Université de Montréal

Expression des récepteurs EphA dans le raphé dorsal néonatal et adulte

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Expression des récepteurs EphA dans le raphé dorsal néonatal et adulte

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Résumé français

Des travaux antérieurs réalisés *in vitro*, dans notre laboratoire, ont montré que des neurones sérotoninergiques (5-HT) dissociés du raphé dorsal (DR) peuvent reconnaître des signaux spécifiques de guidage axonal dans des membranes cellulaires extraites à partir de diverses régions du cerveau néonatal, comme le cortex cérébral (Ctx), le striatum (Str) et le mésencéphale ventral (VM). Cette activité de guidage a été diminuée dans les membranes traitées avec la phospholipase-C spécifique du phosphatidylinositol (PI-PLC), qui enlève les protéines à ancrage membranaire par lien lipidique glycosylphosphatidylinositol (GPI). Un traitement des membranes avec la protéine de fusion EphA3-Fc, qui bloque les éphrines-A a eu un effet similaire, quoique moins grand. Les éphrines-A sont des protéines membranaires à ancrage GPI ayant une haute affinité pour les récepteurs EphA, qui ont des rôles reconnus dans le guidage axonal. Elles représentent ainsi des candidates appropriées pour cette activité de guidage des axones 5-HT. Les objectifs du travail actuel étaient d'examiner la présence d'éphrines-A dans les extraits de membrane de Ctx, de Str et de VM néonatals de même que l'expression de récepteurs EphA dans le DR d'embryons, de nouveau-nés et d'adultes murins. Des transferts western avec des anticorps spécifiques ont démontré la présence des éphrines-A4 et -A5 dans les membranes extraites du Ctx, Str et VM néonatals. D'autre part, l'hybridation *in situ* avec des ribosondes marquées à la digoxygénine a montré l'expression d'EphA4, EphA5 et EphA7 dans la région du DR de rats fœtaux ou néonatals et de souris adultes. D'autres expériences, avec double marquage de la région du DR par immunocytochimie de la 5-HT et hybridation *in situ* des récepteurs EphA, seront nécessaires pour confirmer si les neurones marqués dans la région du DR comprennent des neurones 5-HT, et si tous les neurones 5-HT sont marqués uniformément

avec les 3 sondes. L'hybridation *in situ* en fluorescence (FISH), avec des ribosondes d'EphA pourrait aussi permettre dans le futur d'examiner l'expression de ces récepteurs dans des neurones 5-HT dissociés, en culture.

Mots clés :

Neurobiologie, développement, guidage axonal, raphé dorsal, sérotonine, cortex cérébral, striatum, mésencéphale ventral, éphrines, récepteurs Eph, hybridation *in situ,* transfert western

Résumé anglais

Previous *in vitro* experiments in our laboratory have shown that serotonergic (5-HT) neurons dissociated from the dorsal raphe (DR) region recognize specific axon guidance signals in cellular membranes extracted from various target brain regions, i.e. neonatal cerebral cortex (Ctx), striatum (Str) and ventral midbrain (VM). This axon guidance activity was decreased in the membranes treated with the phosphatidylinositol-specific phospholipase-C (PI-PLC), which removes the glycosylphosphatidylinositol (GPI) anchors, as well as in membranes treated with the fusion protein, EphA3-Fc, which blocks ephrin-As. Ephrin-As, being the GPI-anchored membrane ligands of EphA receptors, represent proper candidates for this axon guidance activity.

The objectives of the present work were to examine the presence of ephrin-A ligands in membrane extracts from neonatal Ctx, Str and VM, as well as the expression of EphA receptors in the DR of embryonic, neonatal and adult rodents. Western blot experiments with specific antibodies showed the presence of ephrin-A4 and ephrin-A5 in membrane extracts of Ctx, Str and VM. *In situ* hybridization experiments with digoxygenin-labelled riboprobes showed a high expression of EphA4, EphA5 and EphA7 in the DR of fetal or neonatal rat, as well as of adult mouse.

Further experiments, with double labelling of the DR with 5-HT immunocytochemistry combined with *in situ* hybridization for EphA receptors, will tell us if the cell bodies labelled in DR include 5-HT neurons, and whether all 5-HT neurons are uniformly labelled with the 3 riboprobes. Fluoroscent *in situ* hybridization (FISH) with EphA riboprobes could also be used to determine the expression of these receptors in dissociated 5-HT neurons in culture.

Key words:

Neurobiology, development, axon guidance, dorsal raphe, serotonin neurons, *in situ* hybridization, ephrins, Eph receptors

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5-HT: 5-hydroxytryptamine, (sérotonine)

CNS: central nervous system (système nerveux central)

CSF: cerebrospinal fluid (liquide cérébro-spinal)

Ctx: cerebral cortex (cortex cérébral)

DR: dorsal raphe (raphé dorsal)

Eph: Eph receptor (récepteur Eph)

FGF: fibroblast growth factor (facteur de croissance des fibroblasts)

GPI: glycosylphosphatidylinositol

GTP: guanosine tri-phosphate

MAO: monoamine oxidase (monoamine oxydase)

PET: positron-emitting tomography (tomographie à emission de positons)

PI-PLC: phosphatidylinositol-specific phospholipase C (phospholipase-C spécifique du phosphatidylinositol)

REM: rapid eye movement (mouvements rapides des yeux)

SCZ: schizophrenia (schizophrenia)

SERT: Serotonin transporter (transporteur de la sérotonine)

Shh: sonic hedgehog

SSRI: selective serotonin re-uptake inhibitor (inhibiteur spécifique de la recapture de sérotonine)

SWS : slow wave sleep (sommeil à ondes lentes)

Str: striatum

VM: ventral midbrain (mésencéphale ventral)

VMAT: vesicular monoamine transporter (transporteur vésiculaire des monoamines)

Dedications:

To my dear father and mother; Nasy and Fariba

My lovely brother and sister; Nazan and Mahbod

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1. Introduction

Serotonin (5-hydroxytryptamine or 5-HT) is an important neurotransmitter, taking part in diverse functions during development and at maturity, in neuronal and non-neuronal cells, including pain perception, sleep/wake cycles and neurodevelopment. Likewise, as exemplified below, disruptions in 5-HT neuronal development or 5-HT neurotransmission appear to be involved in mood disorders, cognitive dysfunction, mental retardation, anxiety and aggression.

Therefore, a better knowledge of the molecules involved in the development of the 5-HT neurons is currently needed. There has been some progress, recently, in the identification of factors influencing the differentiation of 5-HT neurons (see below), but little is currently known about the molecules that guide 5-HT axons to their multiple innervation targets (Sharif Askari, 2006).

Recent work in our laboratory show that 5-HT neurons of the dorsal raphe (DR) can recognize axon guidance signals in membrane purified from different brain regions, i.e., cerebral cortex (Ctx), striatum (Str) and ventral midbrain (VM) (Petit *et al.*, 2005; Sharif Askari, 2006). Some of these signals were sensitive to prior treatment of the membranes with phosphatidylinositol-specific phospholipase-C (PI-PLC), removing GPI-anchored membrane proteins. Among those signals, some were inducing the branching of 5-HT axons. This latter effect of the membranes was inhibited by a prior treatment with a fusion protein comprising the extracellular domain of the EphA3 receptor and the Fc domain of human immunoglobulin (EphA3-Fc); an agent interfering with the binding of ephrin-As with EphA receptors. Thus, these results suggest a role for the ephrin-A and EphA guidance molecules in the branching of 5-HT axons.

