed, the experiments were replicated following the same experimental method (protocol 2, method section) described by Zhu *et al.,* (2006). Moreover, in the literature, it was reported that inhibition of basal p38 MAP kinase activity induces SERT down-regulation in synaptosomes (Samuvel et al., 2005). In this group of experiments therefore, not only anisomycin but also the p38 MAP kinase inhibitor, SB203580, was assessed for impact on 5HT uptake.

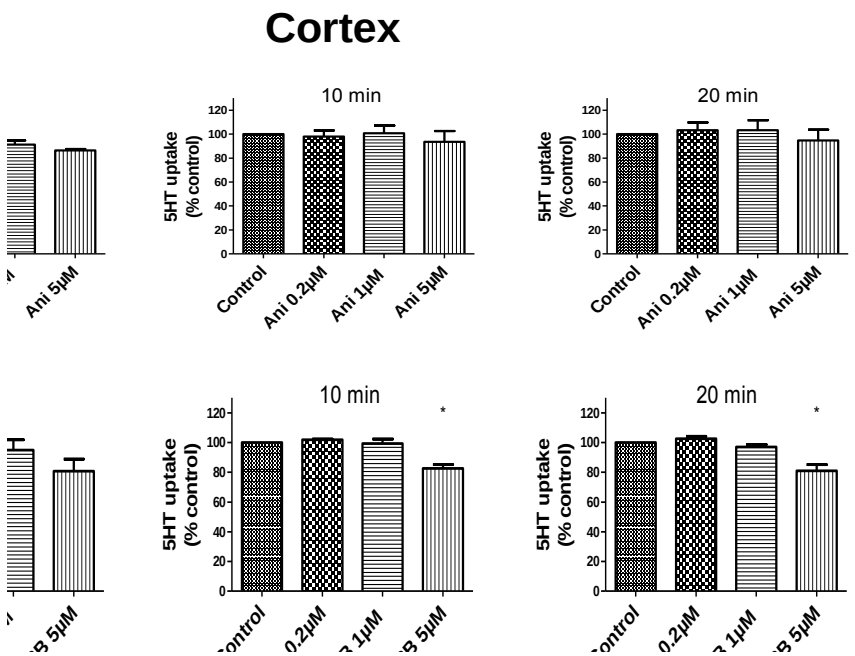


Figure 4. ***5HT uptake on rat cortex synaptosomes in the presence of anisomycin and SB203580 incubated at different end points.*** *0.2, 1, 5 μM anisomycin and SB203580 were incubated for 5, 10, 20 min with synaptosomes before measuring 5HT uptake. Values are expressed as the mean±SEM of three experiments (n=3; one-way ANOVA;* LSD method *Control Vs SB 5μM *P<0.05).*

No impact upon 5HT uptake, however, was identified by anisomycin incubation, confirming a lack of effect of p38 MAP kinase activation on SERT activity in rat cortex irrespective of the methodology used. In contrast, a slight but significant reduction of 5HT uptake was detected when SB203580 was tested at the concentration of 5 μM. This last piece of data was in agreement with literature results suggesting that constitutive activation of p38 MAP kinase may maintain SERT basal activity: hence when this activation was inhibited by SB203580 a reduction in SERT function was observed.

Section 3.1.1 Effect of anisomycin on 5HT uptake in mouse brain synaptosomes

Rat brain synaptosomes treated with anisomycin did not show any modulation of SERT function. However, literature reported an increase of 5HT uptake rate when mouse brain synaptosomes were treated with anisomycin (Zhu et al., 2007). In order to understand if the lack of effect was due to species differences in the modulation of SERT, the rate of 5HT uptake was also measured in mouse brain synaptosomes with or without anisomycin treatment, using identical assay conditions and brain areas (striatum, midbrain) as described (Zhu et al., 2007).

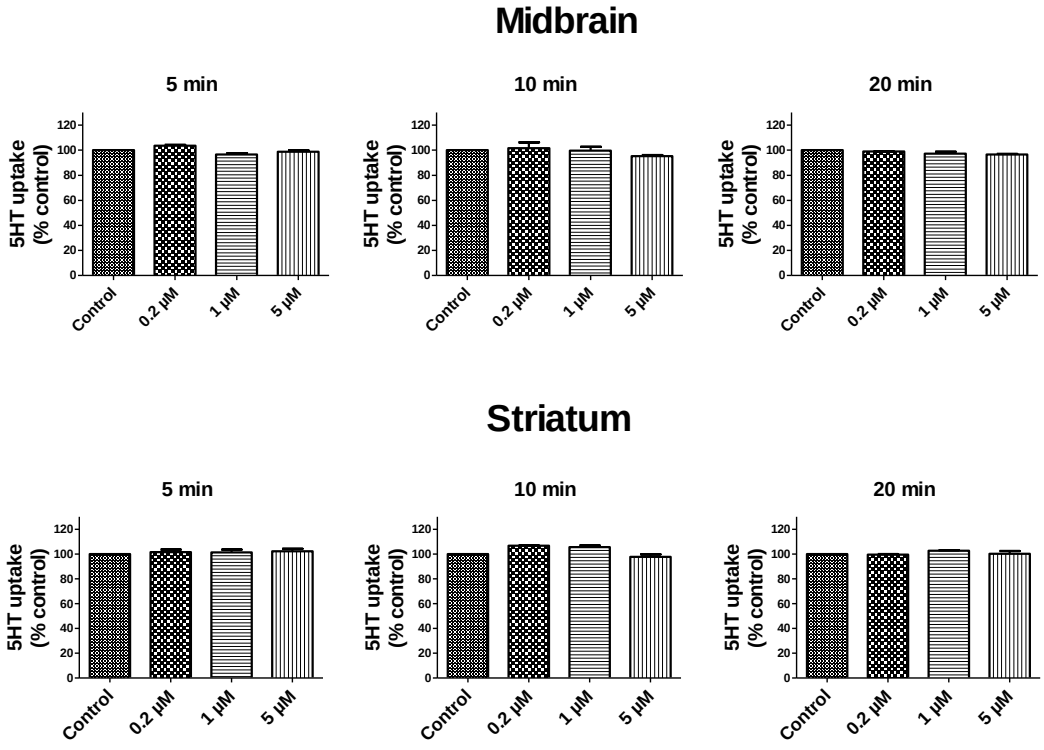


Figure 5. ***5HT uptake on mouse brain synaptosomes incubated with anisomycin at different end points.*** *0.2, 1, 5 µM anisomycin was incubated with synaptosomes for 5, 10, 20 min before measuring 5HT uptake. Values are expressed as the mean±SEM of three experiments (n=3; one-way ANOVA)*

No effect on 5HT uptake was detected in the presence of increasing concentrations of anisomycin in mouse synaptosomes with 5 to 20 min pre-incubations (Figure 5).

In the attempt to understand the reasons that led to the lack of effect of anisomycin or IL-1β on SERT function, further experiments were performed using striatal synaptosomes of individually-housed mice (Figure 6). These studies aimed to clarify if animal housing conditions, provoking a stress, could interfere with cytokine or anisomycin modulation of 5HT uptake.

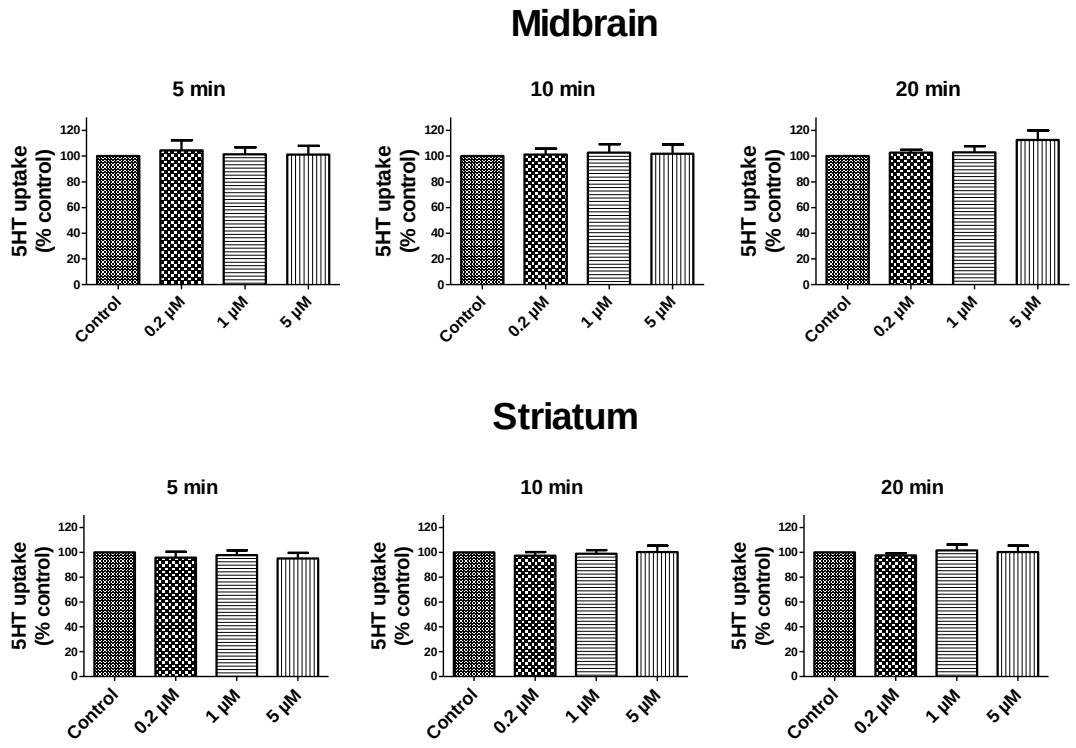


Figure 6. ***5HT uptake in synaptosomes of single housed mice pre-incubated with anisomycin at different end points.*** *1 µM anisomycin was incubated with synaptosomes for 5, 10, 20 min before measuring 5HT uptake. Values are expressed as the mean±SEM of three experiments (n=3; One-way ANOVA)*

In these conditions, no significant effect on 5HT transport was observed as well suggesting that housing conditions did not influence the results.

Section 3.1.2 Influence of interleukin 1ß and anisomycin on p38 MAP kinase activation in rat synaptosomes

Data reported demonstrated that IL-1ß and p38 MAP kinase did not influence SERT function in rat and mouse synaptosomes. The activated form of p38 MAP kinase has a dual phosphorylation in two tyrosine residues (Tyr 180 and Tyr 182). This form is able to phopshorylate and then activate a wide range of substrates that can be classified into protein kinases (MK2, MK3, MNK1/2, PRAK) and transcription factors (ATF1/2/6, Sap1, CHOP etc.). The level of activated p38 MAP kinase can be easily quantified by using specific antibodies that recognize this double phosphorylation. p38 MAPK activation has been shown to occur in response to extracellular stimuli such as UV light, heat, block of protein synthesis, osmotic shock, pro-inflammatory cytokines (TNF- & IL-1) and growth factors (CSF-1) (Roux PP and Blenis J, 2004). In order to assess if p38 MAP kinase could be activated in synaptosomal preparations, rat synaptosomes from cortex and striatum were incubated with IL-1ß (10 ng/ml) or anisomycin (1 µM) for 10, 20, 30 minutes and then phospho-p38 MAP kinase was quantified using an ELISA method. As a positive control, phospho-p38 MAP kinase was also measured in HeLa cells treated with 50 ng/ml IL-1ß or 1 µM anisomycin for 20 minutes. HeLa cells were recommended by the ELISA kit supplier as a cell line where anisomycin treatment results in high activation of p38 MAP kinase.

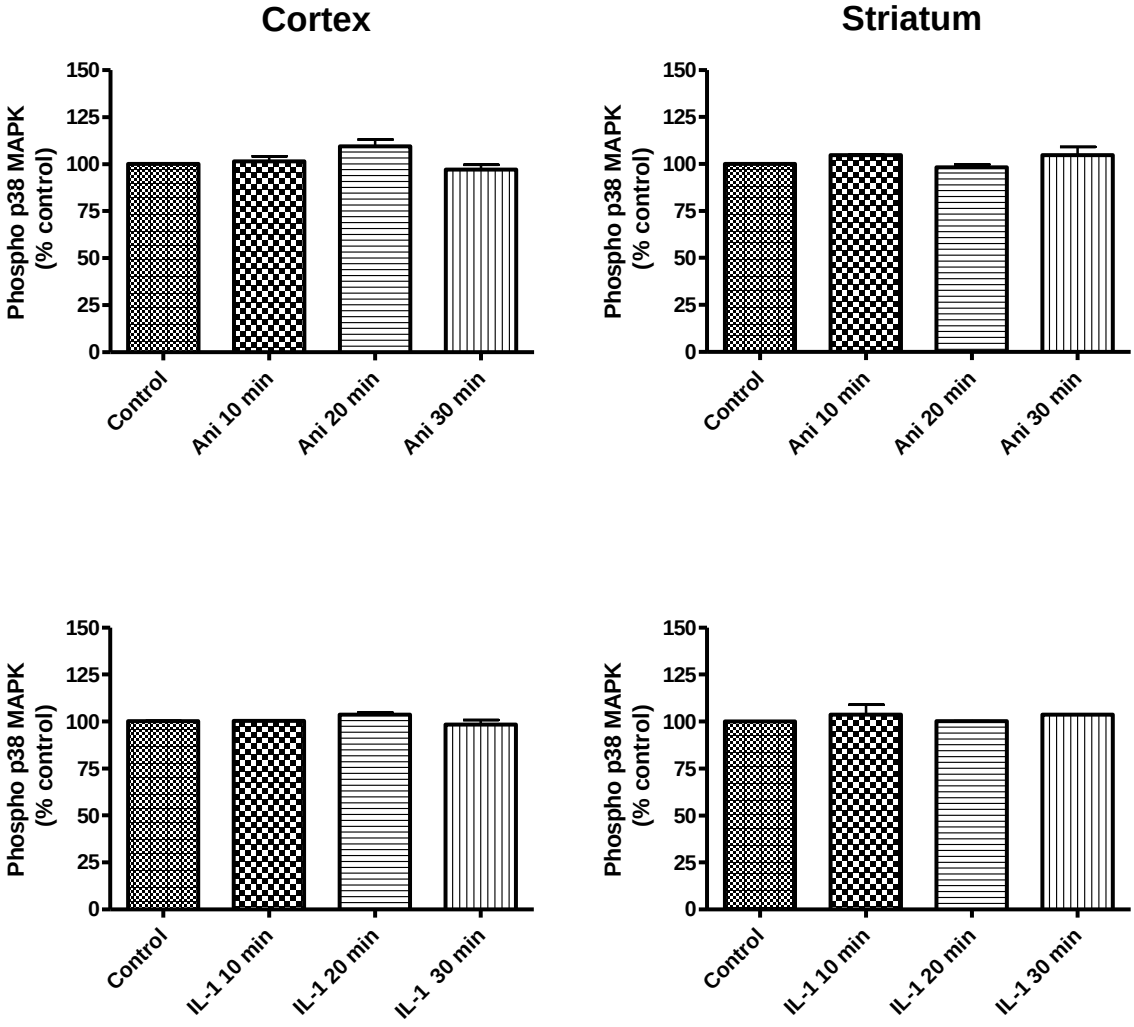


*Figure 7**. Measurement of phospho-p38 MAP kinase in rat synaptosomes from cortex (n=3) and striatum (n=2)** treated with 1 µM anisomycin or 10 ng/ml IL-1ß over an incubation time of 10-30 minutes. Data are expressed as mean±SEM (cortical) or mean±range (striatal) of % activated p38 MAPK Vs control. No significant difference between samples (One-way ANOVA)*

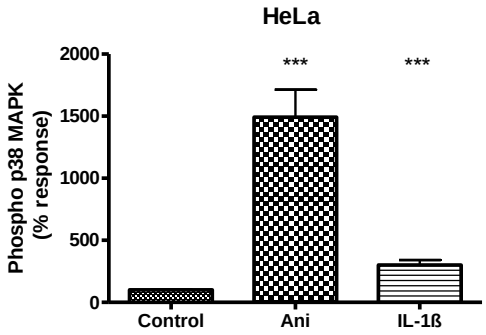


Figure 8. ***Measurement of phospho-p38 MAP kinase in HeLa cells*** *treated with 1 µM anisomycin or 50 ng/ml IL-1ß over an incubation time of 20 minutes (n=3). Values are expressed as average of % activated p38 MAPK Vs control. ***= p<0.001 Vs Control One-way Anova, Dunnett's test).*

IL-1ß and anisomycin did not stimulate the activation of p38 MAP kinase in rat synaptosomes irrespective of the brain region or incubation time (figure 7). In contrast, HeLa cells treated with anisomycin or interleukin 1ß displayed enhanced levels of activated p38 MAP kinase, about 15 fold and 3 fold respectively, compared to control (figure 8). These data supported the hypothesis that in brain synaptosomes, p38 MAP kinase cannot be activated by IL-1ß or anisomycin under these conditions.

Section 3.1.3 Lack of effect of anisomycin on 5HT uptake in human platelets

5HT is expressed and released by many cells that belong to the CNS but also to the immune system. Human blood platelets represent an alternative biological system in which SERT is functionally expressed and can be modulated by anisomycin, as previously reported (Zhu *et al.,* 2005). For this

reason, 5HT uptake was measured in human platelets incubated with increasing concentrations of anisomycin at different end points. (Figure 9)

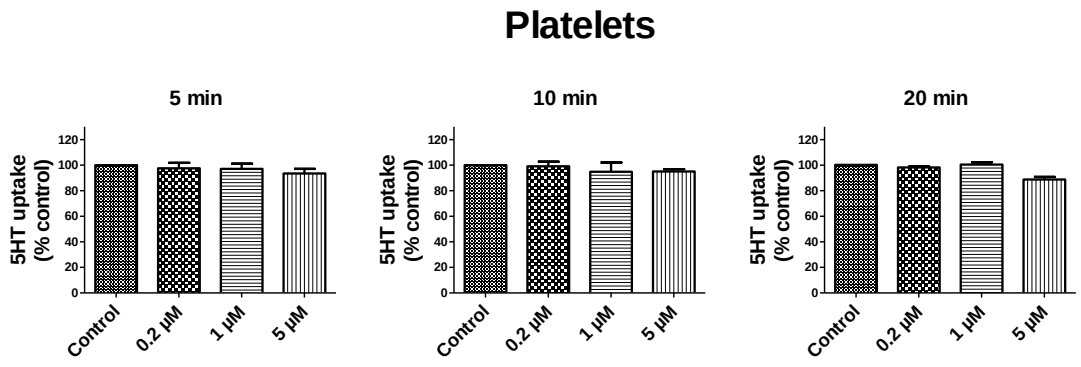


Figure 9. ***5HT uptake on human platelets pre-incubated with anisomycin at different end points and concentrations.*** *1 µM anisomycin was incubated with synaptosomes for 5, 10, 20 min before measuring 5HT uptake. Values are expressed as the mean of two experiments performed in triplicate (n=3; one-way ANOVA).*

In agreement with results in brain synaptosomes, 5HT uptake rate was unmodified by anisomycin, suggesting that no modulation on SERT function in human platelets was associated with anisomycin treatment under these conditions.

Section 3.1.4 Effect of anisomycin on 5HT uptake and p38 MAP kinase in rat and human cell lines expressing SERT

Another system in which anisomycin has demonstrated a modulation of SERT function is in a rat basophilic leucemia (RBL-2H3) cell line (Zhu et al., 2005). These cells have a native expression of SERT and some stimuli activating p38 MAP caused an increase in 5HT uptake. In order to understand if these cells responded to anisomycin with a SERT modulation, I reproduced the same

experiment using the same assay conditions. In parallel, anisomycin was also tested in a LLC-PK cell line expressing human SERT; where the high SERT expression could amplify small changes of transporter function induced by the kinase.

Increasing concentration of anisomycin (0.2-5 μM) was incubated at different end-points and subsequently 5HT uptake and phosphorylation of p38 MAP kinase was measured.

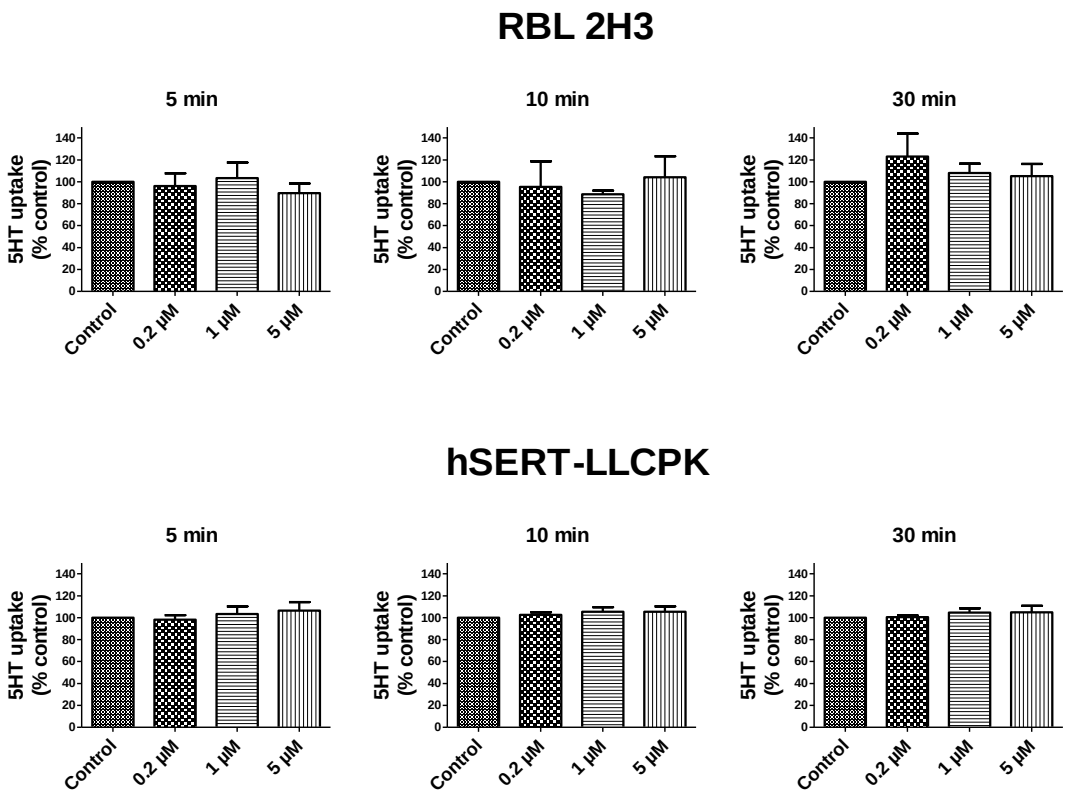


Figure 10. ***5HT uptake on rat basophilic leukemia cells (RBL-2H3) and LLCPK expressing human SERT pre-incubated with anisomycin at different end points.*** *Values are expressed as the mean of at least three experiments performed in triplicate (n=3; one-way ANOVA).*

Anisomycin up to 5 μM did not show any effect upon both cell lines, thus confirming the absences of SERT modulation irrespective of the cell line used (figure 10). In contrast, anisomycin treatment induced a significant increase of p38 MAP kinase phopshorylation in hSERT-LLCPK cells after 30 min from addition. This effect was reversed by SB203580, confirming the specific activation of p38 MAP kinase when cells were incubated with anisomycin (figure 11).

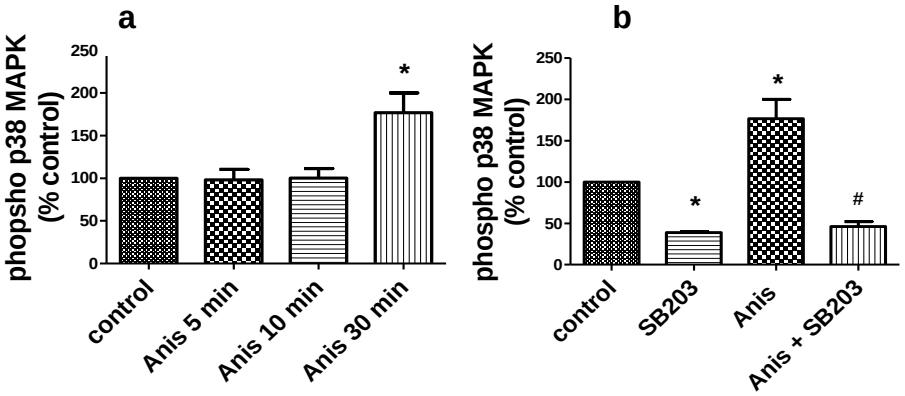


Figure 11. ***Measurement of phospho-p38 MAP kinase in hSERT-LLCPK cells treated with anisomycin.*** *a) 1 μM anisomycin was added over an incubation time of 30 min. b) addition of 10 μM SB203580 for 30 min before the stimulation with 1 μM anisomycin for 30 min. Values generated by three different experiments (n=3) are expressed as average of % activated p38 MAPK Vs control (*= p<0.05 Vs Control; #p<0.05 Vs 1 μM Anisomycin; One-way Anova, Dunnett's test).*

This finding therefore highlighted that the lack of effect of anisomycin on 5HT uptake was not caused by the absence of p38 MAPK activation as it was reported in synaptosomes but it may be due to a lack of interaction between p38 MAPK and SERT

Section 3.2. Effect of LPS on SERT function in an ex-vivo experiment

Data generated with the aim of demonstrating the effect of IL-1ß on SERT function through p38 MAP kinase were negative. However, this mechanism could be complex and tightly regulated so that a simplified *in vitro* system such as synaptosomes or isolated cells may have limited sensitivity. For this reason, a more physiological model was also investigated, in which an inflammatory response was induced directly in the intact brain avoiding tissue manipulation that could alter the interaction. The stimulation was performed by LPS, a potent activator of the inflammatory response that, when injected in the brain, produces a release of pro-inflammatory cytokines for a few hours (Roche et al., 2006). This model is widely used to investigate the effect of neuroinflammation on brain function. Moreover, intraperitoneal administration of LPS stimulates an increase of activated p38 MAP kinase in rat cortex and hippocampus by the formation of reactive oxygen species (Nolan Y et. al., 2003). Taken together these findings suggest that injection of LPS was a suitable intervention for the purpose of this study because it activates both cytokines and the p38 MAP kinase response.

Rats were injected (i.c.v.) with vehicle (artificial cerebral spinal fluid; aCSF) or LPS (20ug/rat) and after two hours, animals were killed and the cerebral cortex, striatum and hippocampus harvested. These tissues were then used to prepare synaptosomes and subsequently 5HT uptake parameters K_m and V_{max} rate were assessed between treated and control animals. In order to confirm that LPS injection evoked a cytokine release, part of the brain tissues were analysed

by Luminex technology to determine the level of IFNγ, TNFα, IL-1α, IL-1β, IL-6, and IL-10.

Animals treated with LPS revealed a [^{3}H]5HT uptake with K_m and V_{max} values of 102.2±19.5 nM and 20.7±2 pmol/min/mg, respectively, in striatum and 34.8±1.9 nM and 7.8±0.7 pmol/min/mg, respectively, in cortex. Animals treated with vehicle showed K_m and V_{max} values of 118.5±27.9 µM and 24.1±6.7 pmol/min/mg in striatum and 33.2±1.1 nM and 7.3±0.4 pmol/min/mg in cortex (Figure 12). Comparison of uptake parameters between the two groups revealed no significant differences, suggesting no change of 5HT uptake rate (Student's t-Test). However, LPS administration caused considerably higher levels of interleukin 1α and TNFα in comparison to vehicle treatment in striatum, cerebral cortex and hippocampus. Interleukin 1ß and IL-6 were also significantly increased in cortex and in hippocampus. In contrast, interleukin 10 and interferon-γ levels did not undergo any significant change in any region investigated (Table 6). Overall, these data revealed that SERT function was not changed in a rat intact brain system during central exposure to inflammatory mediators.

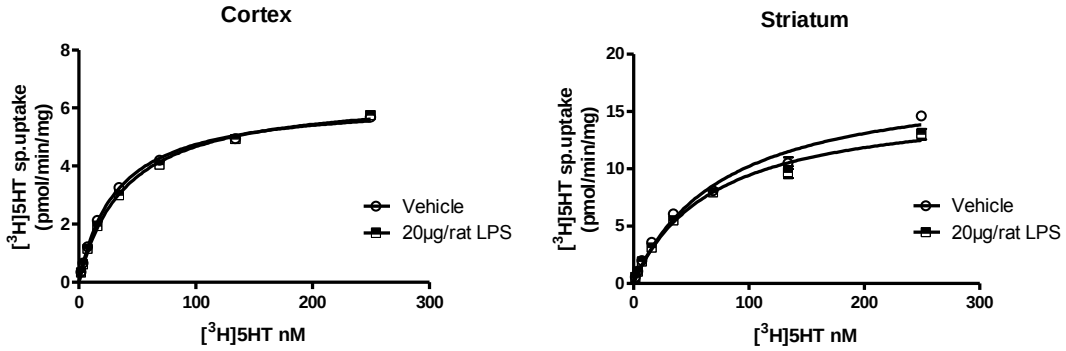


Figure 12. ***5HT uptake on rat synaptosomes in animals treated with vehicle or LPS (i.c.v. 20 µg/rat).*** *Each curve represents the specific [^{3}H]5HT uptake measured in a single animal. Values are expressed as the mean±sem of three uptake measurements.*

	Striatum		Cortex		Hippo	
	Vehicle	LPS	Vehicle	LPS	Vehicle	LPS
IL-1α	**228±86**	**2028 ±1057**$^{\#}$	**103±22**	**1428±404****	**226±116**	**2188±629****
IL-1ß	668±123	1402±468	**602±41**	**1049±128****	**671±121**	**1559±306***
IL-6	690±26	787±61	**528±38**	**684±61**$^{\#}$	**659±37**	**1005±92****
TNFα	**56±8**	**284±132**$^{\#}$	**33±2**	**262±72****	**40±5**	**412±129****
IFNγ	70±16	75±7	41±4	49±2	56±2	53±2
IL-10	530±49	519±4.2	392±35	432±23	409±29	363±39

$^{\#}$*p<0.06, *p<0.05, **p<0.01 Vs Vehicle*

Table 6. Comparison of cytokine levels between animals treated with vehicle and LPS. Values are expressed as mean±sem of pg/ml cytokines measured in the brain tissue of four animals per group (n=4; Student's T-test).

Section 3.3 Expression of 5HT$_7$ receptors in rat cortical astrocytes and activation of p38 MAP kinase

Studies reported in the previous sections indicate that the p38 MAP kinase activator, anisomycin, and the pro-inflammatory cytokine, interleukin-1ß, do not

produce an activation of 5HT uptake in rat and mouse brain, suggesting that SERT function is not altered by inflammatory processes involving p38 MAP kinase. 5HT itself regulates serotonergic transmission in the CNS through a number of auto- and hetero-receptors (Barnes & Sharp, 1999). One such receptor involved not only in the serotonergic transmission but also in the regulation of neuroinflammation appears to be the $5HT_7$ receptor. Some investigations have reported that $5HT_7$ regulates p38 MAPK activation and consequently cytokine release in human U-373 astrocytoma cells (Lieb *et al*., 2005). This mechanism seems present also in human microglia cells where $5HT_7$ receptors regulate IL-6 release (Mahe et. al. 2004). Taken together, it would seem that pro-inflammatory cytokines *via* p38 MAP kinase mechanisms can be regulated by $5HT_7$ receptors, providing a plausible mechanistic link between the immune and serotonergic systems. Experiments described in this section aimed to establish the presence of $5HT_7$ receptors in rat cortical astrocytes and, if their activation produced an increase of cAMP with a consequent activation of p38 MAP kinase, the release of cytokines was assessed.

Section 3.3.1 Increase of intracellular cAMP induced by $5HT_7$ receptors in rat cortical astrocytes

Since $5HT_7$ receptor is positively coupled with Gs proteins, its activation stimulates an increase of intracellular cyclic adenosine monophosphate (cAMP) through adenylate cyclase (AC) activation. This phenomenon was reported in

various recombinant cell systems transfected with human and rat $5HT_7$ receptors (Mahé C *et al.,* 2004; Romero G *et al.,* 2006). Indeed previous investigations showed that 5HT induced an increase of cAMP in thalamic/hypothalamic rat astrocytes while no response was detected in cortex and cerebellum, suggesting no expression of $5HT_7$ receptors in these two areas. However further PCR analysis revealed the presence of mRNA for $5HT_6$ and $5HT_7$ receptors in cortex suggesting the expression of $5HT_7$ receptors in cortex that was too weak to be detected (Hirst WD *et al.,* 1997). The use of a specific assay based on Fluorescence Resonance Energy Transfer (FRET) technology could increase the sensitivity to quantify cAMP at low concentrations. Therefore, to confirm the presence of $5HT_7$ receptors in plasma membranes of rat cortical astrocytes, a measurement of intracellular cAMP was performed with this method. Rat cortical astrocytes were obtained from 2-3 day old pups following the procedure described in the “material and methods” in section 2.8. Previous investigations showed $5HT_7$ receptors expression in astrocytes after 12 days *in vitro* and therefore cells were plated in 96-well plates and cultured for 15 days in normal condition before running the assay (Hirst WD *et al.,* 1997). Since FBS could interfere with 5HT receptor expression due to the presence of serotonin, cells were cultured in normal medium and in dialysed FBS medium. Moreover, cells cultured in normal medium were also kept in FBS-free medium for 24 hours before running the assay.

The specificity of signal was assessed using the selective $5HT_7$ receptor antagonist, SB258719. Cells were treated with 10 μM SB258719 for 15 min at 37°C before the addition of 1 μM 5HT. As positive control, cells were treated in

parallel with 1 μM forskolin. After 30 min, cells were lysed and cAMP concentration was measured.

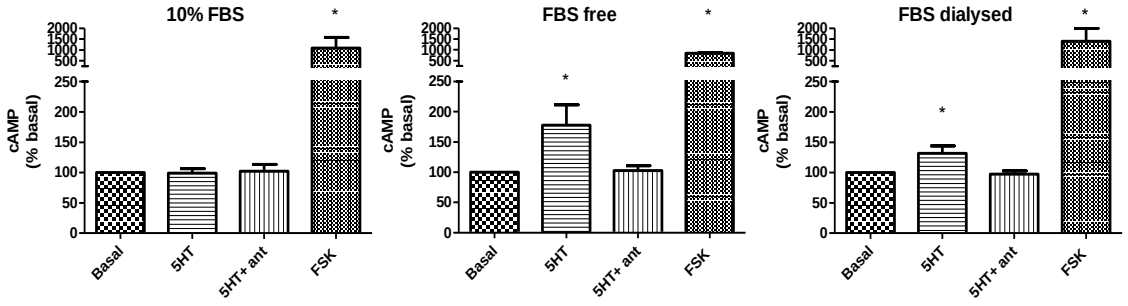


Figure 13. ***Measurement of intracellular cAMP in rat cortical astrocytes****. Cells were treated with 10 μM SB258719 (ant) prior to stimulation with 1 μM 5HT for 30 min . As positive control, 10 μM forskolin (FSK) was also tested. Data were the mean ± SEM of three individual experiments (n=3). Results are expressed as % response vs. basal cAMP levels (one-way ANOVA; Dunnett's test *p<0.05).*

Rat cortical astrocytes treated with 5HT showed a significant increase in cAMP levels when cells were cultured in medium without FBS or with dialysed FBS; increase of 178±33% and 131±13% vs. basal, respectively. No effect was observed in normal 10% FBS medium, conforming the impact of FBS on the 5HT receptor response. FSK increased cAMP levels across all conditions, as expected. SB258719 (10 μM) reversed completely the effect of 5HT, confirming that this response was mediated by $5HT_7$ receptors. Culturing cells for 24 hours in the absence of FBS gave a higher cAMP increase, demonstrating a better response of $5HT_7$ receptors under this condition (figure 13).