The present work was undertaken to further test this hypothesis, by examining the presence of ephrin-A molecules at the level of 5-HT innervation targets (Ctx, Str and VM), as well as the expression of EphA receptors by 5-HT neurons of the DR.

In the following paragraphs, we will first review briefly the basic current knowledge on 5-HT neuronal systems, their roles in some neurological diseases, and the molecules involved in their development.

1.1 Serotonin metabolism and neurotransmission

1.1.1 Biosynthesis of 5-HT

In 1930, Erspamer and colleagues identified a molecule from enterochromaffin cells in the gastrointestinal tract that they named "enteramine". In 1948, Page and colleagues isolated a vasoconstrictor substance in blood serum that they named serotonin (Lozeva-Thomas, 2004; Pucadyil *et al.*, 2005).

Only about 1-2% of total body 5-HT is present in the central nervous system (CNS). Serotonin itself cannot pass the blood brain barrier, and must be synthesized locally. In the CNS, it is synthesized by 5-HT neurons, from the precursor amino acid L-tryptophan, through two enzymatic steps. The first step is hydroxylation of tryptophan that takes place in the neuronal cytosol, catalyzed by the enzyme tryptophan hydroxylase. It is the rate-limiting step in 5-HT synthesis and produces 5-hydroxytryptophan. The second step is catalyzed by aromatic L-amino acid decarboxylase, which decarboxylates 5-hydroxytryptophane to 5-HT. Tryptophan is present in blood at high concentrations depending on dietary supply. The availability of tryptophan in the CNS depends on the balance between cerebral demand and the rate of tryptophan active transport through the blood brain barrier. For this specific transport, tryptophan has to compete with other neutral

amino acids, like phenylalanine, leucine and methionine (Russo *et al.*, 2003). In normal conditions, tryptophan hydroxylase is not completely saturated by tryptophan, so any change in the transport of this aminoacid across the blood-brain barrier may affect the synthesis rate of 5-HT and 5-HT-dependant behaviors or functions (Frazer & Hensler, 1994).

1.1.2 Serotonin storage and release

Like other neurotransmitters, 5-HT is primarily stored in storage vesicles. It is accumulated into the vesicles by the vesicular monoamine transporters (VMAT-1, present in endocrine and paracrine cells in peripheral organs, or VMAT-2, the predominant form in CNS). VMAT-2 may transport 5-HT, dopamine, adrenaline, noradrenaline or histamine. It uses the proton gradient that is present across the vesicular membrane, as the motive force.

The release of 5-HT into the synaptic cleft depends on the firing rate of 5-HT neurons, but is also regulated by somatodendritic and terminal autoreceptors (Squire LR, 2003).

Other molecules may also regulate 5-HT release, such as Inhibitory Protein Factor, or IPF, which has been shown to inhibit the vesicular uptake of 5-HT - or other neurotransmitters -, and thereby influence the size of its quantal release (Tamura *et al.*, 2001).

1.1.3 Inactivation of released 5-HT

Serotonin neurotransmission also depends on its reuptake from the synaptic cleft. Eighty percent of released 5-HT is inactivated by reuptake (active membrane transport) and recycling. The 5-HT transporter (SERT) belongs to a family of Na^+/Cl^--dependent

transporters with 12 transmembrane domains (Iversen, 2006). Inside the nerve terminal, 5-HT is catabolized by mitochondrial monoamine oxidase (MAO) into 5-hydroxyindole acetaldehyde, and then oxidized to 5-hydroxyindoleacetic acid, the main metabolite of 5-HT in CNS (Cooper JR, 1996). Monoamine oxidase exists in two forms, MAO-A and MAO-B, that have different distributions, substrate selectivities and structures (Saura *et al.*, 1992). Monoamine oxidase-A preferentially oxidizes 5-HT and norepinephrine, whereas MAO-B preferentially oxidizes phenyl ethylamine (see Nishi *et al.*, 2006). Interestingly, it is MAO-B, which has a lower affinity for 5-HT, which is expressed in 5-HT neurons (and glia).

1.1.4 Serotonin receptors

There are at least 14 different 5-HT receptors, classified into 7 major types, named 5-HT_1 to 5-HT_7, with subtypes for 5-HT_1 (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}), 5-HT_2 (5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}), and 5-HT_5 (5-HT_{5A}, 5-HT_{5B}) receptors. With the exception of 5-HT3, which is an ionic channel receptor, the others are G-protein-coupled receptors with 7 transmembrane-spanning domains. The 5-HT_1 subtypes and 5-HT_4 activate or inhibit adenylate cyclase, whereas 5-HT_{1C} and 5-HT_2 stimulate the phospholipase-C (PLC) pathway that increases the intracellular levels of diacylglycerol and inositol 1,4,5-triphosphate (Oh *et al.*, 2001).

Among the 5-HT receptors, 5-HT_{1A} has been the most extensively studied. It has been stably expressed into several neuronal and non-neuronal cell lines, and it is the earliest receptor appearing during development, with its highest peak during the prenatal period (Whitaker-Azmitia, 2005). It is encoded by a gene lacking introns. It is thought to have an active role during neural development and a neuroprotective activity against apoptosis and degeneration. It has its highest expression in hippocampus, raphe nuclei, amygdala,

hypothalamus, and cortex and its lowest expression in the basal ganglia, substantia nigra and cerebellum. This pattern of expression is consistent with 5-HT roles in thermoregulation, aggressive and sexual behaviour, mood, appetite and sleep-wake cycle. At the sub-cellular level, 5-HT_{1A} is located in the somato-dendritic region of raphe neurons, where it acts as an autoreceptor inhibiting 5-HT cell firing through inhibition of cAMP. In hippocampus, it is found on postsynaptic neurons, acting as a heteroreceptor. Because of the various known functions of 5-HT_{1A}, this receptor has been considered as a pharmacological target for several disorders, notably neuro-developmental impairments such as autism (Stamford *et al.*, 2000; Pucadyil *et al.*, 2005).

The 5-HT_2 family subtypes, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}, are similar in structure, pharmacology and signal transduction pathway. The genes of 5-HT_{2A} and 5-HT_{2B} receptors have 2 introns, whereas there are 3 for 5-HT_{2C}. All 3 bind to phospholipase-C and mobilize intracellular calcium. Antagonists of 5-HT_2 have been considered as potential treatment for schizophrenia, anxiety, sleep disorders and migraine. Receptor 5-HT_3 is a ligand-gated ion channel. It is highly expressed in the dorsal vagus complex, in brainstem, a center initiating and coordinating the vomiting reflex; and thus, 5-HT_3 antagonists are used as antiemetic medication. Its overall expression is low in the forebrain. The 5-HT_4 receptor, modulates dopamine and acetylcholine release in CNS, and its activation increases cognitive performance, with also some adverse reactions, like anxiety (Barnes & Sharp, 1999).

Receptors 5-HT_5, 5-HT_6, and 5-HT_7 were discovered and cloned about ten years ago, but there is still little known about their functions or signaling pathways. There is some evidence that 5-HT_6 might be involved in appetite, cognition, learning, psychosis, or convulsions (Glennon, 2003).

1.2 Serotonin impairments in neurological or psychiatric diseases

Serotonin, being an ubiquitous neurotransmitter in the CNS, has naturally been involved in various neurological and psychiatric disorders. We will briefly discuss the current state of knowledge for a few of these disorders.