Section 3.3.2. p38 MAP kinase phosphorylation and interaction with $5HT_7$ receptors in rat cortical astrocytes

On confirmation of the expression of $5HT_7$ receptors in rat cultured astrocytes, the interaction between p38 MAP kinase and $5HT_7$ receptors was investigated by measuring specific p38 MAP kinase phosphorylation after 5HT stimulation. Rat astrocytes cultured for 15 days and kept in FBS-free medium for 24 hours were pre-treated with SB258719 for 15 min at 37°C and then 1 μM 5HT was added. Cells were lysated after 5 min and the amount of phosphorylated p38 MAP kinase was quantified using a specific ELISA assay.

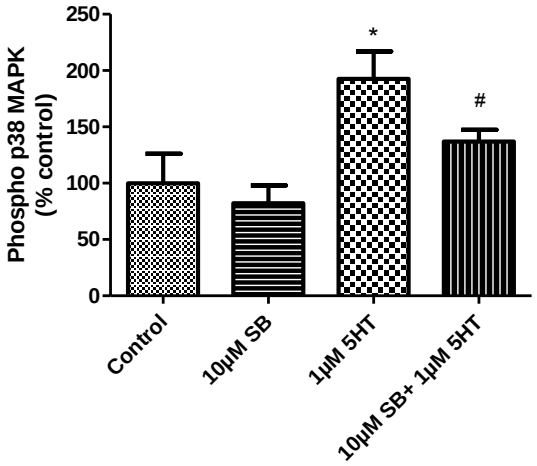


Figure 14. ***Effect of 5HT on p38 MAP kinase phosphorylation on rat astrocytes****. Cells were treated with 10 μM SB258719 for 10 min before stimulation with 1 μM 5HT for 5 min. Data were the mean ± SEM of at least seven individual experiments (n=7; one-way ANOVA followed by post-hoc comparisons (LSD method) between Basal Vs 5HT and 5HT Vs SB+5HT; *p<0.05 Vs Basal; #p=0.05 Vs 5HT).*

Rat astrocytes showed a significant increase of p38 MAP kinase phosphorylation after 1 μM 5HT addition and the effect was reversed by 10 μM

SB258719 (figure 14). These findings revealed an interaction between $5HT_7$ receptors and p38 MAP kinase not only in astrocytoma and microglia as previous reported in the literature (Lieb et al., 2005; Mahe et al., 2004) but also in cultured astrocytes, supporting the role of 5HT *via* $5HT_7$ receptors in the regulation of brain inflammation.

Section 3.4 Expression of $5HT_7$ receptors in hippocampal and cortical neurons

The interaction between $5HT_7$ receptors and p38 MAP kinase in rat astrocytes supports the hypothesis of a role of $5HT_7$ receptors in the regulation of neuro-inflammation. The literature reported that this interaction was observed in microglia cells and in astrocytomas and the current study confirmed this mechanism in rat astrocytes. $5HT_7$ receptors are also expressed in neurons expecially in hippocampus with a link to neuronal excitability (Tokarski *el al.,* 2003) and LTP (Roberts AJ *et al.,* 2004). However, no data have been reported on the interaction between $5HT_7$ receptors and p38 MAP kinase in neurons and if this mechanism may be linked with neurotrasmission. Therefore, this potential interaction was investigated in rat primary neurons obtained from rat hippocampus and cortex. In order to obtain a pure culture of neurons, rat embryos at day 18/19 of gestationwere used according to the procedure described in “materials and methods” section. The purity of the rat primary neurons cultures after 8-10 days *in vitro* was evaluated using the neuronal marker NeuN, the astrocyte marker, GFAP and the general nuclear marker,

DAPI. The percentage of cortical NeuN and GFAP positive cells vs DAPI cells was 66% and 1.7% respectively while the percentage of hippocampal NeuN and GFAP positive cells vs DAPI was 78.3% and 10.2% respectively.

In these brain regions, serotonergic neurons express different subtypes of 5HT receptor. Oligonucleotides recognising the sequence of $5HT_7$, $5HT_4$, $5HT_6$ receptor transcripts were used to investigate expression of Gs-protein coupled 5HT receptors subtypes.

Neurons were grown for 8-10 days in normal medium at 37°C, 5% CO_2 and then total mRNA was extracted and analysed by RT-PCR. Positive controls represented amplification of cDNA obtained from hippocampus of adult rat brain and negative controls represented an amplification of cDNA from cultured neurons in which the RT enzyme was excluded from the reverse transcriptase step. Data revealed that negative controls did not result in any product, thus confirming the absence of DNA from genome or other non-specific contaminations. PCR products of positive controls and both cultured hippocampal and cortical neurons yielded bands of the correct size corresponding to $5HT_7$, $5HT_4$ and $5HT_6$ receptors' cDNA (Figure 15).

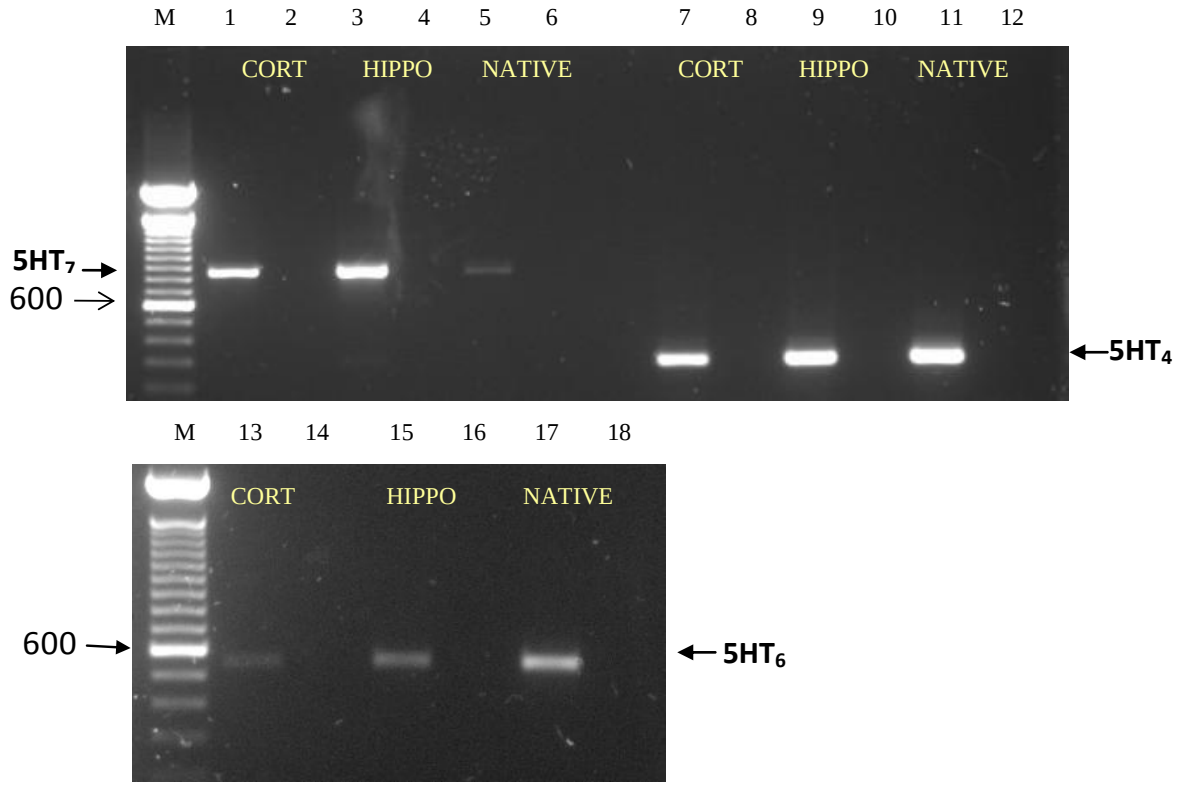


Figure 15. ***Representative reverse transcriptase PCR*** *of cDNA from adult rat hippocampus (lanes 5,6,11,12,17 and 18) and cultured neurons obtained from hippocampus (lanes 3,4,9,10, 15 and 16) and cortex (lanes 1,2,7,8, 13 and 14) of E18/19 rat embryos. Primers specific for $5HT_7$ receptor gene (lanes 1-6), $5HT_4$ receptor gene (lanes 7-12) and $5HT_6$ receptor gene (lanes 13-18) were used to amplify DNA fragments of 850, 303 and 538 bp respectively. Lanes 2,4,6,8,10,12,14,16,18 show negative controls where the RT enzyme was excluded from the RT step. Each line of ladder (M) represents a band size of 100 bp. Identical results were obtained by three independent neurons preparations (n=3).*

Although PCR is not a quantitative analysis of mRNA expression, this piece of data confirms the presence of mRNA for $5HT_7$, $5HT_4$ and $5HT_6$ receptors in cortical and hippocampal primary neurons. The characterization of functional $5HT_7$ receptor expression in membranes was assessed subsequently.

Section 3.4.1 Characterization of 5HT$_7$ response in cortical and hippocampal embryonic derived neurons by measuring intracellular cAMP levels

In order to verify if 5HT receptors coupled to Gs protein were functionally expressed in cortical and hippocampal embryonic derived neurons, intracellular cAMP concentration was measured in neurons after treatment with 5HT (1 μM) for 30 minutes. In parallel, neurons were also treated for 30 minutes with Forskolin (10 μM), a potent activator of adenylate cyclase.

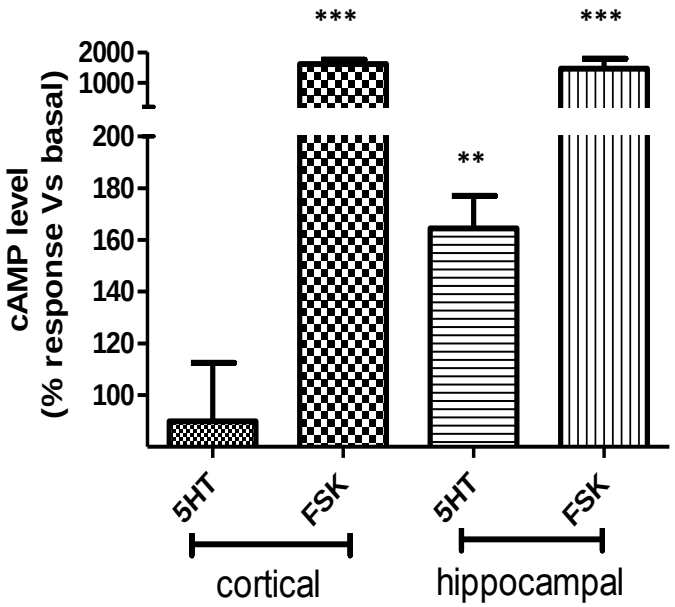


Figure 16. ***Measurement of intracellular cAMP in cortical and hippocampal neurons stimulated with 5HT (1 μM) or forskolin (10 μM) for 30 min****. Data are the mean ± SEM of three individual experiments (n=3). Results are expressed as % response vs. basal cAMP levels (one way ANOVA, Dunnett's test; **p<0.01, ***p<0.001).*

Cortical neurons did not show a robust response to 5HT, while an increase in cAMP levels of about 1618±140% compared to the basal level was measured

after forskolin treatment. Hippocampal neurons displayed a consistent response when treated with 5HT or with forskolin and the increase of intracellular cAMP vs. basal levels was 145±15% and 1462±324%, respectively (Figure 16, one-way ANOVA, response vs. basal, **P<0.01, ***P<0.001). This result indicated that cortical neurons did not express detectable functional 5HT receptors coupled to adenylate cyclase, although a considerable mRNA expression was detected, while hippocampal neurons expressed 5HT receptors able to increase intracellular cAMP.

The presence of 5HT receptors in hippocampal neurons was further investigated by measuring cAMP levels after treatment with increasing concentrations of 5HT for 30 min. 5HT showed a concentration-dependent increase of cAMP. Data was fitted to a four-parameter logistic equation giving a pEC_{50} of 7.2±0.2 and Hill slope of 1.2±0.2 The maximal response to 5HT was an increase of cAMP of 180% compared to basal levels (Figure 17).

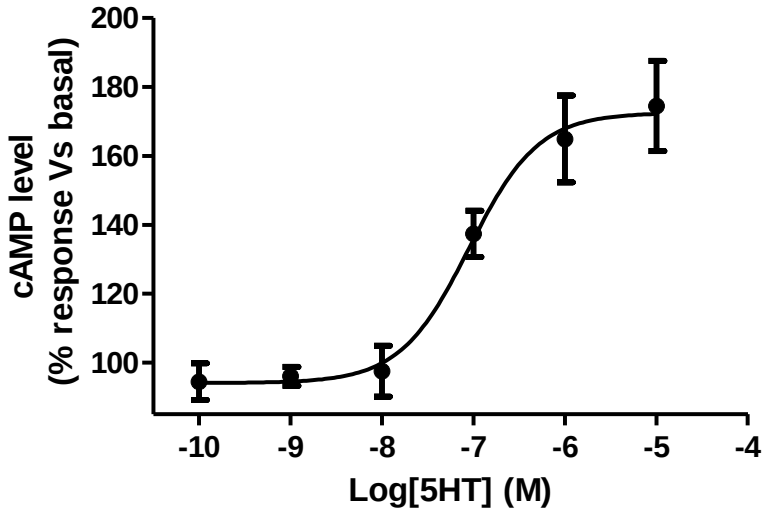


Figure 17. ***Concentration-response curve to 5HT in hippocampal cultured neurons.*** *Hippocampal cultured neurons were treated with increasing concentrations of 5HT (10 µM-0.1nM). Intracellular cAMP was measured 30 min after addition. Data were generated in three different experiments (n=3) and represent the percentage of accumulated cAMP±SEM vs. basal levels.*

In order to understand which Gs protein-coupled 5HT receptors were involved in this response, the selective $5HT_7$ receptor antagonist SB258719, the $5HT_4$ receptor antagonist SB399885 and the $5HT_6$ receptor antagonist GR113808 were tested. cAMP increase induced by 5HT (0.1 µM) was fully reversed by SB258719, with a pIC_{50} of 7.95 ±0.09 (n=3). In contrast, the selective antagonists SB399885 and GR113808 (either at 1 µM) did not reverse 5HT impact upon cAMP levels, suggesting that cAMP increase induced by 5HT was principally mediated by $5HT_7$ receptors (Figure 18). The selective $5HT_7$ receptor antagonist SB258719 also antagonized in an apparently competitive manner the response to 5HT with a pKb of 8.23±0.1 (Figure 19), confirming a profile of

competitive antagonism for this compound, as previously observed (Thomas *et al.,* 1999).