1.2.1 Migraine

Migraine is a chronic neurological disorder manifesting as attacks of severe unilateral and pulsatile headaches associated with nausea, photophobia and increased reactivity to sensory stimuli. The crisis is aggravated by physical activity, and may be accompanied by an aura. It has a one year prevalence of 13% and is more prevalent in women (Linde, 2006).

In migraine subjects, the plasma levels of 5-HT are lower, and those of 5-hydroxyindoleacetic acid (5-HIAA) higher than in normal individuals between attacks, whereas the reverse occurs during migraine attacks. It thus seems that blood 5-HT metabolism increases in migraine subjects during headache-free periods, but decreases during attacks (Ferrari *et al.*, 1989).

Tryptophan depletion in migraine patients increases headache and nausea episodes in comparison with normal individuals (Drummond, 2006). Receptors 5-HT_{1B} and 5-HT_{1D} play important roles in migraine modulation, via mechanisms known to mediate vascular smooth muscle constriction and to inhibit vasoactive peptide synthesis. Treatment with 5-$HT_{1B/1D}$ receptor agonists, like sumatriptan and zolmitriptan, decreases the attack rates in migraine headache subjects. Serotonin can also produce dilatation of intra and extra cranial vessels via 5-HT_7 receptors. The mRNA of 5-HT_7 is highly expressed in cranial vessels and 5-HT_7 activation may induce excitatory activity in the nervous system and cause

hyperalgesia and neurogenic inflammation. Most of the 5-HT receptor antagonists used in prophylaxis of migraine have a high affinity for 5-HT_7 receptors. Reduction of this receptor can also decrease the rates of migraine attacks in susceptible individuals (Terron, 2002).

1.2.2 Serotonin in sleep disorders

For the first time, in 1958, Bradley found out that injection of 5-HT in the lateral ventricles of a cat, induced a short period of arousal followed by drowsiness and sleep. Jouvet, in 1967, found that lesions of the raphe nuclei region induced a lack of sleep, while the size of the lesions correlated positively with sleep loss and negatively with remaining brain 5-HT (Portas *et al.*, 2000).

Serotonin is viewed as a sleep and a wake neurotransmitter. The levels of extra cellular 5-HT, and the discharge rates of 5-HT neurons in DR increase during waking periods, and decrease progressively during slow wave sleep (SWS) to reach a minimum level during paradoxical sleep, or rapid eye movement (REM) sleep. During SWS, the decrease in 5-HT levels is mediated by an activation of inhibitory GABA in the DR region. In REM phase, histamine and phenylephrine antagonists reverse the inhibition of 5-HT neurons, but GABA antagonists are without effect during this phase (Adell *et al.*, 2002).

Different mechanisms take part in the activation or inhibition of 5-HT neurons during the sleep/wake cycle (Portas *et al.*, 2000). The inhibitory effect of cholinergic neurons is mediated via 5-HT_{1A} receptors on 5-HT neurons of the DR, during the REM phase. Local activation of 5-HT_{1A} in DR region augments REM sleep in both rats and cats. Agonists of 5-HT_{1B}, dose-dependently increase the waking periods and reduce REM sleep, without effect on the SWS phase. Receptors 5-HT_{1B} are located on cholinergic, glutamatergic and GABAergic axonal terminals. Serotonin also acts on 5-HT_{2A} and 5-HT_{2C}

receptors located on thalamic reticular nucleus neurons, inducing arousal. The 5-HT_2 receptor is also involved in the regulation of SWS and respiratory control during sleep. Serotonin has a facilitatory effect on SWS, via $5HT_{2B}$ receptors, and an inhibitory effect mediated by 5-HT_{2A}. Agonists of 5-HT_2 also increase sleep apnea attacks during SWS. (Popa *et al.*, 2005)

Narcolepsy, a well-recognized type of hypersomnia, starts with excessive daytime sleepiness that can lead to cataplexy in a few years. Cataplexy is defined as a skeletal muscle atonia, usually triggered by emotional stimuli, that usually lasts about 2 min. The inhibitory pathways of descending motor neurons are excessively activated during cataplexy attacks (Young & Silber, 2006). PET scan experiment using a 5-HTI_{1A} radioligand in narcoleptic patients showed that the binding potential of this receptor was increased significantly during sleep versus wakefulness, in whole brain and, specifically, in temporal, mesial temporal, and cingulate cortex. This finding demonstrates that there is increased availability of 5-HTI_{1A} receptor in the sleep phase in narcoleptic humans. (Derry *et al.*, 2006).

Sleepwalking (somnambulism) is a special type of parasomnia characterized by several motor behaviours, including walking, running and aggressive behaviours that start during stages 3- 4 of SWS. Sleepwalking is more common in children, and could thus be assumed to be a development disorder. During sleepwalking periods, the firing rate of 5-HT neurons increases and, interestingly, paroxetine (a SSRI), which increases SWS, can also induce sleepwalking episodes. Febrile illness can be a precipitating factor for somnambulism. During fever, the total brain concentration of 5-HT increases, and could be the trigger for sleepwalking episodes (Juszczak & Swiergiel, 2005).

1.2.3 Depression

Major depressive disorder is an important clinical problem with lifetime risk of 15-20% of the general population. The prevalence is twice in women than in men and is associated with a high risk of suicidal behaviour (15%) (Albert & Lemonde, 2004). Symptoms include depressed mood or reduced interest, non reactive mood, helplessness, somatic and psychic anxiety, hopelessness, change in sleep and appetite (decrease or increase), impaired concentration and overexpression of anger.

The relationship between 5-HT and depression was realized for the first time in 1975 when reserpine, a monoamine depleting agent (used to decrease blood pressure) induced depressive symptoms in susceptible patients. Altered 5-HIAA concentration in the CSF of depressed cases and suicide victims supported the 5-HT hypothesis. Tryptophane-free diet is also known to induce a relapse of depressive symptoms in patients that have been treated with an SSRI.

Detection of susceptible genes for affective disorders, like major depression, is complicated by the multiple phenotypes, small effect of individual genes and interaction between environmental and genetic factors. Genetic polymorphism of 5-HT-associated molecules, like SERT or MAO, are considered as candidates in affective disorders, including major depressive disorders (Cryan & Leonard, 2000).

The 5-HT_{1A} receptor has been implicated also in the pathology of major depression. Positron-emitting tomographic (PET) imaging using a 5-HT_{1A} radioligand showed that this receptor's binding potential is reduced in depression, particularly in midbrain raphe (Drevets *et al.*, 1999).

The hypothalamic-pituitary-adrenal axis is dysregulated in a large proportion of depressed patients, leading to increased secretion of cortisol. This hypercortisolemia may contribute to aetiology of depressive symptoms. Chronic administration of corticosterone in rats induced reduction in sensitivity of 5-HT_{1A} in DR, whereas such effect was not reported in rats with acute administration corticosteroids (Fairchild *et al.*, 2003).

Serotonin dysfunction is also implicated in suicidal behaviour, in depressed cases. A decrease in 5-HT_{1A} signal transduction (coupling with adenylate cyclase) was reported in suicide victims (Hsiung *et al.*, 2003).

Some transcription factors might potentially be involved in altering the levels of brain 5-HT_{1A}. For example, Freud-1 is a transcription factor with a helix-loop-helix DNA binding domain, a calcium-dependent binding domain, a PKC conserved region and a phospholipid binding domain. Its mRNA and protein have been detected in the raphe nuclei, cerebral cortex, hippocampus, and hypothalamus and they co-localize with 5-HT_{1A}. An intact calcium binding domain is necessary for the function of Freud-1. Alteration in the level of Freud-1 expression, or activation of Freud-1 by decreased levels of intracellular calcium, down regulates the expression of 5-HT_{1A} receptors. Thus, altered expression of such factors might be implicated in depressive disorders, even if the 5-HT_{1A} gene itself is not affected (Albert & Lemonde, 2004).