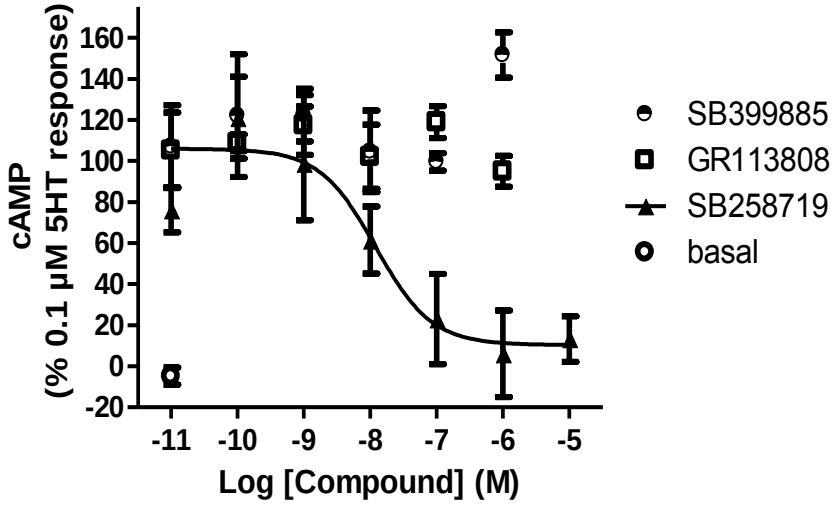


Figure 18 ***Effect of selective 5HT receptor subtype antagonists SB258719 ($5HT_7$), SB399885 ($5HT_4$) and GR113808 ($5HT_6$) on the increase of cAMP levels induced by 5HT****. Hippocampal neurons were treated with antagonists for 15 min followed by the addition of 0.1 µM 5HT. Intracellular cAMP was measured 30 min after agonist addition. Data were generated by three different experiments (n=3) and represent the percentage of increase of cAMP±SEM after 5HT addition.*

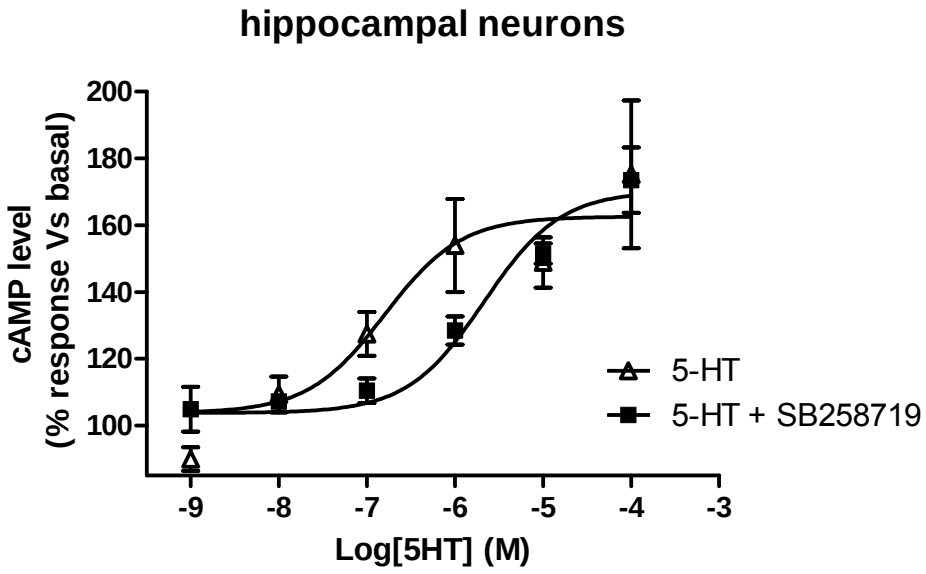


Figure 19. ***Concentration-response curve of 5HT alone and in the presence of SB258719****. Hippocampal cultured neurons were treated 15 min with 100nM SB258719 before the stimulation with 5HT. Data were generated by three different experiments (n=3) and represent the percentage of accumulated cAMP±SEM vs. basal levels.*

Section 3.4.2 Influence of $5HT_7$ receptors on CREB expression and phosphorylation in hippocampal neurons

One of the most relevant effects following an increase of intracellular cAMP is the phosphorylation of CREB at serine 133 (CREB[133]), which is necessary for the activation of CRE-dependent gene expression. In order to evaluate if the increase of cAMP induced by $5HT_7$ receptors is translated in a signal transduction pathways, the phosphorylation of CREB was evaluated. Cultures were treated with 1 μM 5HT at different time points ranging between 5 and 60 min. In parallel, cultures were also incubated with 10 μM forskolin for 30 min.

After treatment, cells were lysed and the amount of phospho-CREB[133] and total CREB were measured using ELISA assays.

The time course analysis of 5HT response showed a significant increase of phospho-CREB[133] of about 168±23% vs. basal 5 min after addition. This signal was transient and returned to basal level after 10 min. Forskolin also induced an increase of CREB phosphorylation of about 193±7% vs. basal levels 30 min after addition (Figure 20).

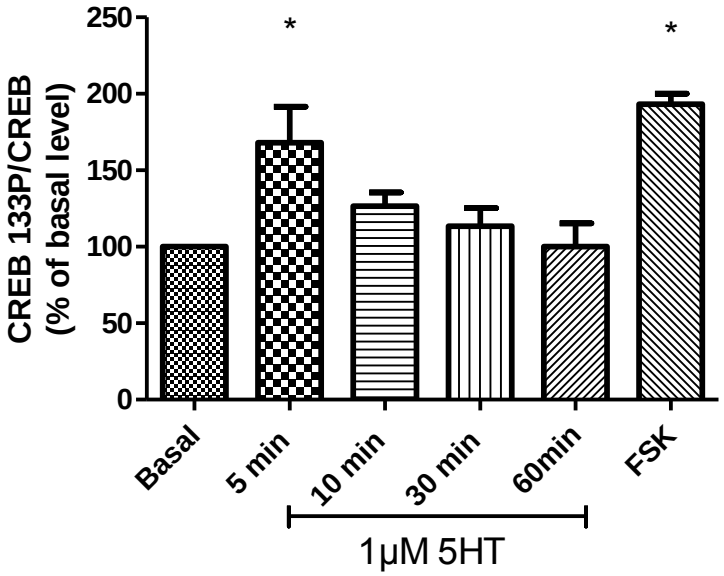


Figure 20. ***Effect of 5HT on CREB phosphorylation at different time points.*** *5HT was added to cultures for different incubation times (5, 10, 30, 60 min) and then the amounts of phospho CREB[133] and total CREB were measured using an ELISA assay. 10 µM forskolin incubated for 30 min was used as control. Data were the mean±SEM. of three different experiments (n=3) and represent the pCREB[133]/ total CREB ratio of treated neurons vs. basal (one-way ANOVA; Dunnett's test. *p<0.05 Vs Basal).*

In order to investigate if the increase of CREB phosphorylation induced by 5HT was mediated by $5HT_7$ receptors, cultured hippocampal neurons were treated for 15 min with increasing concentrations of the $5HT_7$ antagonist SB258719 prior to stimulation with 1 μM 5HT for 5 min. Data analysis revealed that SB258719 significantly reduced CREB phosphorylation induced by 5HT in a concentration-dependent manner confirming the mediation of $5HT_7$ receptors (Figure 21). Interestingly, SB258719 did not reverse completely the 5HT response even at 10 μM although the calculated inhibition of 10 μM SB258719 should be around the 99% (value extrapolated by pKb generated in the cAMP assay).

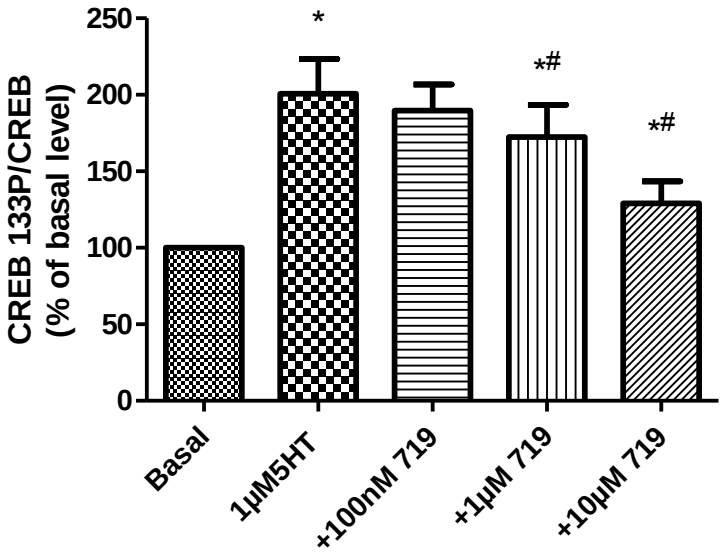


Figure 21. ***Effect of the selective $5HT_7$ antagonist SB258719 on CREB phosphorylation induced by 5HT.*** *Cultured neurons were treated with SB258719 for 15 min and then 1 μM 5HT was added for 5 min. The amount of phospho CREB[133] and total CREB were then measured using ELISA assays. Data were the mean±SEM of three different experiments (n=3) and represent the pCREB[133]/ total CREB ratio of treated neurons vs. basal (one-way ANOVA; post doc comparison (LSD method) Vs Basal and Vs 5HT. *p<0.05 Vs Basal, $^{\#}$p<0.05 Vs 1 μM5HT).*

Section 3.5 Influence of 5HT$_7$ receptors on p38 MAPK expression and phosphorylation in hippocampal neurons

Once the pharmacological characterization of 5HT$_7$ receptors in hippocampal cultured neurons was completed, the interaction between 5HT$_7$ receptors and p38 MAP kinase in hippocampal neurons was investigated. Hippocampal neurons were treated with 1 μM 5HT at different end points ranging between 5 and 60 min. In parallel, cells were also treated with Forskolin for 30min as positive control. Cells were then lysated and the amount of phosphorylated p38 MAP kinase was quantified using an ELISA assay.

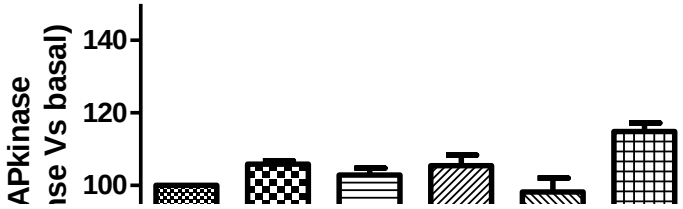


Figure 22. ***Effect of 5HT on p38 MAP kinase phosphorylation at different time points.*** *5HT was added to cultured neurons for various incubation times (5, 10, 30, 60 min) and then the amount of phospho p38 MAP kinase was measured using an ELISA assay. In parallel, neurons were treated with 10 μM forskolin incubated for 30 min. Data were the mean±SEM of three different experiments (n=3) and represent the percentage of response vs. basal levels (one-way ANOVA).*

Data showed that neither 5HT nor Forskolin (FSK) changed the level of phospho p38 MAP kinase at any of the tested time points, suggesting that although $5HT_7$ receptors are expressed in hippocampal neurons and produce a functional response (increase of cAMP level and CREB phosphorylation), they do not interact with p38 MAP kinase under these conditions (Figure 22).

Section 3.6 Influence of $5HT_7$ receptors on GluR1 expression and its post-translational modification

The fact that $5HT_7$ receptors do not interact with p38 MAP kinase in neurons suggests a cell-dependent function of $5HT_7$ receptors with a different role in neurons compared to that observed in astrocytes. The presence of $5HT_7$ receptors in hippocampus was previously described as a regulator of synaptic transmission (LTP) and neuronal excitability (Tokarski *el al.,* 2003). Synaptic strength is tightly regulated by the phosphorylation of GluR1 receptors (AMPAR) and their cellular distribution (Santos et al., 2009). Basically, AMPA receptors are formed by different subunits which are encoded by four genes (such as GluR1, GluR2, GluR3, and GluR4, Hollmann, and Heinemann, 1994). Hippocampal neurons expressed mainly GluR1, GluR2 and GluR3 and the preferred configurations were two identical heterodimers of GluR1/2 and GluR2/3. When AMPAR received the stimulus, GluR1 was phosphorylated at multiple sites (Ser831, Ser845 etc) and this determined a migration of AMPAR to the synapses. The amount of AMPARs at the level of synapses determined the modulation of glutamatergic transmission.

In order to understand if $5HT_7$ receptors may affect GluR1 phosphorylation or expression in hippocampal neurons, cultures were treated with 1 μM 5HT or 10 μM FSK for 5 min. Cells were lysed and proteins in the extract were separated by SDS-PAGE. Immunoblot analysis was then performed with antibodies that recognize phopshorylated GluR1 at Ser845, GluR1 and GAPDH.

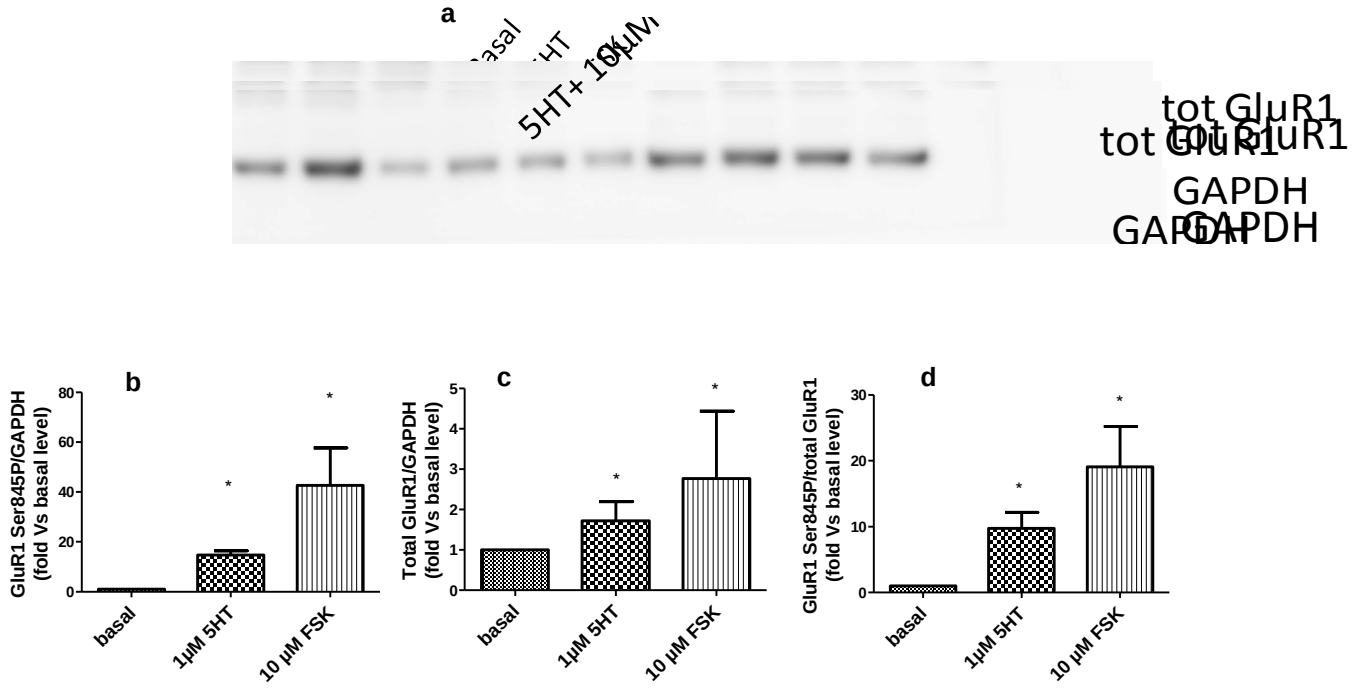


*Figure **23 Effect of 5HT and FSK on phopshorylated GluR1 at Ser 845 and total GluR1 in primary hippocampal neurons**. (a) Representative western blot of neurons treated with 5HT and FSK for 5 min. Cells extracts were analysed with anti-phosphoGluR1(845), anti-GluR1, and anti-GAPDH antibodies. (b-c-d) Quantification of phosphorylated GluR1(Ser845) and total GluR1. Ratio of GluR1(Ser845P)/GAPDH, totGluR1/GAPDH and GluR1(Ser845P)/totGluR1 are expressed as fold increase±SEM. with respect to basal levels in three different experiments (n=3; ANOVA followed by post hoc comparisons (LSD method) Vs basal *P<0.05 vs. Basal).*

These experiments showed that both 5HT and FSK significantly increased the amount of phosphorylated GluR1 by about 14.7±1.7 and 42.7.1±14.9 fold compared to basal levels. Moreover, both compounds induced an increase of total GluR1 of 1.73±0.5 and 2.8±1.7 compared to basal levels, respectively. The graph in figure 23d represents the ratio between phosphorylated and total GluR1 reflected the data reported above.

Since the PCR analysis suggested that different kinds of 5HT receptors were expressed under these conditions, a further group of experiments was performed to assess if this effect was specific to the activation of $5HT_7$ receptors.

Before the stimulation with 1 μM 5HT for 5 min, neurons were treated with increasing concentrations of different classes of 5HT antagonists (the selective $5HT_7$ receptor antagonist SB258719, the selective $5HT_4$ receptor antagonist GR113808 and the selective $5HT_6$ receptor antagonist SB399885) for 15 min and then analysis of phosphorylated GluR1 expression was performed. In addition, neurons were also treated with the selective $5HT_7$ receptor partial agonist, AS-19 (1 μM) for 5 min in the presence or in the absence of 10 μM SB258719 pre-incubation as above.

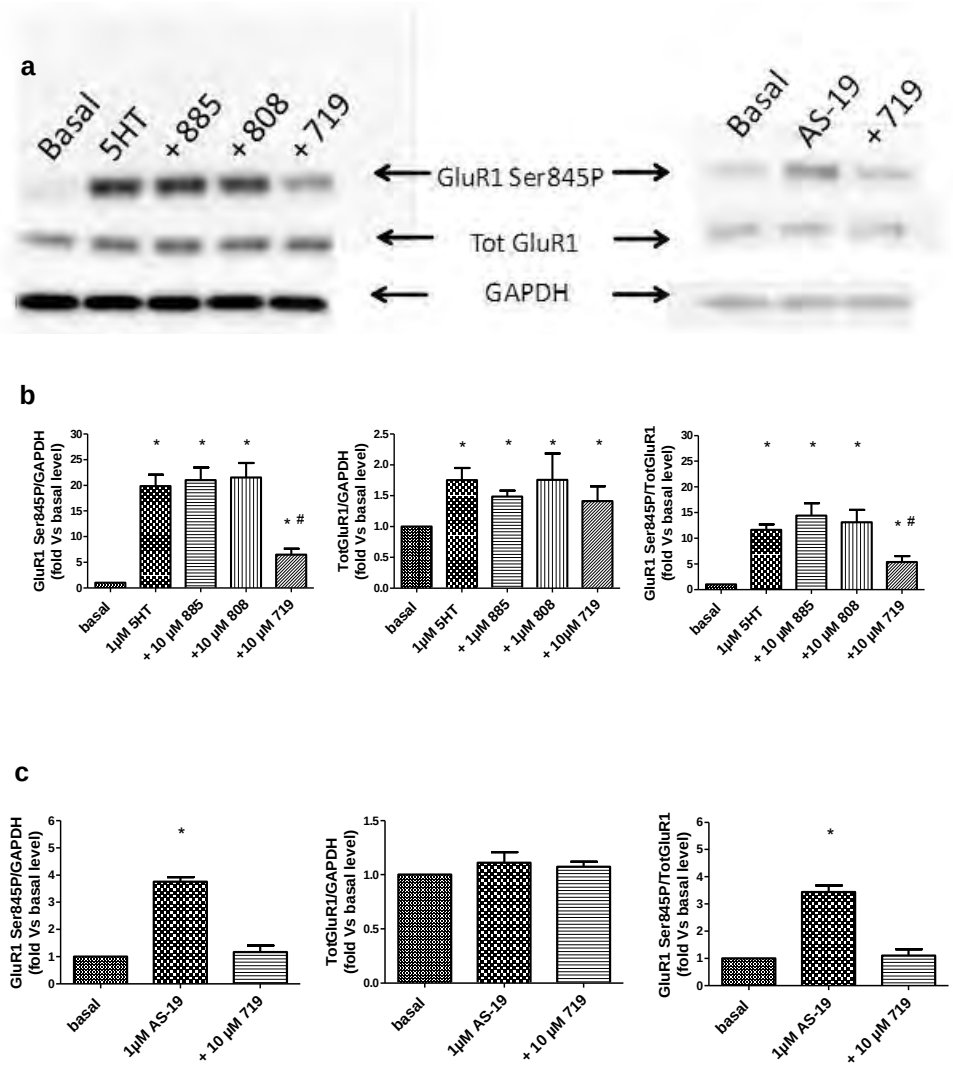


Figure 24 ***Effect of agonists and antagonists of 5HT receptor subtype es on phosphorylated GluR1 at Ser 842 and total GluR1****. (a) Representative western blot of neurons treated for 15 min with vehicle (DMSO 0.1%), 1 μM SB399885 ($5HT_6$ ant.), 1 μM GR113808 ($5HT_4$ ant.) or 10 μM SB258719 ($5HT_7$ ant.) followed by treatment with 1 μM 5HT (left hand panel) or 1 μM AS19 (right hand panel) for 5 min. (b-c) Quantification of phosphorylated GluR1(Ser845) and total GluR1. Ratio of GluR1(Ser845P)/GAPDH, totGluR1/GAPDH and GluR1(Ser845P)/totGluR1 are expressed as fold increase±SEM with respect to basal levels in three different experiments (n=3; ANOVA followed by post hoc comparisons (LSD method) Vs basal Vs 5HT or AS-19, . *P<0.05 vs. Basal; #p<0.05 Vs 1 μM 5HT or AS-19;*

These data revealed an increase of GluR1 phosphorylation when 5HT was added (11.6 ±1.1 fold *vs* basal) and this effect was not changed by GR113808 (13.1 ±2.4 fold *vs* basal) nor SB399885 (14.4 ±2.4 fold vs. basal), while it was reversed by SB258719 (5.3 ±1.2 fold *vs* basal). In addition, 5HT induced a significant increase of total GluR1 expression that was not reversed by the investigated antagonists, suggesting that $5HT_7$, $5HT_6$, $5HT_4$ receptors were not involved in this latter effect. Moreover, the selective $5HT_7$ receptor agonist AS-19 induced a significant increase of GluR1 phosphorylation (3.4±0.2 foId vs basal) that was reversed by SB258719 (1.1±0.2 fold vs basal). No effect was observed on total GluR1 expression. Overall, this data suggest that 5HT increased GluR1 phosphorylation at Ser 845 and this modulation was mediated by $5HT_7$ receptors.

Section 3.6.1 Influence of $5HT_7$ receptors on AMPAR trafficking

One of the effects produced by GluR1phosphorylation at Ser 845 is a modulation of receptor trafficking. Recent studies suggested that GluR1 phosphorylation at Ser 845 primes AMPAR for synaptic incorporation because it induces a transport of AMPA on the surface membrane of synapses (Gomes et al., 2004). In order to understand if the increase of GluR1 phosphorylation induced by $5HT_7$ receptors changed GluR1 expression in the plasma membrane, a biotinylation assay was performed. Basically, hippocampal neurons were treated with 5HT at different time points (5, 10 and 30 min) at 37°C. After one wash with cold PBS, cells were treated with biotin, which is a

membrane-impermeable reagent that hence labels all proteins present at the cell surface. These proteins were then isolated by affinity chromatography on streptavidin columns followed by an analysis of membrane-associated GluR1 and phosphorylated GluR1 at Ser 845. Membranes-associated GluR1 was normalized for pan-Cadherine proteins which are a family of single chain glycoprotein receptors mediating calcium dependent cell-cell adhesion. They are expressed principally in the plasma membranes and then anti pan-Cadherine antibodies are used as a specific plasma membrane marker (Geiger et al., 1990).

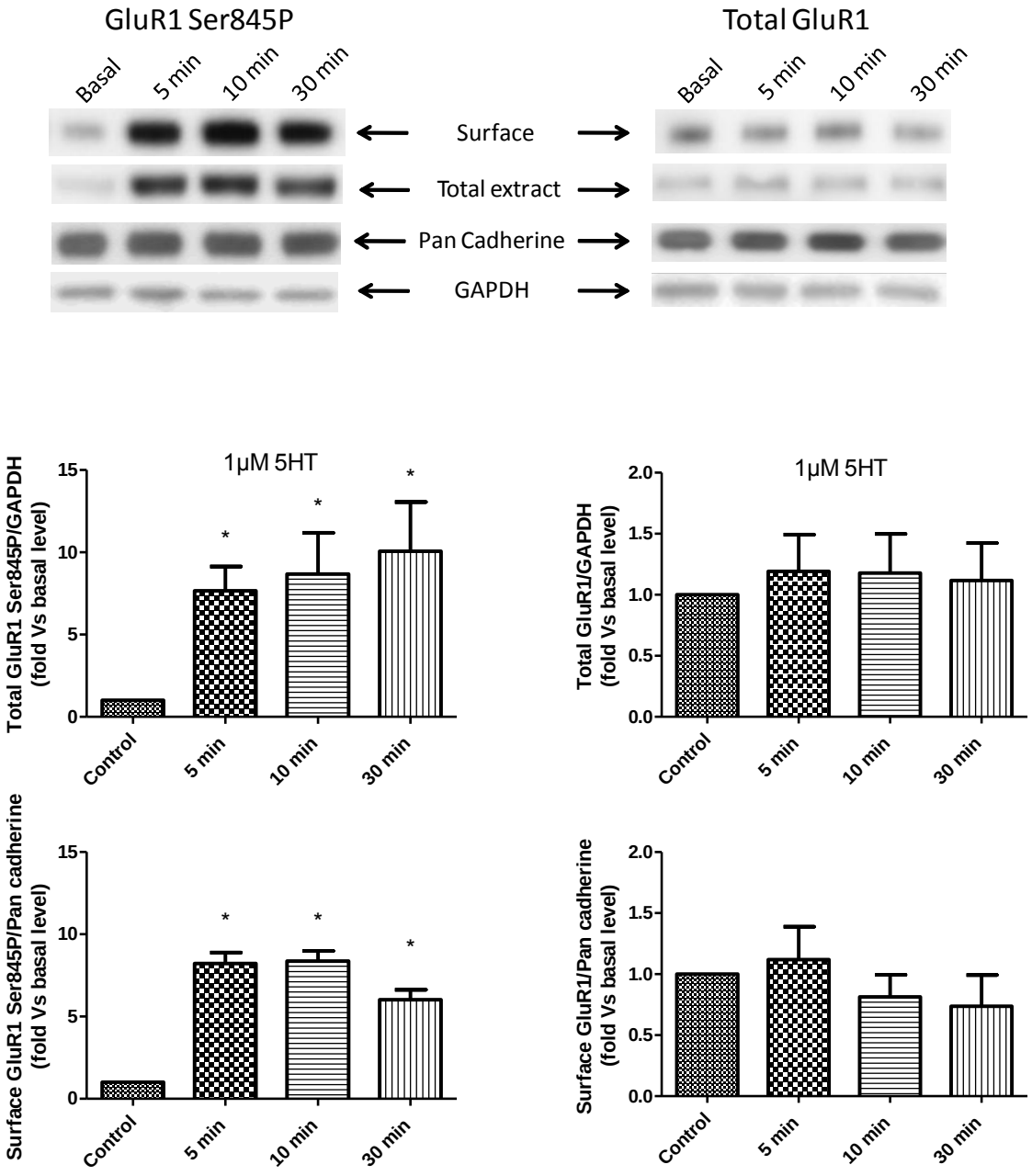


Figure 25 ***Effect of 5HT on surface phopshorylated GluR1 at Ser 842 and surface total GluR1****. (a) Representative western blot of neurons treated with 1 µM 5HT at different time points. Surface proteins were isolated using biotinylation assay as described in the methods. Extracts were analysed using anti-phosphorylated GluR1 at Ser845, anti-GluR1, anti-pan cadherine and anti-GAPDH antibodies. (b) Quantification of phosphorylated GluR1(Ser845) and total GluR1 in the total extract and in the surface fraction. Amount of proteins were normalised using GAPDH and pan-cadherine in the total extract and in the surface fraction respectively. Value are expressed as fold increase±SEM. respect to basal of three different experiments (n=3; One-way ANOVA, Dunnett's test; *P<0.05 vs. Basal).*

5HT produced a clear increase of phopshorylated GluR1 both in total extract and in surface fraction at all time points. This effect was already detectable at 5 min after addition and was stable up to 30 min. In contrast, no difference in GluR1 expression was detected in both extracts, suggesting that GluR1 phosphorylation induced by 5HT was not followed by a change of GluR1 expression in the plasma membrane.

Literature described that GluR1 phosphorylation at Ser845 is mediated by protein kinase A (PKA). which isnormalyc activated by an increase of intracellular cAMP. Further analysis had the aim to confirm if the increase of phopshorylated GluR1 at Ser845 was due to $5HT_7$ receptors through the activation of PKA. Hippocampal neurons were treated with H89, a potent inhibitor of PKA, and SB259719 for 15 min before stimulation with 5HT for 5 min at 37°C. Biotinylation assay and analysis of GluR1 phosphorylation were then performed as described before.

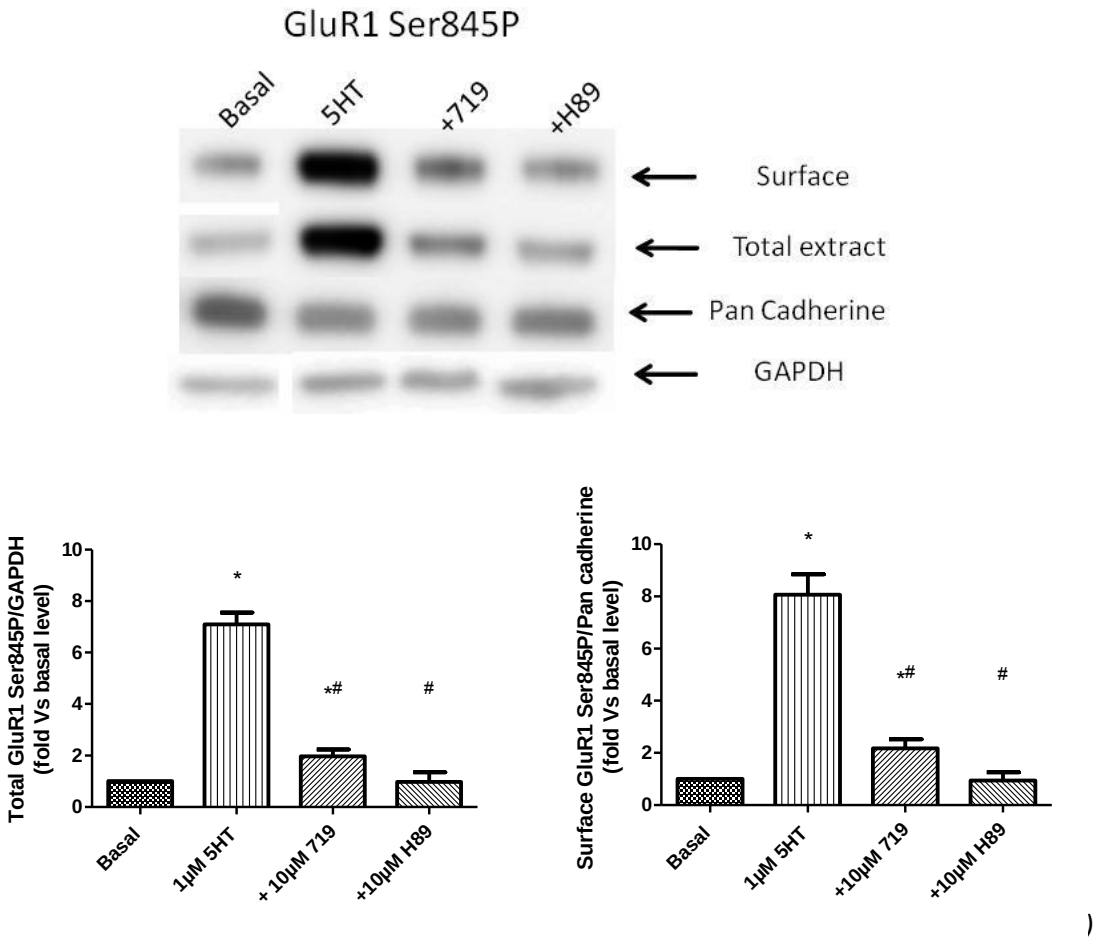


Figure 26. ***Representative western blot of neurons treated with SB258719 and H89 for 15 min followed by 1 µM 5HT treatment for 5 min****. Surface proteins were isolated using biotinylation assay as described in the methods. (b) Quantification of phosphorylated GluR1(Ser845) in total extract and in surface fraction. Amount of proteins were normalised using GAPDH and pan-cadherine in the total extract in the surface fraction, respectively. Value are expressed as fold increase±SEM. with respect to basal of three different experiments (n=3; One-way ANOVA followed by post hoc comparisons (LSD method) Vs Basal or 5HT,*P<0.05 vs. Basal, #P<0.05 Vs 1 µM 5HT;).*

5HT increased GluR1 phosphorylation in the surface fraction (8.1±0.8 vs. basal) and the effect was blocked by SB258719 (2.2±0.3 vs. basal) and H89 (0.9±0.3 vs. basal). Similarly, a significant increase of phosphorylated GluR1 induced by

5HT in the total extract (7.1±0.4 vs. Basal) was reversed by SB258719 (2.0±0.3 vs. Basal) and by H89 (1.0±0.4 vs. Basal). These results show that GluR1 phosphorylation in plasma membranes of hippocampal neurons was mainly induced by $5HT_7$ receptors via PKA activation.

Section 3.6.2 PDE_4 and PDE_{10} regulate GluR1 phopshorylation induced by $5HT_7$ receptors

PDEs are a family of enzymes that control the degradation of intracellular cAMP and cGMP. They present specific subcellular compartmentalizations and modulate the signalling cascades triggered by these two molecules. Previous experiments demonstrated that activation of $5HT_7$ receptors in hippocampal neurons altered cAMP levels resulting in increased GluR1 phosphorylation. A further investigation was then performed to understand if PDE4 and PDE10 may modulate GluR1 phosphorylation induced by $5HT_7$ receptors in hippocampal neurons, since these enzymes are expressed in the hippocampus of adult rat brain (Menniti et al., 2006). Neurons were treated with rolipram (10 μM; PDE4 inhibitor) or papaverine (10 μM; PDE 10 inhibitor) for 30 min and then stimulated with 5HT (1 μM) for 5 min.