Sleep deprivation is an effective treatment for mood swings in depressive patients. There are several mechanisms underlying the effects of sleep deprivation on depressed cases. In rat, sleep deprivation causes a down regulation of norepinephrine and 5-HT transporters, and increases the availability of NE and 5-HT in the synaptic space. This effect is the same that has been observed during intake of certain antidepressants in several brain regions (Hipolide *et al.*, 2005). Also consistent with an antidepressant effect of sleep

deprivation is that sleep deprivation also increases 5-HT levels in hippocampus (a region highly implicated in the pathophysiology of depression) in rat models (Lopez-Rodriguez *et al.*, 2003). After 24 hours of sleep deprivation, the highest 5-HT increase, in comparison with normal controls, was observed in the hypothalamus, in line with the known role of this area in regulating sleep/wake cycles and with the participation of 5-HT in this function (Senthilvelan *et al.*, 2006).

1.2.4 Schizophrenia

Schizophrenia (SCZ) is a catastrophic disease with a prevalence of 0.5 to 1.0 percent of the population. The profound and pervasive cognitive and emotional disturbances that characterize SCZ suggest a serious underlying brain disease affecting multiple functions and systems. Clinical symptoms of SCZ are divided in two categories: positive symptoms like delusion, hallucination, disorganized speech and bizarre behaviour, and negative symptoms such as anhedonia, poverty of speech, flat affect, social withdrawal and psychomotor retardation. The etiology of the disorder is complex. Genetic, early environmental risk factors (maternal infection, obstetric complications, later spring/winter birth) and late environmental factors (immigration status, chronic cannabis use, stress), uniquely or in combination, are believed to give rise to the disorder (Winograd-Gurvich *et al.*, 2006).

Several genes have been potentially associated with SCZ, including neuregulin-1 dysbendin-1, and Disrupted-in-Schizophrenia-1 (DISC-1) (Ishizuka *et al.*, 2006; Ross *et al.*, 2006).

The classical hypothesis for SCZ is the dopamine hypothesis, implicating a hyperactivity of dopamine receptors (D2) in the mesencephalic projections to the limbic

striatum. Nevertheless, a 5-HT hypothesis of SCZ has emerged, when it was found that LSD (d-lysergic acid diethylamide) had a strong inhibitory effect on 5-HT neurons of the DR, and that the clinical presentation of LSD-like hallucinations was very similar to SCZ. There is a potent correlation between the affinity of hallucinogenic drugs for 5-HT_{2A} receptors and the level of hallucination in human subjects. Hallucinogens increase the rate of glutamate release in the neocortex, via activation of 5-HT_{2A} receptors. Glutamate release activates NMDA-type receptors and induces excitatory activity in cerebral cortical neurons, a pattern similar to that observed in the hallucinating phase of SCZ (Aghajanian & Marek, 2000). Moreover, the amounts of 5-HT_{1A} and 5-HT_{2A} are altered in SCZ (respectively increased and decreased) and certain polymorphisms for 5-HT_{2A} gene have been associated with SCZ (Miyamoto *et al.*, 2003).

Positron-emitting tomographic studies, in at risk individuals, showed decreased expression of 5-HT_{2A} in prefrontal cortex. This pattern of alteration was also observed in psycho-affective disorders, indicating that, perhaps, these two categories share common 5-HT impairments in prefrontal cortex (Hurlemann *et al.*, 2005). The typical antipsychotics that block D2 receptors only decrease the positive symptoms of SCZ, whereas atypical antipsychotics, like Clozapine whose effect is mediated by 5-HT_{2A}, is a potent medication for the control of negative symptoms. It thus seems that there is a correlation between 5-HT_{2A} receptors and negative symptoms in SCZ (Akhondzadeh, 2001; Sawa & Snyder, 2002).

Post-mortem studies showed altered levels of other 5-HT receptors in the cerebral cortex of subjects with SCZ, but it is not always clear that this alteration results from the antipsychotic medication, or participates in the pathophysiology of SCZ. A recent study showed a significant decrease in the binding capacity of a radioligand, SB269970, specific

for 5-HT_7, in Brodmann's area 9 of SCZ patients that were never treated with such medication. The same study reported no significant differences in the binding of sumatryptan, which specifically binds both 5-HT_{1D} and 5-HT_{1F}. It was thus concluded that 5-HT_7 might be involved in pathophysiology of SCZ (Dean *et al.*, 2006).

1.2.5 Down Syndrome

Down syndrome is a common chromosomal anomaly, with a prevalence of 1 in 800 live births. It results from the trisomy of chromosome 21, and is the most common genetic cause of mental retardation. The risk of having an affected child increases with maternal age. The affected children show early mental retardation and aberrant behavior, as well as cognitive dysfunction with Alzheimer's disease-type neurodegeneration in early adulthood.

Down syndrome is accompanied with lower levels of 5-HT in blood, CSF and brain (post-mortem). The gene coding for 5-HT_{1A} is located on chromosome 21. In normal fetuses, the levels of 5-HT_{A1} reach a peak in hippocampus and cerebral cortex, at approximately 24 weeks of gestation. In affected fetuses, the levels of 5-HT_{1A} are below those of the normal individuals at 24 weeks of gestation and remain low in the brain of neonates (Okado *et al.*, 2001; Stasko *et al.*, 2006).

In affected adults, there are region-specific anomalies in 5-HT innervation. Serotonin levels are decreased in caudate nucleus and temporal cortex, whereas higher amounts were reported in occipital and frontal cortex (Seidl *et al.*, 1999; Whitaker-Azmitia, 2001). The level of SERT is also significantly increased in prefrontal cortex, but not in cerebellum (Gulesserian *et al.*, 2000).

S100β is an astroglial-derived, calcium-binding protein that was shown to have trophic effects on 5-HT neurons. The gene coding for S100β is also located on

chromosome 21, and the levels of this protein are elevated in the blood and brain of Down syndrome cases. Transgenic mice over-expressing S100β show some morphological changes similar to Down syndrome cases, like overdevelopment and early loss of dendrites in the hippocampus. In behavioural experiments, these animals show learning and memory deficits and exhibit more approaches to novel and harmful objects in comparison with normal mice. Recently, the amniotic levels of S100β have been proposed for prenatal diagnosing of Down syndrome (Whitaker-Azmitia, 2001; Bell *et al.*, 2003).

The brain in Down syndrome is characterized by decreased numbers of cortical neurons, deformed dendritic trees and spines, defects in cortical lamination and abnormal synapses. In normal individuals, spine density and size of pyramidal dendritic trees increase with postnatal development, reach to peak at around 1 year, and then decrease to reach the adult value. Synaptogenesis follows a similar pattern in cerebral cortex. In Down syndrome patients, the overproduction of spines does not happen and spine density decreases gradually in the postnatal period. Because of this neuro-developmental defect, brain size in Down syndrome is normal at embryonic ages, and near normal in neonates, but decreases during, as well as after the first year of life (Okado *et al.*, 2001; Nelson *et al.*, 2006).