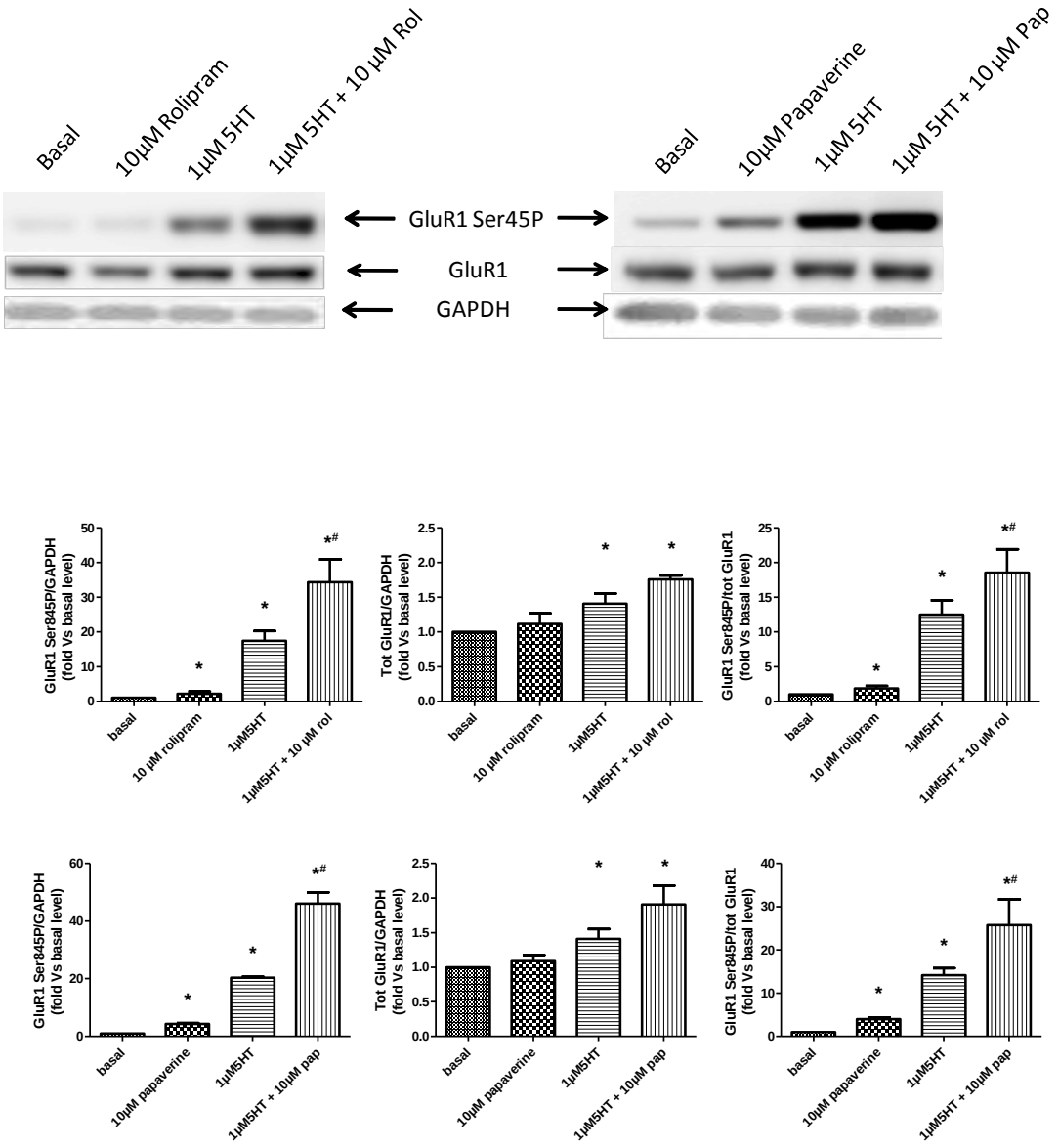


Figure 27 ***Effect of rolipram and papaverine on phopshorylated GluR1 at Ser 842 and total GluR1.*** *(a) Representative western blots of neurons treated for 30 min with 10 μM Rolipram or 10 μM Papaverine followed by 1 μM 5HT treatment for 5 min. (b) Quantification of phosphorylated GluR1 and total GluR1. Ratio of GluR1(Ser845P)/GAPDH, totGluR1/GAPDH and GluR1(Ser845P)/totGluR1 are expressed as fold increase±SEM with respect to basal levels of three different experiments (n=3; One-way ANOVA followed by post hoc comparisons (LSD method) Vs Basal or 5HT,*P<0.05 vs. Basal,#P<0.05 Vs 1 μM 5HT ().*

Rolipram, papaverine and 5HT significantly increased phosphorylated GluR1 of 1.9±0.4, 4.0±0.3 and 13.3±1.8 folds compared to basal levels, respectively. Pre-incubation with rolipram or papaverine potentiated 5HT-induced phosphorylation by 18.5±3.4 and 25.8±6.0 fold, respectively. Rolipram and papaverine did not show any effect on total GluR1 and the increase of total GluR1 induced by 5HT was not affected by PDE inhibitors. These data identify that PDE4 and PDE10 are actively involved in GluR1 phosphorylation induced by $5HT_7$ receptors in hippocampal neurons.

Section 3.6.3 Influence of $5HT_7$ receptors on Ca^{2+} intake mediated by AMPA

AMPAR is a ionotropic transmembrane receptor activated by glutamate that controls cell permeability to calcium, sodium and potassium ions. PKA activation and consequent increase of GluR1 phosphorylation at Ser 845 could affect receptor trafficking and potentiate AMPA function (Oh MC *et. al.,* 2006). To understand if GluR1 phosphorylation modulated by $5HT_7$ receptors affect physiological function of AMPAR, Ca^{2+} intake induced by AMPA was measured in hippocampal neurons after exposure to 5HT or FSK.

Labelling was performed by incubating neurons with the Ca^{2+} indicator dye Fluo4 for 45 min at 37°C in HBSS buffer. Once labelled, cells were incubated with 10 µM 5HT or 10 µM FSK for 10 min at 37°C and then stimulated with increasing concentrations of AMPA (10 nM – 15 µM).

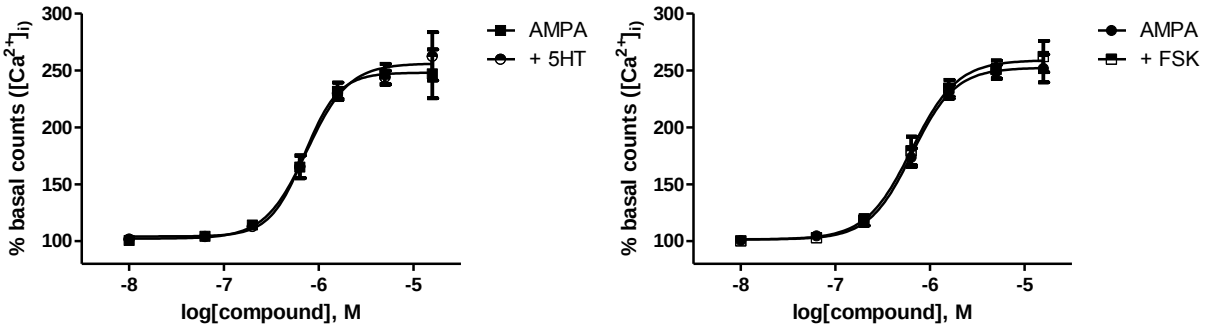


Figure 28. ***Effect of 5HT and FSK in $[Ca^{2+}]_i$ influx induced by AMPA****. 10 μM 5HT or 10 μM FSK were added to cultured neurons for 10 min and then the increase of intracellular $[Ca^{2+}]_i$ induced by AMPA was measured with FLIPR TETRA ($[Ca^{2+}]_i$). Data are expressed as percentage of basal counts and represent the mean±SEM of at least three experiments (n=3).*

AMPA produced a concentration-dependent increase of Ca^{2+} with a pEC_{50} of 6.14±0.05 which was not altered by 5HT (6.12±0.07) nor FSK (6.20±0.07). Similarly, maximal response to AMPA was not changed after the exposure to 5HT or FSK (one-way ANOVA, Dunnett's test).

Chapter 4 Discussion

Section 4.1 Interaction between interleukin 1, p38 MAP kinase and SERT in native tissue and recombinant cell lines

Available evidence supports the notion that an altered immune response may influence CNS function, resulting in the development of depressive illness (Raison at al., 2006). One of the mechanisms that may explain this link is that pro-inflammatory cytokines, elevated in depressed patients, have been shown to have a direct modulation of SERT function by p38 MAP kinase activation *in vitro (Zhu et al., 2005, 2006)*. Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases which respond to extracellular stimuli and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis (Cuenda & Rousseau, 2007). The role of cytokines *via* p38 MAP kinase in the modulation of SERT has been demonstrated in mice synaptosomes, in rat embryonic raphe RN46A cells and in recombinant cells expressing human SERT. where p38 MAP kinase may activate SERT by directly changing functional activity rather than expression levels. Such effects were reversed in the presence of the p38 MAP kinase selective inhibitor, SB203580, confirming that the regulation of neuronal SERT activity induced by cytokines is mediated by p38 MAP kinase linked pathways (Zhu *et al.,* 2007).

p38 MAP kinase belong to the MAPK superfamily and is activated by numerous cytokines as well as by environmental and chemical stressors in many cases triggering inflammatory and apoptotic pathways. Anisomycin, a protein

synthesis inhibitor derived from *Streptomyces griseolus*, induces an activation of p38 MAP kinase phosphorylation in a variety of cells including neutrophils (Nahas et al., 1996), mast cells (Zhang et al., 1997), and myocardial cells (Mcnicol, 1996). This p38 MAP kinase activation induced by anisomycin was followed by the activation of SERT in an SB203580-sensitive manner in recombinant cell line and in native tissue mimic the effect observed on SERT by cytokines (Zhu et. al. 2005). Other studies demonstrated that inhibition of p38 MAP kinase by PD169316 or SB203580 reduced 5HT transport in rat midbrain synaptosomes and that the constitutive active form of MKK3b (upstream kinase that phosphorylates p38 MAP kinase) co-transfected with SERT in HEK-293 cells increased the 5HT transport. Additionally, decreasing p38 MAP kinase expression by siRNAs in this recombinant HEK-293 reduced 5HT transport and surface SERT expression, indicating a basal SERT activity maintained by an constitutively active form of p38 MAP kinase (Samuvel et al., 2005).

Based on what was reported in the literature, in the present studies rat and mouse cortical synaptosomes were treated with IL-1β and anisomycin at different concentrations and time points followed by a measurement of 5HT uptake. In contrast to literature reports, the current work has failed to see such effects in both rat and mouse synaptosomes irrespective of methodology and brain area used. Two possible hypothesis for this discrepancy were evaluated in mice brain synaptosomes in which literature reported this activation. The first involved animal housing that may induce sufficient stress to endogenously activate p38 MAP kinase thus masking the effects of IL-1β and anisomycin on 5HT uptake. Therefore, further 5HT uptake studies were performed by using

brain synaptosomes from striatum and midbrain of individually-housed mice. Using these conditions, still no effect was observed, suggesting that there was no activation of SERT function by anisomycin irrespective to the housing conditions.

On the other hand, it could be that the synaptosome preparation itself altered the activation of p38 MAP kinase. Literature has reported that the anisomycin effects in rat raphe cells and human platelets were more pronounced (~100 and ~ 60% above basal) than those observed in mouse brain synaptosomes (~20% above basal), supporting the hypothesis that the mechanics of synaptosomal preparations may indeed alter the basal activity of p38 MAP kinase and hence limit the responsiveness to anisomycin (Zhu *et al.,* 2007).

In order to assess if the treatment with anisomycin was able to promote the change in the status of p38 MAP kinase from non active (non-phosphorylated) to active form (dual tyrosin-phosphorylated), synaptosomes were treated with IL-1β or anisomycin and then analysed by ELISA to quantify the phospho-MAP kinase in various samples. The data revealed that neither IL-1ß nor anisomycin could activate p38 MAP kinase in rat synaptosomes from cortex and striatum, while in a parallel experiment performed in HeLa cells, both compounds produced a large effect. These new findings suggested that in my synaptosome preparation, neither IL-1ß nor anisomycin have the capacity to induce p38 MAP kinase activation and consequently no downstream signal is produced that could act on transporter function.

Alternative biological systems in which SERT function was modulated by anisomycin *in vitro* were human blood platelets and rat basophilic leukemia cells RBL-2H3 (Zhu *et al.,* 2005). Platelets treated with 1μM anisomycin for 10 min showed an increase of 5HT uptake which was reversed by the presence of p38 MAP kinase inhibitor, SB203580. RBL-2H3 treated with 1μM anisomycin for 10 min showed a reduction of SERT Km for 5HT (1057 ±145 versus 628 ±70μM, $p<0.05$) without a significant change in maximal transport capacity (Vmax). In order to understand if the reduction of Km reflected a change of 5HT affinity, the Ki of 5HT was estimated in the [^{125}I]RTI-55 competitive binding assay in whole cell. Under this conditions, 1μM anisomycin incubated for 10 min reduced significantly the apparent 5HT Ki for [^{125}I]RTI-55 (1.31 ± 0.26 μM in the vehicle control, 0.24 ± 0.005 μM in the presence of anisomycin, $p<0.05$) and this effect was blocked by SB203580. The study then supported the hypothesis that p38 MAP kinase impacts SERT not by a change in surface trafficking but by a catalytic modulation of pre-existing surface-resident transporters. These findings were then used to set up an *in vitro* human blood platelet and RBL-2H3 cell uptake assays to assess the effects of the p38 MAP kinase on 5HT uptake in a system perhaps less prone to mechanical and behavioural stress. In addition, anisomycin was also tested in LLC-PK cells expressing human recombinant SERT (hSERT-LLCPK). This cell line was used because the high expression of SERT could better highlight the possible interaction with p38 MAP kinase. In all these *in vitro* systems, anisomycin did not potentiate the SERT function, comparable to results observed in synaptosomes. The analysis of p38 MAP kinase phosphorylation in the hSERT-LLCPK confirmed that anisomycin

induced an activation of p38 MAP kinase; nevertheless, a change of SERT function was not induced. The conditions used in these assays were identical to those used in the literature on RBL-2H3 cell line in which a change of SERT function was observed.

Overall, the discrepancy between the literature and these results in the present thesis were not clarified although many approaches were used. SERT is likely to be phosphorylated on multiple domains and different kinase and phosphatase may act in concert at different steps in the trasporter's life cycle to change the trafficking or the catalytic activity. The lack of effect of anisomycin in brain synaptosomes, platelets and cell lines could be then explained by the fact that crucial factors for the activation of p38 MAP kinase and its modulation on SERT are not present in these systems.

Moreover, p38 MAP kinase is regulated by stress and little changes of this status during sample manipulation could interfere with its activity. For these reasons an *in vivo* or *ex vivo* study could be more suitable to further explore this complex mechanism. However, the fact that p38 MAP kinase activation observed in hSERT-LLCPK did not produce a change of SERT function may suggest a different regulation of human SERT compared to rodents which may involve different secondary mediators other than p38 MAP kinase

Section 4.2 Infiammatory cytokines do not link with a regulation of serotonin transporter in rats treated with LPS

In animal models, treatment with LPS can elicit a series of behavioural changes called “sickness behaviour” such as reduction of food intake and withdrawal from the physical and social environment. Sickness is an adaptive response to infection by pathogens and fully reversible once the pathogen has been cleared. Considering the temporal aspects of symptom expression, sickness behaviour develops rapidly after administration of LPS in animals and usually peaks 2–6 h post-treatment. These effects are always associated with an increase of plasma cytokines and then the activation of inflammatory response. Sickness behaviour gradually resolves after 6h but this high level of cytokines in the brain evolves into depressive-like behaviour 24h post-LPS (Dantzer et al., 2008, Capuron and Miller, 2011). The indications that depression-like behaviour remained after sickness behaviour came from experiments in which LPS-treated mice displayed increased immobility in the tail-suspension test and in the forced-swim test 24 hours after treatment, a time point when motor activity had returned to normal (Frenois et al., 2007). The effect of cytokines on behaviour are similar to those showed by depressive patients such as anhedonia, fatigue, loss of energy and then this model, is often used to understand the link between cytokines and the pathophysiology of psychiatry disease. An expansive literature has proposed that cytokines can alter serotonergic neurotransmission in *in vitro* systems by the modulation of SERT function. However, a clear link between inflammation effectors and 5HT transporter activity in *in vivo* or *ex vivo* experiments was not well documented. Moreover, my previous *in vitro*

investigations showed no link between IL1β, p38 MAP kinase activation and SERT suggesting that a different approach had to be used to better understand this mechanism. A recent study reported that LPS induced a dose- and time-dependent alteration in SERT activity, measured *ex-vivo* in synaptosomes from C57BL/6 (inbred) and CD-1 (outbred) mice (Zhu et al., 2010). This effect was similar across different brain areas (frontal cortex, hippocampus, striatum and midbrain) and then was not dependent on tissue used. Moreover, SERT stimulation by LPS induced depressive-like behaviours because it was paralleled by increased immobility in both the tail suspension test (TST) and the forced swim test (FST). The stimulation of SERT activity and induced immobility were absent when LPS was administered to interleukin-1 receptor (IL-1R)-deficient mice and in the presence of SB203580. These data therefore confirmed that p38 MAP kinase and IL-1β receptors pathway are involved in the modulation of SERT and this caused a depressive-like behaviour in mice.

The same *ex vivo* approach was used in this PhD study where LPS was injected directly into the rat brain and then, after two hours, SERT function was measured in parallel with cytokine levels. Although pro-inflammatory cytokines involved in the modulation of SERT, such as IL-1ß and TNFα, showed a significant increase, a modulation of SERT activity in term of K_m and V_{max} was not detected, confirming again that a signalling cascade induced by cytokines does not affect the rat SERT function *in vitro* and *in vivo*. The results obtained using these different methodologies were quite unexpected because no effect on SERT was induced by the cytokines signalling pathways or p38 MAP kinase activation. These negative data can be explained by a potential influence of the

specific approaches chosen in this study, for example, the use of IL-1β as the reference cytokine in the modulation of SERT activity. This cytokine has an important role as mediator of the neurochemical alterations that occur in response to an immunological challenge (Dantzer et al 2006). Indeed, literature reported this direct modulation of SERT function (Zhu et al, 2005). However, clinical and preclinical evidence shows that other potential cytokines (IL-6, IL-2 and IFNγ) are important in the development of depressive-like symptoms and can modulate the level of some monoamines including 5HT (Song et al., 2002, La costa 2000, Anisman et al., 2007; Sato et al., 2006) Moreover, TNFα demonstrated a direct activation of SERT in a cell line (Mossner et al., 1998) and in mice synaptosomes (Zhu et al 2005).These findings suggest that IL-6, IL-2, IFNγ and TNFα may also modulate significantly serotonergic transmission in particular SERT function/expression. Another variable of this study was the brain regions (cortex, hippocampus and midbrain) in which the *in vitro* and *in vivo* study was conducted. They are relevant for many psychiatry diseases and monoamine transporters are highly expressed in these areas. Moreover, many effects following LPS or cytokines injection in animals were reported in these regions (Anisman et al., 2008, Hayley et al 2001). However, other regions may be more involved in the interaction with cytokines such as the basal ganglia. Basal ganglia and the subgenual and dorsal aspects of the anterior cingulate cortex (ACC) seem to be areas in which neurocircuits are affected by cytokines (Capuron et al., 2011). In particular, IFNγ increased glucose metabolism in nasal ganglia and cerebellum while it was decreased in dorsal prefrontal cortex (Capuron et al., 2007). Dopamine neurons are present in basal ganglia

especially in striatum (Smith and kieval 2004) and p38 MAP kinase showed a direct interaction with the dopamine transporter (Zhu et. al, 2005) suggesting a possible modulation of the dopaminergic system induced by cytokines in basal ganglia. The time of treatment both in *in vitro* and *ex vivo* experiments could be another important variable that needs to be discussed. Many *In vitro* data suggest a time dependent-effect of cytokines and p38 MAP kinase activation on monoamine transporter function (Zhu et. al., 2005) Moreover, in animal models, the duration of treatment seems a key factor in the change of neurochemistry induced by cytokines administration. For example, this was reported for IFNα and IL-1β where acute *vs* chronic administrations showed a different changes of monoaminergic levels and their metabolites in mice brain (Sato et al., 2006; Anisman et al., 2008). LPS treatment performed in this PhD lasted two hours and this produced a big cytokine increase without effect on serotonin uptake. However, it may be that a longer incubation or chronic treatment with LPS induced long-lasting change of the serotonergic system including change of SERT expression and consequently modification of 5HT level in the brain.