1.2.6 Autism

Autism is a neuro-developmental disorder with a prevalence of 1-2 per 1000 with a large range of severity. There are more than 15 candidate genes associated with autism. However, environmental factors are also important, as concordance of disease in monozygotic twins is less than 100%, and the phenotypic expression of the disease differs widely, even between monozygotic twins (Santangelo & Tsatsanis, 2005).

Autism is characterized by a triad of behavioral defects in social skills, language development and stereotyped behaviour. Cognitive deficits, affective flatness, lack of interest and perseveration are usually present (Boylan *et al.*, 2006).

Early findings of hyperserotoninemia have been reported in 30% of autistic patients. On the other hand, trytophan depletion in patients can increase stereotyped autistic behaviour, although measurement of 5-HIAA in cerebrospinal fluid (CSF) showed no difference between autistics and normal subjects. Functional neuro-imaging studies showed impaired regional 5-HT synthesis in the cerebral cortex of autistics, compared with normal subjects. Positron-emitting tomographic studies in boys with autism showed unilateral decreases in 5-HT synthesis in the frontal cortex and thalamus, and augmented levels in contralateral dentate nucleus of cerebellum. These 3 regions are anatomically connected via the dentato-thalamo-cortical pathway (taking part in sensory integration and speech skills) that is apparently impaired in autistic patients. These regional anomalies in 5-HT synthesis could explain why total 5-HIAA concentrations in CSF are not different between normal subjects and autistic cases. Altogether, the evidence supports that impairment in 5-HT systems could participate in autistic behaviour and phenotype (Scott & Deneris, 2005; Lam *et al.*, 2006).

There are both environmental and genetic hypotheses for autism. There is a higher prevalence of autistic disorders in children that have been exposed to intrauterine drugs that increase 5-HT levels, like cocaine and possibly alcohol (Lam *et al.*, 2006). It has also been reported that plasma 5-HT levels in mothers of autistic children are significantly lower than those of mothers of normal newborns. Thus, low maternal plasma 5-HT, during fetal development of the CNS, can itself be a risk factor for autism (Connors *et al.*, 2006).

One of the most likely candidate genes for autism is the *SERT* gene (locus SLC6A4). The 5-HT transporter is a transmembrane protein with a high affinity for 5-HT. A polymorphism has been reported for the promoter of this gene: a short allele (with a frequency of 43%) and a long allele (with a frequency of 57%). The short allele was associated with severely autistic patients, while the long allele was associated with mild/moderate cases (Tordjman *et al.*, 2001). Specific 5-HT reuptake inhibitors (SSRI), which block SERT, reduce the hyperactive, compulsive and stereotyped behaviours in autism. Risperidone, an antipsychotic that blocks dopamine and 5-HT post-synaptically also reduces anxiety, aggression and self-injury behaviours in autistic patients.

Another candidate gene is *Reelin*, coding for a protein, that acts as an extracellular matrix protein responsible for correct lamination of the cerebral cortex during the embryonic period and involved in cell signaling and synaptic plasticity in the adult life (Fatemi, 2002). Post-mortem studies showed that Reelin signaling is impaired in frontal cortical and cerebellar areas of autistic brains in comparison with control subjects (Fatemi *et al.*, 2005). The anatomical defects in *Reelin* knockout mouse resemble those reported in individuals with autism (such as cerebellar hypoplasia and decrease in number of Purkinje cells).

Engrailed is still another candidate gene. It is a transcription factor taking part in the development of the midbrain and cerebellum, during the embryonic and early postnatal period. *Engrailed* genes (1, 2) control the development of 5-HT neurons in the DR and noradrenergic neurons in locus cœruleus. In *Engrailed* double knockout mice, the population of 5-HT and noradrenergic neurons of midbrain/hindbrain are highly reduced. Mutations of *Engrailed* produces a cerebellar phenotype similar to that described in autism (Bartlett *et al.*, 2005; Simon *et al.*, 2005).

Brain imaging studies in affected children showed that they have smaller than normal brain size at birth, but that the brain grows faster in the first years of life, resulting in increased cortical volumes. This abnormal growth then slows down, so in late adolescence the autistic brain has the same size as the normal. Magnetic resonance imaging, in newborns autistics showed increases in cerebral cortical gray matter, as well as cerebral and cerebellar white matter (Boylan *et al.*, 2006). In SERT knockout mice, the excessive levels of 5-HT induce alterations in arborisation and segregation of thalamocortical terminals. In this model, a reduction in 5-HT clearance, and an over-stimulation of 5-HT_{1B} receptor were reported as potentially responsible for a decrease in thickness of cortical layer IV. The SERT mutant animals also showed some other neuroanatomical abnormalities reminiscent of those found in autistic brains, like increased neuronal packing densities in hippocampus, amygdala and entorhinal cortex, and augmented numbers of neurons in cerebral cortex. The association between increased cortical gray matter and reduction in SERT also supports the role for extracellular 5-HT in stimulating abnormal brain growth and macrocephaly in autistic patients (Altamura *et al.*, 2006).

1.3 Roles of 5-HT in development

Evidence from biochemical, pharmacological and clinical studies indicate a role for the 5-HT systems in CNS development. A direct involvement of 5-HT has been disclosed in the development of the architecture of the somatosensory cortex. Indeed, 5-HT depletion in newborn rat caused a significant reduction in the size of vibrissae-related barrels of thalamocortical afferents. But this reduction was not associated with a reduction of the brain or cortical size, or a decrease in somatosensory cortex dimensions (Bennett-Clarke *et*

al., 1994; Gu, 2002). These changes are reminiscent of defects described in the autistic brain (Bauman & Kemper, 2005).

Reelin is among the genes potentially associated with autism (see above). Now, 5-HT axons innervate the marginal zone during the prenatal period and make synaptic contacts with the soma and proximal dendrites of reelin-producing Cajal-Retzius cells, as early as E17 (Janusonis *et al.*, 2004). Blockade of the 5-HT input, using 5-methoxytryptamine, a 5-HT_1 and 5-HT_2 receptor antagonist, led to altered reelin levels in the brain of newborn pups and resulted in a disruption of the laminar and columnar organization of the cerebral cortex, also similar to defects described in autistic cerebral cortex (Bauman & Kemper, 2005).

An excess of 5-HT (in knock-out MAO-A mice) also causes abnormal branching of thalamocortical axons in layer IV of the somatosensory cortex. In double TrkB and MAO-A knock-out mice, there was an even more severe phenotype than in either mutant animals (a widespread tangential and radial expansion of thalamocortical axons). These observations indicate that 5-HT and TrkB signalings may act together to cluster thalamocortical axons in layer IV (Vitalis *et al.*, 2002).

Chronic tryptophan restriction in rat was reported to have adverse effects on the density of dendritic spines in pyramidal neurons of hippocampal CA1 region, and on the dendritic arborization and number of dendritic spines of pyramidal neurons in the third layer of prefrontal cortex. These changes correlated with defects in short term memory (Feria-Velasco *et al.*, 2002). Serotonin depletion by para-chloroamphetamine (ρCA), in P14 rats, also induced reductions in the number and length of dendritic spines of dentate gyrus granule cells, suggesting that 5-HT has a neurotrophic action on these neurons (Yan *et al.*,

1997; Faber & Haring, 1999). This neurotrophic action of 5-HT was apparently mediated by 5-HT_{1A} receptors, since subcutaneous injection of the 5-HT_{1A} antagonist, NAN-190, also resulted in permanent loss of dendritic spines. This result shows that the first two weeks of life constitute a critical period for the action of 5-HT on developing granule cells.