Despite the number of variables described above, the important finding emanating from this study is that the hypothesis in which cytokines alter the serotonergic system by modulation of SERT function through p38 MAP kinase was not confirmed and then this may be not a relevant mechanism in the pathology of depression induced by altered immune response. We demonstrated that direct activation of p38 MAP kinase or the stimulation of immune response in brain by LPS did not change the function or the expression of SERT. Serotonin is certainly connected with the effect of cytokines in the

alteration of brain function but probably 5HT participates more actively to the control of cytokines level which is the object of the second part of this PhD and will be discussed later (section 4.6). The hyphothesis describing the modulation of SERT and other monoamine transporters by cytokines lead to consideration of the selective serotonin reuptake inhibitors (SSRI) such as fluoxetine as a potential therapy in the treatment of psychiatry diseases associated with the abnormal immune response. However, this hypothesis should be revisited since findings generated in this study did not confirm the interaction between cytokines, p38 MAP kinase and SERT. A mechanism that can suggest an alternative efficient therapy in the pathophysiology of depression induced by cytokines which is well supported by *in vivo* and *in vitro* study is the activity of cytokines on indoleamine 2,3-dioxygenease (IDO). This enzyme converts the main precursor of serotonin, tryptophan (TRP), into kynurenine (KYN) and it has been demonstrated to be activated by cytokines, reducing TRP availability and consequently decreasing 5HT concentrations in the brain. In parallel, a cytokine-induced augmentation of KYN increases the neurotoxic components that may also produce an increase in the NMDA receptor agonist quinolinic acid in the brain. A depletion of TRP along with an increase of quinolinic acid and consequently an altered glutamatergic neurotransmission are strongly associated with depression (Muller & Schwarz, 2007).

Recently, literature reported that peripheral administration of LPS to mice led to increased expression of IDO in the brain along with IL-1β, TNFα, IFNγ and this effect was associated with an evidence of sickness behaviours measured by increased duration of immobility in both the forced-swim and tail suspension

tests. This effect was abolished by the administration of 1MT, a competitive inhibitor of IDO that normalized IDO expression without changing the increase of cytokine levels. The increase of the level of kynurenic acid after LPS administration strongly suggested that the increased ratio between kynurenic acid and tryptophan rather than a change in tryptophan levels was involved in causing the sickness behaviour. This hypothesis was also confirmed by the finding that KYN injected in rat brain dose dependently caused depressive-like behaviour (O'Connor et al., 2008).

A significant effect on TRP concentration was highlighted in patients with malignant melanoma treated with IFNα and IL-2 where TRP blood concentration significantly decreased during the therapy. This reduction was consistent with the development and intensity of mood and cognitive impairment symptoms (Capuron *et al*., 2002). Moreover, clinical studies of patients with chronic hepatitis C (HCV) infection treated with IFN-α showed that in blood TRP availability was significantly reduced over the therapy although IFN-α had no effect on CSF TRP concentrations.. The concentration of KYN increased significantly and there was a correlation between the increasing level of KYN and the rise in the severity of depressive symptoms, suggesting that IDO activated by IFN-α is involved in the pathophysiology of depression changing TRP levels (Raison *et al*., 2010).

Section 4.3 Interaction between $5HT_7$ receptors and p38 MAP kinase in glial cells

The neurotransmitter serotonin is involved in a number of processes through the central nervous system and this function is mediated by a large family of receptors. The $5HT_7$ receptor was the last addition to the large serotonin subfamily of G-protein coupled receptors. Considering the number of papers published in the serotonin receptor subtypes field, the $5HT_7$ receptor is not the most studied, being surpassed by $5HT_{1A}$, $5HT_{2A}$, $5HT_3$, and $5HT_4$ receptors. This might be explained by the lack for a long time period of selective $5HT_7$ receptor ligands that would help researchers to understand the pathophysiological role of this receptor. Thus, since antagonists and, to some extent, agonists became available as well as $5HT_7$ receptor-knockout mice, our understanding of the role of the $5HT_7$ receptor in normal and pathological processes has improved. One of the mechanisms that has been recently described but is not still clear is the involvement of $5HT_7$ receptors in the inflammatory process and this effect on the CNS functions. Previous investigations in primary rat hippocampal astrocytes treated with 5HT showed inductions of mRNA of IL-6 gene after 1 hour but the receptors subtypes involved were not explored in details (Pousset et al., 1996). The induction of IL-6 synthesis by 5HT was shown also in human vascular smooth muscle cells (Ito et al., 2000). Finally, other investigations suggested that $5HT_7$ receptors regulate the release of IL-6 from astrocytoma and microglia *via* p38 MAP kinase (Lieb et al., 2005; Mahe et al., 2004).

Lieb et al. (2005) showed that astrocytoma U373 MG treated with 100nM 5HT for a period of 120 min showed a significant increase of phospho p38 MAP kinase with a maximal response at 10 min. The release of IL-6 induced by 5HT and the p38 MAP kinase activation was fully reversed by 10μM $5HT_7$ receptors antagonist SB202190 confirming the interaction between $5HT_7$ receptors and p38 MAP kinase in U373 MG cells. Experiments performed in this PhD study revealed that $5HT_7$ receptors were expressed in rat cortical astrocytes and were functionally coupled with an increase of intracellular cAMP. Moreover, serotonin *via* $5HT_7$ receptors increased the p38 MAP kinase phosphorylation after 5 min from stimulus confirming that the signalling cascade of $5HT_7$ receptors involved p38 MAP kinase activation. All these data confirmed the importance of $5HT_7$ receptors in the regulation of serotonergic function in astrocytes and suggested an important role in the control of cytokine release through p38 MAP kinase.

p38 MAP kinase is an important regulator of cytokine intracellular signals especially in those processes that induce interleukin release. p38α was first isolated as a 38-kDa protein rapidly tyrosine phosphorylated in response to LPS stimulation (Han et al., 1994). Four splice variants of the p38 MAP kinase family have been now identified: p38α, p38β, p38γ (ERK6, SAPK3) and p38δ (SAPK4) (Zarubin and Han, 2005). The ELISA used was not selective for a specific p38 MAP kinase isoform and hence it is unknown which isoforms interact with $5HT_7$ receptors. The biological consequence of p38 MAP kinase activation can be various because different kinases, transcription factors and gene expression are activated downstream of p38 MAP kinase. A strong link has been established between p38 MAP kinase pathway and apoptosis, control of cell cycle,

cardiomyocyte hypertrophy, development and cell differentiation (Zarubin and Han, 2005). However the principal pathway involved with p38 MAP kinase is inflammation. Rheumatoid arthritis, Alzheimer's disease and inflammatory bowel disease are all postulated to be regulated in part by the p38 MAPK pathway (Johnson and Bailey, 2003; Hollenbach et al., 2004). The activation of the p38 MAPK pathway induces the production of proinflammatory cytokines (IL-1β, TNF-α and IL-6)(Salituro et al., 1999; Lee et al., 2000); induction of enzymes such as COX-2 (Badger et al., 1998); expression of intracellular enzymes such as iNOS, a regulator of oxidation (Da silva et al., 1997). In order to further confirm the role of $5HT_7$ receptors in the release of inflammatory factors in brain *via* p38 MAP kinase, studies are ongoing to determine the level of IL-6 in medium of cortical astrocytes after a stimulus with serotonin or AS19 at different time points. These results will be the basis of further investigations to indentify which cytokines are secreted following the activation of $5HT_7$ receptors in cortical astrocytes and the pathways involved such as PKA, PKC, Erk1.

Little is known about the role of $5HT_7$ receptors in inflammatory events especially in the CNS system. Considering the influence of cytokines on neuronal transmission associated with behavioural changing, it can be speculated that the role of $5HT_7$ receptors in the regulation cytokines may be linked with the antidepressive effect of $5HT_7$ receptors antagonists in *in vivo* models. Further experiments would be focused to better understand if a treatment with $5HT_7$ receptor antagonists could therefore decrease the level of cytokines in the brain and then dampen the negative impact of inflammatory

events in brain function. Moreover, microglia cells, derived from peripheral macrophages, secrete Th1 cytokines such as IL-12, whereas astrocytes inhibit the production of IL-12 and other type 1 cytokines and secrete the Type 2 cytokine IL-10. The type1/type2 imbalance in the CNS which could be caused by the imbalance in the activation of microglia and astrocytes, has already been observed in depression (Muller & Schwarz, 2007). $5HT_7$ antagonist could then have an effect in the balance of type1/tyep2 cytokines which is may be linked with depressive status.

Section 4.4 Expression of $5HT_7$ receptors in hippocampal neurons which control the activation of CREB but not p38 MAP kinase

Literature reports that a number of 5HT receptors subtypes that are positively coupled with adenylate cyclase, such as $5HT_4$ and $5HT_6$ and $5HT_7$ receptors, and these are expressed in hippocampus. The increase of cAMP induced by these 5HT receptors and the following signalling cascade may generally affect neuronal activity through a protein kinase activation, or change of gene expression. Previous studies revealed the expression of $5HT_7$ receptors in cortical astrocytes with a positive link with cAMP pathway and p38 MAK kinase. So far, no published data are available concerning the role of $5HT_7$ receptors in neurons and its possible interaction with inflammatory processes involving p38 MAP kinase.

The study performed in this PhD thesis characterized the expression of Gs-coupled 5HT receptors in primary cultured neurons, showing that the mRNAs

encoding for $5HT_6$, $5HT_4$, $5HT_7$ receptors are expressed in cortical and hippocampal neurons after one week in culture. However, further explorations revealed that cortical neurons did not respond to a treatment with 5HT, while hippocampal neurons showed a significant increase of cAMP levels with a pEC_{50} of 7.2±0.2. This value is in agreement with previous reports showing a 5HT-induced cAMP increase with a potency of 6.68±0.08 in rat cultured astrocytes (Hirst et al, 1997) and of 7.7±0.1 in guinea-pig hippocampal membranes (Thomas et al., 1999). In order to better define which receptors were actively involved in mediating 5HT stimulation of adenylate cyclase in hippocampal neurons, different antagonists were tested. The selective $5HT_6$ and $5HT_4$ receptors antagonists SB399885 (Mikami et al., 2008) and GR113808 (Hirst et al., 2006) did not antagonize the effect of 5HT, thus suggesting that neither $5HT_4$ nor $5HT_6$ receptors were involved. In contrast, $5HT_7$ receptor antagonist SB258719 completely blocked this response with a pIC_{50} of 7.95±0.09. Moreover, a shift of 5HT response curve produced by SB258719 confirmed a pK_B value of 8.23±0.1 with a competitive antagonist profile. This compound was fully characterized in various assays both in recombinant cells and native tissue. In binding experiments on HEK293 expressing recombinant human $5HT_7$ receptors and in guinea-pig hippocampal membranes, affinity values of SB258719 were 7.5 (n=2) and 7.2±0.1 (n=3) respectively (Thomas et al.,1998 and 1999) In cAMP functional experiments, SB258719 reversed the 5HT response with pK_b of 7.57±0.08 and 7.21±0.12 and 7.2 ±0.1 at human, rat rand guinea-pig $5HT_7$ receptors respectively (Mahe et al., 2004, Thomas et al.,

1999). All these data are in line with those obtained in this study, confirming the functional expression of $5HT_7$ receptors in hippocampal neurons.

All these data confirmed the expression of $5HT_7$ receptors in hippocampal neurons while $5HT_6$ and $5HT_4$ receptors do not appear to contribute to the cAMP signalling under these conditions. In this study, PCR primers did not discriminate the splice variants and therefore it is uncertain which $5HT_7$ isoforms are expressed which could have a different interaction with adenylate cyclase. The lack of 5HT response in cortical neurons could be explained by two reasons: the absence of Gs-coupled 5HT receptors expression in membranes under these conditions or the presence of Gi-coupled 5HT receptors that are negatively associated with adenylate cyclise balancing out an overall response. The lacks of selective agonists for the different classes of 5HT receptors complicate this analysis. However, the use of uncoupling agents such pertussis toxin (PTX) that blocks the responses mediated by protein Gi or selective antagonists for the different families of 5HT receptors, for example $5HT_1$ receptor antagonists, may help to explore these hypotheses.

In the attempt to understand the events downstream of 5HT stimulation, CREB and p38 MAP kinase phosphorylation were also measured. These experiments revealed an increase of phosphoCREB[133] induced by 5HT with a maximal effect 5 min after stimulation. The $5HT_7$ receptor antagonist, SB258719 significantly inhibited this response, confirming that $5HT_7$ receptors not only activated adenylate cyclase but were also directly involved in the phosphorylation of CREB[133]. This data confirmed the role of $5HT_7$ receptors in the regulation of CREB phosphorylation in hippocampal neurons (Mahgoub et

al., 2006). It can be noted that 10 μM SB258719 did not block completely the increase of CREB suggesting the presence of secondary signalling pathways activated by 5HT that are not inhibited by SB258719. In contrast, p38 MAP kinase was not affected by $5HT_7$ receptors stimulation over a period of 60 min. All these findings generated by using pharmacological and biochemical tools demonstrated that $5HT_7$ receptors regulate cAMP signalling in hippocampus with a modulation of CREB function. Increasing CREB phsphorylation and then its activation would be expected to alter the expression of specific target genes. Among the many potential target genes regulated by CREB are those for brain-derived neurotrophic factor (BDNF) and its receptor, TrkB (Duman et al.,1995, Condorelli et al., 1994). Taking into account these findings, it would be interesting to understand if serotonin *via* $5HT_7$ receptors alters the levels of BDNF and TrkB in hippocampus.

The analysis of p38 MAP kinase phosphorylation after the activation of $5HT_7$ receptors revealed lack of activation of this kinase in neurons over a period of 60 min. This was in contrast with those reported in astrocyte and astrocytoma cells that showed an activation of p38 MAP kinase 5 min after 5HT addition. Status of p38 MAP kinase was also unchanged using forskolin suggesting that an increase of cAMP was not an upstream signal for p38 MAP kinase activation in neurons. These data suggested that in both neurons and glia cells $5HT_7$ receptors couple with an increase of intracellular cAMP and activation of CREB, whereas the activation of $5HT_7$ receptors induced an activation of p38 MAP kinase only in glial cells and not in neurons. As described before, p38 MAP kinase exists in different isoforms and their activation can be specifically

controlled through different regulators and coactivated by various combinations of upstream regulators. In addition, the activation mechanisms of p38 MAP kinase may vary in different cells under various physiological or pathological conditions (Zarubin and Han., 2005). For this reason, it is complex to understand why in neurons p38 MAP kinase is not influenced by $5HT_7$ receptors. However, it may be hypothesized that neurons and glial cells express different cofactors that specifically link $5HT_7$ receptors with the activation of p38 MAP kinase. Finally, this work highlighted a different cell signalling of $5HT_7$ receptors in the CNS between glial cells and neurons.

Section 4.5 Serotonin induced a post-transcriptional modulation of AMPA receptors by $5HT_7$ receptors

The previous studies reported the expression of $5HT_7$ receptors in neurons and that their activation results in accumulation of intracellular cAMP and activation of CREB. The present study investigated also the interaction between $5HT_7$ receptors and AMPA receptors (AMPAR). $5HT_7$ receptors are involved in the synaptic transmission regulating the neuronal excitability and LTP in hippocampus (Roberts et al., 2004). Synaptic strength is tightly regulated by mechanisms that include a change in AMPA receptor (AMPAR) phosphorylation and their cellular distribution (Santos et al., 2009).