In the cerebellum, 5-HT axons and receptors appear early after birth, and influence dendrite formation on Purkinje cells. In vitro studies on cerebellar slices suggested that the dendritic growth of Purkinje cells was promoted by a 5-HT_{1A} receptor agonist and inhibited by a 5-HT_{2A} receptor agonist (Kondoh *et al.*, 2004).

1.4 Organization of the 5-HT systems

Studies on the development of 5-HT neurons showed that they develop early in fetal life, in two separate clusters in brainstem: a rostral one, just caudal to the mesencephalic flexure, that gives rise to almost all ascending 5-HT fibers, and a caudal cluster in the medulla oblongata that gives rise to the majority of descending fibers. The 5-HT neurons in brainstem have been classified into 9 groups, B1-B9. The B1, B2/4 and B3 groups constitute the caudal cluster, whereas the B5/8, B6/7 and B9 groups form the rostral cluster. Group B1 is located in nucleus raphe pallidus, which is the most ventrally placed 5-HT neuron group, in the medulla oblongata. Groups B2 to B4 are in nuclei raphe obscurus and raphe magnus and in the periventricular gray of medulla oblongata. Groups B5 to B8 are located in the midbrain/pontine medial raphe and dorsal raphe nuclei, and B9 is a lateral extension of B8, in the medial lemniscus (see Harding *et al.*, 2004; Cordes, 2005).

From the brainstem, 5-HT neurons have extensive projections to virtually all areas of brain and spinal cord. The present work concerns the ascending projections of the DR, notably those that innervate the cerebral cortex, the striatum and the ventral midbrain.

These axons initially enter the medial forebrain bundle and then divide into several pathways to innervate their target structures, including the olfactory bulbs, hypothalamus, thalamus, septal area, striatum, hippocampus and cerebral cortex. The basal ganglia receive dense 5-HT projections from the B7 group, more concentrated in the posteromedial striatum and globus pallidus. The same 5-HT neurons also send collaterals to the ventral midbrain. Most cytoarchitectural areas of cortex receive their 5-HT innervations from B7 and B9, which innervate the entire cerebral cortex, with particular subdivisions of neurons innervating specific cortical or subcortical regions. For example, 5-HT neurons in the lateral wing of DR innervate the primary visual cortex, as well as the superior colliculus or lateral geniculate thalamic nucleus. In contrast, group B9 innervates the outer cortical layers of most cortical regions (Harding *et al.*, 2004).

1.5 Molecules influencing the differentiation of 5-HT neurons

1.5.1 Sonic hedgehog and Fibroblast Growth Factors

The differentiation of 5-HT - and dopamine - neurons is controlled by several signals expressed by dorso-ventral or antero-posterior compartments. Sonic hedgehog (Shh) is a signalling protein that is expressed by the notochord and floor plate, as well as prechordal mesoderm, and has a major role in the dorso-ventral patterning of the neural tube, early in the development of the CNS (Cordes, 2005). In vitro experiments, using explants of the midbrain/hindbrain, or of more caudal hindbrain have shown that the expression and signalling of both Shh and FGF8 are necessary for the differentiation of the rostral 5-HT cell groups, whereas, differentiation of the caudal groups, which are farther from the brainstem isthmus, the source of FGF8, only depends on Shh. FGF2 and FGF4 were also shown to induce the differentiation of 5-HT neurons in more rostral, midbrain

explants, perhaps to the expense of dopamine neurons, suggesting that FGF4 in midbrain may change the fate of neuronal progenitors to become 5-HT neurons. These findings suggest that these 3 signals act in concert to produce rostral 5-HT neurons (Ye *et al.*, 1998).

1.5.2 Gli transcription factors in the differentiation of 5-HT neurons

One of the downstream components of the Shh signalling pathway, during embryogenesis, is the Gli transcription factor family. Gli1, -2 and -3 are the major members of this group that are expressed throughout the neural plate prior to floor plate induction (although Gli3 is weak or absent from the midline). In Gli2 knockout mice, the floor plate does not form in the midbrain, hindbrain and spinal cord, although the notochord is present and expresses Shh. Despite the absence of a floor plate and lack of Shh expression in the ventral midline of the neural tube, the pattern of cell differentiation along the dorsoventral axis, outside the ventral midbrain appears normal in Gli2 homozygotes, suggesting the signals from the notochord can be sufficient for the early dorso-ventral differentiation of the ventral spinal cord and hindbrain. In the Gli2 knockout mice, the number of 5-HT and dopaminergic neurons that were generated in the region flanking the floor plate, was markedly reduced, indicating either a direct requirement for Gli2 in the precursors of these cells or a requirement for a normal floor plate for a normal development of these neurons (Matise *et al.*, 1998).

At early ages, Gli1 is expressed throughout the neural tube, including ventral midline cells and receives the Shh signals from notochord. Ectopic expression of Gli1 in the dorsal midbrain and hindbrain leads to activation of ventral neural tube markers and to the formation of ectopic dorsal clusters of 5-HT and dopaminergic neurons in the neural tube (Hynes *et al.*, 1997).

1.5.3 Other transcription factors involved in 5-HT neuron development

Transcription factors involved in 5-HT neuron development may be divided into 2 groups: I) the factors that are necessary to generate 5-HT neuron precursors, like Nkx2.2, Nkx6.1, or Mash1; and II) factors that are required for 5-HT subtype selection and ultimate differentiation of 5-HT neurons, like Mash1, Gata2, Gata3, Lmx1b, Pet1, and Otx2.

Nkx2.2 (a homeodomain transcription factor that acts downstream of Shh signaling) is necessary for initiating the specification of all 5-HT neurons in the raphe, except the DR. In Nkx2.2 knockout mice, only DR 5-HT neurons are present and all others are missing (Cordes, 2005).

The earliest 5-HT neurons that develop adjacent to the mid-hindbrain boundary at the ventral midline are in rhombomere 1 (r1). This group of 5-HT neurons sends ascending projections to the forebrain. During 5-HT neuron development in chick embryo, Nkx2.2 cooperates with another transcription factor, Nkx6.1, to induce the expression of the zinc-finger transcription factor Gata2 in ventral r1. Gata2 is sufficient to activate transcription factors Gata3, Lmx1b and Pet1 and induce 5-HT neurons in r1. Gene knockout studies showed that 5-HT neurons in r1 are specified normally in Gata3 mutant embryos, but are completely absent in mid-hindbrain explants derived from Gata2 null mice. These data suggest that Gata3 is unable to specify 5-HT neurons in the absence of Gata2. The loss of 5-HT neurons in the caudal raphe nucleus of Gata3 knockout mice indicates that Gata3 has a role in the development of caudal 5-HT populations (Craven *et al.*, 2004; Scott & Deneris, 2005)

Lmx1b is a LIM homeobox-containing gene that is expressed bilaterally in the ventral part of hindbrain, including the floor plate, at E10 in mouse. At E13.5 and later

stages, Lmx1b is present in the raphe nuclei and adjacent reticular formation. The expression pattern of Lmx1b is similar to the 5-HT expression pattern in the hindbrain, during embryonic development. In Lmx1b knockout mice, the differentiation of 5-HT neurons is completely interrupted. The expression of Shh and Nkx2.2 are normal in these animals, supporting the idea that Lmx1b acts downstream of theses two genes. However, Gata3 expression is largely lost in Lmx1b mutant mice in the caudal raphe nuclei, indicating that it acts downstream of Lmx1b in the development of some caudal 5-HT neurons (Cheng *et al.*, 2003; Ding *et al.*, 2003).

The transcription factor Otx2 determines the mesencephalic, versus metencephalic (cerebellum/pons) territories during embryogenesis. It is involved in positioning and maintaining the isthmic organizer at the border between midbrain and anterior hindbrain. Otx2 expression regulates the location and size of dopamine and 5-HT neuron populations. If Otx2 is expressed more caudally, the isthmic organizer also shifts caudally, and the number of midbrain dopamine neurons augments, at the expense of rostral 5-HT neurons of B7, or DR. If Otx2 expression is reduced, the isthmic organizer shifts rostrally and the rostral 5-HT population expands into the rostral region (Brodski *et al.*, 2003).

Otx2 expression is maintained long after this organizer is established. Experiments on Otx2 knockout mice revealed that Nkx2.2 and Shh are abnormally co-expressed in midbrain and hindbrain neuronal progenitors of these mice and give rise to ectopic 5-HT neurons (Nkx2.2 expression being normally limited to hindbrain progenitors). Ectopic Nkx2.2 may be responsible for the abnormal formation of 5-HT neurons in the midbrain. In agreement with this hypothesis, removal of Nkx2.2 in Otx2 mutant mice, resulted in the disappearance of ectopic midbrain 5-HT neurons. These results suggested that Otx2

normally prevents 5-HT development in the midbrain by limiting the expression of Nkx2.2 (Vernay *et al.*, 2005).

Ascl1 (or Mash1) is a basic helix-loop-helix protein that is expressed along with Nkx2.2 in neural precursors of hindbrain that generate 5-HT neurons later during development. Ascl1 together with Nkx2.2, is also required for the phenotypic specification of 5-HT neurons in the neuroepithelium. Experiments on Gata3 mutant mice also showed that Gata3, Lmx1b and Pet1 act downstream of these transcription factors (Pattyn *et al.*, 2004).

1.5.4 Molecules that influence the growth of 5-HT axons

Although major progress has been made recently in the identification of factors specifying the fate of 5-HT neurons, little is currently known about the molecules that guide 5-HT axons to their targets. This subject has been reviewed by my colleague, B. Sharif Askari, in his own Master's thesis (Sharif Askari, 2006). I will thus only present a brief summary of the question..

Some trophic factors have been shown to have some effect on the 5-HT axonal projections in the forebrain. One of them is brain-derived neurotrophic factor (BDNF), which may promote terminal 5-HT sprouting in the hippocampus and whose receptor, TrkB, mRNA is expressed in DR 5-HT neurons (Madhav *et al.*, 2001). Brain-derived neurotrophic factor was shown to promote the sprouting of 5-HT axons after local chemical insult in cerebral cortex, and to induce the expression of 5-HT markers, such as tryptophan hydroxylase (Rios *et al.*, 2006).

Another trophic factor, S100β, a glia-derived calcium binding protein, was also shown to have a trophic activity on 5-HT neurons in cell culture, where it enhanced the

neurite extension and 5-HT uptake capacity of neurons dissociated from the mesencephalic raphe (Azmitia *et al.*, 1990). However, experiments on mice with mutant S100β showed no significant difference in the number or morphology of 5-HT neurons compared with wild type animals (Nishiyama *et al.*, 2002).

Glial cell line-derived neurotrophic factor (GDNF) family was also shown to have a trophic activity on 5-HT neurons from the ventral mesencephalic area, increasing their soma size, number of primary neurites and length of 5-HT axons (Ducray *et al.*, 2006).

Nevertheless, the mechanisms of 5-HT axonal guidance to their various targets remain largely unknown. Previous in vitro experiments in our laboratory suggested that GPI-linked cell membrane proteins may influence the guidance of 5-HT axons. Some candidate membrane proteins that could act as guidance cues include the ephrins (see below), semaphorins (Tamagnone *et al.*, 1999), netrins (Barallobre *et al.*, 2005), cell adhesion molecules like cadherins or immunoglobulin CAMs (Litwack *et al.*, 2004) and Slits (Bagri *et al.*, 2002)

Up to now, Slit-1 and Slit-2 are the only axon guidance cues to have been involved in the guidance of ascending 5-HT axons to forebrain. Slit proteins, in combination with their receptors, the *roundabouts* or Robos, are known as a midline repellents regulating midline commissural formation. In *slit1* and *slit2* double mutant animals, the ascending fibers of DR 5-HT neurons normally projecting to the forebrain, are displaced ventrally as they course through the diencephalon. These data suggest that the loss of Slit function affects the development of the 5-HT axons, while they grow rostrally into the forebrain (Bagri *et al.*, 2002). In addition, 2 Robo receptors, Robo2 and Robo3 interact with the zinc transcription factor, *eagle*, to regulate 5-HT neuron differentiation. Loss of *robo2/3*

function causes a loss of SERT expression in 5-HT neurons in embryos (Couch *et al.*, 2004).

Recent neurochemical studies showed that the 5-HT levels in the striatum of mice expressing a dominant-negative form of the EphA5 receptor, were significantly reduced in comparison with wild type animals. No change was detected, however, in the frontal cortex or hippocampus of these animals. These mutants also demonstrated behavioural deficits (two-way active avoidance learning) compared to controls (Halladay *et al.*, 2004). Thus, with the recent observations of Sharif Askari, in our laboratory, this evidence suggests that ephrins and their receptors might be involved in 5-HT axon guidance or growth in forebrain.

1.6 Ephrins and Eph receptors

Eph receptors belong to the superfamily of tyrosine kinase receptors and take part in various aspects of morphogenesis, angiogenesis and tumorigenesis in different organs. Eph receptors and their ephrin ligands are widely expressed in the developing and adult nervous system. They are implicated in contact repulsion and attraction between adjacent cell surfaces and in the guidance of migrating neurons and growing axons. Ephrins are subdivided into 2 subclasses: ephrin-As (A1-A6) are attached to the outer cell surface by a glycosylphosphatidylinositol (GPI) anchor and ephrin-Bs (B1-B3) are transmembrane proteins. Ephrin-As in general have a high affinity for EphA receptors (A1-A10), whereas ephrin-Bs prefer EphB receptors (EphB1-B6). The latter rule has 2 exceptions: EphA4 having also a high affinity for ephrin-B2 and -B3, and EphB2 for ephrin-A5 (Klein, 2001; Blits-Huizinga *et al.*, 2004; Himanen *et al.*, 2004).

1.6.1 Ephrin and Eph signaling pathways

Eph receptors are type-1 transmembrane proteins. Their extra-cellular part contains a highly conserved N-terminal domain, followed by a cysteine-rich region, and then by 2 fibronectin type III repeats (involved in receptor dimerization and *Cis* interactions with other proteins like NMDA receptors). Their intracellular region contains a juxta-membrane domain, a conserved tyrosine kinase domain, a sterile α-motif (SAM) domain and a PDZ-binding domain (Himanen & Nikolov, 2003).

There are 3 types of signaling pathways associated with Eph receptors: **forward signaling**, **reverse signaling**, and **cross-talk signaling**.