Phosphorylation of AMPAR occurs at three serine residues located in the intracellular C-terminus: serine 831 (ser831) can be phosphorylated by protein kinase C (PKC), and CaMKII; serine 845 (ser845) is a protein kinase A (PKA)

and cGMP-dependent protein kinase II phosphorylation site and serine 818 (ser818) is a substrate for PKC. Both Ser831 and Ser845 are important signals to deliver AMPARs into synapses and then determine the duration of synaptic activity. LTP induction increases the CaMKII-dependent phosphorylation of GluR1 at Ser831 (Mammen *et al.,* 1997). However, mutation on GluR1-Ser831 that prevented this phosphorylation, did not change the delivery of the receptor to synapses by active CaMKII (Hayashi *et al.,* 2000). In contrast, mutation of Ser845 prevented the delivery of GluR1 to synapses (Esteban *et al.,* 2003a) suggesting that Ser845 is critical for the delivery of AMPAR into synapses. In hippocampal neurons, I did not demonstrate a direct activation of PKA by $5HT_7$ receptors but cAMP increase and the following CREB phosphorylation observed after the stimulus suggested that PKA could be activated as well by $5HT_7$ receptors. This activation may trigger the Ser845 phosphorylation on AMPAR and then induce a change of AMPAR trafficking or expression which may be important for the control of neuronal plasticity in hippocampus. This study revealed that 5HT and AS-19 increased GluR1 phosphorylation at PKA site (Ser845) of about 12 and 3.5 folds respectively. These levels of activity reflected the intrinsic activity of these agonists established in the cAMP assay, suggesting a direct correlation between the level of cAMP and GluR1 phosphorylation. These effects were reversed by SB258719 but not by SB399885 and GR113808, confirming the involvement of $5HT_7$ receptors. The total GluR1 expression was also increased by 5HT but the above antagonists did not reverse this effect, suggesting that $5HT_7$, $5HT_6$ and $5HT_4$ were not involved. To the best of our knowledge this study was the first to demonstrate

an activation of GluR1 phosphorylation by $5HT_7$ receptors. Time course revealed that the GluR1 phosphorylation was maximal after just 5 min and remained at this level up to 30 min. The duration and onset of phosphorylation is regulated by the kind of kinase and phosphatase which are carried close to the effectors when receptors are activated. Previous work showed that $5HT_7$ receptors increased CREB phosphorylation in hippocampal neurons but these effects lasted maximum 5 min from stimulation. The different duration observed in the increase of GluR1 phosphorylation compared to CREB phopshorylation may suggests that $5HT_7$ receptors activate different kinase or phosphatase pathways specific for CREB or GluR1. The functional role of Ser 845 phosphorylation has not been fully understood although studies from literature hypothesized a specific mechanism by which phosphorylation of ser845 should increase AMPAR in the synaptic site which is important for the control of LTP (Wang et al., 2005). Basically, under basal conditions, GluR1 is expressed in the internal pools and on the surface membranes of neurons. The amplitude of synaptic potentiation is regulated by the distribution of these receptors that shift from the extrasynaptic to synaptic region during neuronal transmission (Passafaro et al., 2001). Ser845 phosphorylation induces an increase of surface GluR1 in the extrasynaptic AMPAR and then modulates the synaptic plasticity by regulating the pool of AMPARs available for synaptic incorporation (Oh et al., 2006). GluR1 phosphorylation was induced by D1 dopamine receptors which are coupled with AC. D1 receptor agonist increases GluR1 phosphorylation at the protein kinase A phosphorylation site (Ser845) in nucleus accumbens cell cultures (Chao et al., 2002), and cultured hippocampal neurons (Gao et al.,

2006). Moreover, a D1 receptor agonist on hippocampal pyramidal neurons accelerates AMPAR externalization in extrasynaptic sites through a pathway that depends mainly on PKA but also involves CaMKII (Gao et al., 2006). The effect of D1-PKA pathway on AMPAR trafficking plays an important role in the modulation of long-term synaptic changes observed in rats and in mice by dopamine (Huang et al.,2004). Based on these findings, further studies were designed to understand if $5HT_7$ receptors may mimic the effect of D1 receptors in hippocampus to control AMPA trafficking. After the stimulation with 5HT, proteins expressed on the surface of neurons were isolated and analysed by western blot. Results showed a significant increase of GluR1 phosphorylation in the surface pool while expression of total surface GluR1 was not altered. Moreover, the increase of surface GluR1 phosphorylation induced by 5HT was reversed by SB258719 and H89 confirming the involvement of $5HT_7$ receptors through the activation of PKA. These data then showed that $5HT_7$ receptors activate GluR1 phosphorylation at Ser845 in the surface pool *via* PKA without inducing GluR1 migration to the plasma membrane. The impact of this phosphorylation on AMPAR function in hippocampus remains to be explored since it did not seem to have an apparent effect on AMPAR trafficking. However, it may have an effect on AMPAR function determining a change of synaptic plasticity. This is better discussed in the section 4.5.2.

Section 4.5.1. PDE4 and PDE10 potentiate the effect of $5HT_7$ receptors on AMPAR in hippocampal neurons

Activation of adenylate cyclase and following accumulation of cAMP takes place in specific microdomains in the cells. These microdomains are created by physical interaction between different components of these signal cascade and structural elements of the cells. PDEs are a family of enzymes that degrade the cAMP and cGMP and dampen quickly the signal cascades induced by cAMP or cGMP as second messengers. PDE participate in the regulation of temporal and spatial dimension of these microdomains because they are sequestered and anchored to sites near the receptors that trigger the signal cascade. PDE's expression in the brain is tightly regulated and it is distributed in many regions where serotonin is present, such as hippocampus and cortex (Menniti et al., 2006). PDE4 is the principal family involved in the degradation of cAMP throughout the body (Houslay et al., 2003). In the CNS, PDE4 is expressed in striatum, cortex and hippocampus within soluble intracellular compartments (Perez-Torrez et al., 2000). Studies aimed to understand the physiological role of PDE4 described that, in hippocampus, it regulates the increase of CREB phosphorylation (Monti et al., 2006) and facilitate the induction of LTP (Barad et al., 1998). PDE10 enables to metabolize cAMP and cGMP and it is bound to the membranes (Menniti et al., 2006). It is expressed principally in striatum but is also present at low density in extrastriatal neurons, including those of the cortex and hippocampus (Seeger et. al., 2003). Further studies confirmed the expression of PDE4 and PDE10 in hippocampus where the chronic administration of rolipram (PDE4 inhibitor) and papaverine (PDE10 inhibitor)

increased CREB mRNA levels suggesting a role in the modulation of cAMP cascade. Moreover, a co-administration of rolipram with the 5HT reuptake inhibitor, imipramine induced a more rapid increase of CREB than either treatment alone suggesting an interaction between PDE and serotonergic system (Nibuya et al., 1996). However, the molecular mechanism that described the interaction between PDEs and serotonergic system and the functional significance in hippocampus were not clearly described. The present studies had the aim to understand if $5HT_7$ receptors expressed in hippocampal neurons interact with PDE4 and PDE10 in the regulation of GluR1 phosphorylation. Experiments showed that rolipram and papaverine increased the level of GluR1 phosphorylation and when they were co-incubated with 5HT there was a significant potentiation of 5HT response. This study revealed therefore a positive interaction between PDE4 and PDE10 with $5HT_7$ receptors in hippocampus.

Considering the key role of PDE4 and PDE10 in the modulation of cellular signalling pathway in the brain, they are a target for many CNS diseases. In particular PDE4 participates actively to cognitive process such as long-term memory formation, attention and executive function while there are some indications for the use of PDE10A inhibitors for the treatment of schizophrenia (Menniti et al., 2006). Increasing evidence suggests a role of PDE4 in depression since rolipram showed an antidepressant effect in humans (Zeller, 1984). Subsequent work in preclinical models suggests that the antidepressant effects of PDE4 inhibition results from an up-regulation of brain-derived neurotrophic factor (BDNF) and the subsequent facilitation of neurogenesis in

the hippocampus (Nakagawa et al., 2002). However, this PhD study suggested that behavioural effects induced in *in vivo* by PDE4 and PDE10 inhibitors may result in part by an increase in $5HT_7$ receptor function in hippocampus.

Section 4.5.2. Physiological effect of Ser845 phopshorylation induced by $5HT_7$ receptors on AMPAR function

The physiological impact of AMPAR phosphorylation induced by $5HT_7$ receptors on hippocRl neurons remains to be explored. Moreover, a recent publication reported that Ser845 phosphorylation on AMPAR induced by D1 receptors changed the AMPA current and channel open probability in spinal motoneurons using a whole-cell patch clamp techniques (Han and Whelan, 2009). This mechanism was independent of AMPAR mobilization because these effects were not blocked by the use of botulinum toxin C which is a blocker of receptor insertion. Moreover, inhibiting PKA with H89 completely abolished the effect of D1 agonist, which confirmed the involvement of PKA in the effect of D1 receptors stimulation on AMPAR current (Han and Whelan., 2009). The physiological effect of PKA phosphorylation on AMPAR was previously investigated in transiently transfected HEK-293 cells with human GluR1 using whole-cell patch clamp recording techniques. Perfusion of glutamate onto the transfected HEK-293 cells resulted in the rapid activation of inward current that desensitized in the continuous presence of agonist. Once stable baseline recordings was obtained, purified PKA was perfused into the recording pipette. This intracellular perfusion of PKA resulted in 40% potentiation of the peak amplitude of the whole cell glutamate-gated current. This potentiation

developed over 15 min after introduction of PKA into the pipette and remained stable for the duration of the recording. No change in the rate of desensitization or in the steady state response was seen following the addition of PKA. These findings suggest that PKA phosphorylation may modulate not only AMPAR trafficking but also AMPAR function. Hence, it can be speculated that phosphorylation induced by $5HT_7$ receptors act on AMPAR conductance rather than effecting AMPAR trafficking. AMPAR is an ionotropic glutamate receptor allowing the passage of sodium and potassium. The AMPAR also is permeable to calcium (Ca^{2+}) and hence regulates Ca^{2+} influx into neurons. This is the mechanism responsible for the NMDA receptor-indipendent long term potentiation in amygdala (Mahanty and Sah, 1998) and hippocampus (Feldmeyer et al., 1999). An assay that allows measurement of Ca^{2+} influx mediated by AMPAR uses a fluorometric imaging plate reader (FLIPR)/Ca^{2+} assay. I developed a FLIPR/Ca^{2+} assay on cultured hippocampal neurons with the aim to measure the effect of AMPA on Ca^{2+} influx and then understand if 5HT *via* $5HT_7$ receptors influences this activity. In parallel, FSK was also tested on Ca^{2+} influx evoked by AMPA because FSK showed a stronger GluR1 phosphorylation compared to 5HT and hence an effect on AMPAR function may be more evident. AMPA showed a concentration dependent increase in Ca^{2+} influx with a pEC_{50} of 6.14±0.05. Neither 5HT nor FSK changed significantly the pEC_{50} of AMPA or the maximal AMPA response suggesting that although the activation of $5HT_7$ receptors changed AMPAR phosphorylation, it did not have a specific effects on Ca^{2+} influx evoked by AMPA under the current assay conditions.

A different approach should be used to better understand the impact of $5HT_7$ receptors activation on AMPAR function. One possible method could be the use of a whole-cell patch clamp techniques that allows the fine measurement of AMPAR current in hippocampal neurons in the presence of $5HT_7$ receptor agonist or antagonist. However, it cannot be excluded that the effect of $5HT_7$ receptors on AMPAR function may require the involvement of additional signals such as the phoshorylation of Ser818 by PKC or Ser831 by CaMKII (Santos et al., 2009).

Section 4.6. Different functions of serotonin in hippocampus: neuroinflammation and synaptic plasticity.

The hypothesis of the interaction between inflammation and serotonergic transmission has been long discussed but the mechanism involved in this complex pathway has not been well clarified yet. The role of p38 MAP kinase and cytokines in the modulation of SERT function for the control of serotonin level was not supported by this study. Instead, important findings have been generated that confirmed the hypothesis of the influence of serotonin on p38 MAP kinase function in primary astrocytes *via* $5HT_7$ receptor activation. This activation is principally mediated by an increase of intracellular cAMP which determined a direct activation of PKA. Previous studies in mouse embryonic fibroblasts, NIH 3T3, showed that PKA represented the predominant pathway after cAMP increase and p38 MAP kinase becomes activated by PKA in the activation of CREB (Delghandi et al., 2005). These findings suggested that PKA may mediate p38 MAP kinase and then participate in the control of serotonin-

dependent cytokines release in primary astrocyte. The pathway suggested is: $5HT_7$ receptors→cAMP→PKA→p38 MAP kinase→cytokines release. This mechanism then suggests that $5HT_7$ antagonists may work as anti-inflammatory drugs and may contrast the negative effects of abnormal regulation of the immune response on behavior. There are many data that suggest that anti-inflammatory drugs have a positive impact on antidepressive therapy. For example the COX-2 inhibitor, celecoxib, increased significantly the antidepressant efficacy of the norepinephrine reuptake inhibitor, reboxetine (Muller et al., 2006). This study then suggested that $5HT_7$ antagonists alone or in combination with antidepressives may dampen the immune response in the brain and finally ameliorate depressive symptoms associated with inflammation. The influence of p38 MAP activation induced by $5HT_7$ receptors may be also connected with the serotonin synthesis, since a report showed an increase of p38 MAP kinase activity was associated with decreased cerebrospinal fluid (CSF) concentrations of the serotonin metabolite, 5-HIAA, in early life stress of juvenile rhesus monkeys (Sanchez et al., 2007). $5HT_7$ receptor antagonists may block the p38 MAP kinase activation induced by cytokines to prevent the decrease of serotonin levels.

Not only microglia and macroglia but also neuronal cells release cytokines (Bergamaschi et al., 2006). However, $5HT_7$ receptors expressed in hippocampal neurons did not induce p38 MAP kinase activation suggesting no control of cytokines release from neurons. Instead, this study highlighted a different role of $5HT_7$ receptors in neurons which was associated to a modulation of GluR1 phosphorylation at Ser845 with the contribution of PKA, PDE_4 and PDE_{10}. The

pathway suggested then is: $5HT_7$ receptors→cAMP→PKA→GluR1[Ser831]. Despite, this, the study did not demonstrate clearly an altered expression of AMPAR induced by $5HT_7$ receptors, so it can be speculated that GluR1 phosphorylation may increase the presence of AMPAR at the synaptic level and then modulate synaptic plasticity. Since PDE_4 and PDE_{10} are involved in this mechanism, this effect can be potentiated by the use of specific PDE_4 or PDE_{10} inhibitors such as Rolipram. Manipulating synaptic AMPAR trafficking has significant effect on learning, memory and drug addiction (Kessels and Malinow, 2009) and a dysfunction of the regulation of extracellular glutamate levels has been implicated in a number of neurological and psychiatric diseases including Alzheimer's disease, Parkinson's disease, Huntington's disease, schizophrenia, obsessive-compulsive disorder, and mood disorders (major depressive disorders and bipolar disorder) (Zarate and Manji et al., 2008). Moreover, chronic therapy with antidepressives showed an increase of AMPA expression in neurons (Martinez-Turrillas et al., 2002) and positive AMPA modulators has been suggested as an effective treatment for major depression. There is not a clear mechanism explaining why positive AMPA modulators may be useful in depression although they should slow the desensitization and/or deactivation of AMPA in the presence of an agonist (Hashimoto 2009). Finally, a hypothesis can be formulated that $5HT_7$ receptor agonists increase AMPA receptor phosphorylation thus increasing the glutamatergic neurotransmission mediated by AMPAR at the synaptic level and this can be beneficial for neurocognitive disorders involving the glutamatergic response. However, it can not be excluded that an exaggerated stimulation of AMPAR can produce also an excessive

glutamate response which may induce the development of cytotoxicity *via* NMDA or non-NMDA receptors as suggested by literature (Larm et al., 1997). Overall, further investigations need to be performed to better understand the potentiality of $5HT_7$ receptors as novel targets for neuropsychiatric disorders.

Section 4.6. Conclusion

The present study had the principal aim to demonstrate the molecular interaction between some mediators of inflammation (cytokines and p38 MAP kinase) with the serotonergic system *in vitro* and *in vivo*. Two different pathways were investigated: SERT modulation induced by cytokines and the role of $5HT_7$ receptors in the control of cytokines release *via* p38 MAP kinase. The present study demonstrated that incubation with IL-1β or the activation of p38 MAP kinase did not have any effect on 5HT uptake in rodent native brain tissue (cortex, midbrain and striatum), in human platelets and in cell lines, in contrast to literature reports (Zhu et al., 2010). The lack of effect may be due to the absence of key factors for the activation of p38 MAP kinase in these systems and the consequent modulation on SERT. For this reason a different approach was then used in which a release of cytokines was induced directly in the rat brain through an i.c.v. LPS treatment followed by the analysis of SERT function. Although pro-inflammatory cytokines are involved in the change of animal mood such as IL-1ß and TNFα and displayed a significant increase in cortex and striatum, a modulation of SERT activity in term of K_m and V_{max} was not detected, confirming again that no interaction between cytokines, p38 MAP

kinase and SERT function *in vitro* nor *in vivo* was evident. However, this study revealed a positive interaction between $5HT_7$ receptors and p38 MAP kinase in glial cells supporting the role of 5HT *via* $5HT_7$ receptors in the regulation of cytokines release in the brain. However, this pathway was not present in hippocampal neurons where $5HT_7$ receptors did not activate p38 MAP kinase but instead increased the AMPAR and CREB phosphorylation at PKA dependent phosphorylation sites. The effect on AMPAR was reversed by the specific $5HT_7$ antagonist, SB258719 and PKA inhibitor, H89, confirming the specificity of response for $5HT_7$ receptors and the involvement of PKA in the mediation of AMPAR phosphorylation. PDE4 and PDE10, which are expressed in hippocampus, impact $5HT_7$ receptors functions since their specific inhibition potentiated the effect of 5HT on AMPAR phosphorylation. Further experiments highlighted that $5HT_7$ receptors changed the surface AMPAR phosphorylation without a specific effects on Ca^{2+} influx evoked *via* AMPAR. Noteworthy, this is the first report that showed a positive interaction between $5HT_7$ receptors and AMPAR which will hopefully stimulates new investigations concerning the role of $5HT_7$ receptors in neuronal plasticity.

Chapter 5 References

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