In **forward signaling**, the first step is the binding of an ephrin ligand and an Eph receptor located on closely apposed cell surfaces. The activated Eph starts downstream signaling through autophosphorylation that regulates actin dynamics via small guanosine tri-phosphatases (GTPase) of the Rho family (Martinez & Soriano, 2005). These GTPases act as molecular switches, being inactive in the GDP-bound state, and active in the GTP-bound state. Guanine nucleotide exchange factors (GEFs) that promote the exchange of GDP for GTP, facilitate this activation pathway, whereas this process is inhibited by GTPase activating proteins (GAPs). The most important members of the Rho family are RhoA, Rac1 and Cdc42. RhoA regulates stress fiber and focal adhesion formation and cell contractibility, whereas Rac1 and Cdc42 respectively take part in the formation of the protrusive structures, lamellipodia and filopodia (Noren & Pasquale, 2004).

Ephexin is a GEF, linking EphA to RhoA. When an EphA binds to an ephrinA, the catalytic activity of ephexin increases and induces activation of RhoA and down regulation of Rac1 and Cdc42 and leads to cell morphology changes (Shamah *et al.*, 2001).

EphB receptors bind to other exchange factors, like intersectin or kalirin that activate Rac2 and Cdc42, respectively. In hippocampal neurons, EphB interactions with these exchange factors regulate dendritic spine morphogenesis. EphB2 also cooperates with neural Wiscotte-Aldrich syndrome protein (N-WASP), to activate intersectin. N-WASP binds to actin polymerising complex AP 2/3 in the presence of activated Cdc42 and induces actin filament assembly and branching. Kalirin is predominantly expressed in the nervous system. In immature hippocampal neurons, attachment of ephrin-B1 to EphB induces accumulation of kalirin in the postsynaptic region to induce dendritic spine morphogenesis. Phosphorylated kalirin itself regulates the activation of Rac1 and Pak (a Rac1 downstream effector). Activated kalirin, Rac1 and PAK are necessary factors for the induction of dendritic spine maturation via ephrin-B signaling. Eph receptors also regulate the activity of the Ras family of GTPases that are involved in several developmental processes, including axon guidance and cell adhesion. In most cells, Eph receptors negatively control phosphorylation of the Ras-MAP kinase pathway (Noren & Pasquale, 2004).

The **reverse signalling** pathway is induced following the activation of an ephrin ligand (then acting as a receptor) by an Eph receptor. For ephrin-Bs, this pathway is phosphotyrosine- or PDZ-dependent. Ephrin-B phosphorylation can be mediated by a tyrosine kinase of the Src family. Ephrin-A ligands, although deprived of any intracellular domain, also have reverse signalling potentials. For example, activation of ephrin-A2 or -A5 by EphA3 results in a β2–integrin-dependent increase in laminin adhesion capability of the cell expressing the ephrin (Martinez & Soriano, 2005).

Cross-talk signaling, or interaction between Ephrin/Eph and other proteins on the same cell surface, in *Cis*, is thought to be important in synaptogenesis and cell adhesion.

For example, during embryonic life in mice, EphB2 and -B3 cooperate with the tyrosine kinase Ryk to bind to the cell junction-associated, PDZ-domain-containing protein, AF6. This attachment is important in cell binding. Ryk knockout mice present a cleft palate phenotype similar to that observed in EphB2/B3 double knockout mice; indicating a cooperation among these proteins in palate formation (Murai & Pasquale, 2003). EphB receptors also induce aggregation and phosphorylation of NMDA receptors in synapses and initiate postsynaptic maturation during development (Martinez & Soriano, 2005).

1.6.2 Functions of ephrin/Eph in CNS

1.6.2.1 Segmentation and tissue formation:

Several Ephs and ephrins are expressed in a segmented pattern in hindbrain rhombomeres and in somites, and have active roles in the pattern formation of these areas by both forward and reverse signaling pathways. EphA4 and EphB2-B3 are highly expressed in rhombomeres r3 and r5, and their potential ligands, ephrins-B1-B3, are present in r2, r4 and r6. Reverse signaling keeps EphA4 expressing cells at the boundary of r2, r4 and r6 by a repulsion mechanism. The ligand/receptor interaction at the interface of adjacent rhombomeres has an important role in establishing the precise border between cell populations and organization of each segment subunit. Disruption of ephrin/Eph interactions or signalisation, causes major defects in hindbrain segmentation (Martinez & Soriano, 2005).

1.6.2.2 Cell migration and adhesion:

Ephrins and Ephs control both cranial and trunk neural crest cell migration. In early stages of development the trunk neural crest cells of rodents and chick migrate through the

anterior, but not the posterior half of each somite. The presence of ephrin-B1 and -B2 in the posterior half of each somite represents a repulsive signal for neural crest cells that express EphB receptors. In *Xenopus*, forward signaling of EphA4 acts as a repulsive signal and guides branchial neural crest cells: in a dominant negative mutant of EphA4, only the EphA4 expressing neural crest cells are affected, not those expressing the ephrins (Davy *et al.*, 2004).

1.6.2.3 Synaptogenesis:

Ephrins and Ephs are expressed in adult as well as developing nervous system, and play critical roles in modulating synapse maturation and function. They are located in both axonal and dendritic compartments (Bouvier *et al.*, 2007; Tremblay *et al.*, 2007). Ephrins may also be present in pre- and post-synaptic compartments, as well as in astrocytes. Expression of ephrins on glia may have an active role in neuron-glia cross-talk (Murai & Pasquale, 2004). Dendritic spines have a very dynamic structure, they go through remodelling even in mature neurons. Actin neurofilaments have a leading role in the shape and motility of spines and, as mentioned before, several Eph signaling pathways modulate actin neurofilaments remodelling and can take part in structure formation of dendritic spines in adult and developing neurons (Murai & Pasquale, 2004).

EphB receptors regulate the maturation of filopodia into dendritic spines. They induce the clustering and also modulate NMDA-dependant calcium influx (by activating a pathway via Src kinase family) and have important roles in synaptic transmission (Martinez *et al.*, 2005).

Triple EphB1, -B2 and -B3 knockout mice fail to form mature spines by postnatal day 21, and instead retain immature filopodial processes. These triple knockout mice

displays altered clustering of both F-actin and post synaptic density-95 protein (PSD-95) in their long, thin dendritic protrusions. PSD-95 and the afferent terminals, labeled by presynaptic marker synaptophysin, then distribute along the dendritic shafts, instead of their usual association with spines (inside or apposed to), as in wild-type mice, indicating abnormal synapse formation (Lippman & Dunaevsky, 2005).

1.6.2.4 Axonal guidance and formation of topographic maps

Both ephrin-As and -Bs take part in retinotectal mapping by chemorepellent as well as chemoattractant signaling. In retinal ganglion cells, EphA receptors are expressed in a low nasal (or rostral for laterally positioned eyes) to high temporal (or caudal) levels; while ephrin-As have a low rostral to high caudal expression in the visual tectum. During development, nasal retinal ganglion cell axons project to the caudal tectum, while temporal retinal ganglion cell axons project to rostral tectum. EphB receptors and ephrin-B ligands also contribute to the organization of the dorso-ventral axis of the retino-tectal projection, but by chemoattractive mechanisms: ventral retinal ganglion cells, with higher levels of EphBs, project to the medial part of the colliculi, expressing high levels of ephrin-Bs (Lemke & Reber, 2005). Similar mechanisms are involved in the formation of retino-thalamic maps (Feldheim *et al.*, 1998).

A particular mechanism exists for the formation of the thalamocortical fibers. The axons from rostral thalamic nuclei project to the rostral neocortex, while caudal (visual) thalamic nuclei innervate caudal visual cortical areas. This pattern is established during the growth of thalamic fibers in the basal forebrain, towards the cerebral cortex, when EphA4 receptors on thalamic fibers meet with a graded distribution of ephrin-A5 in the ventral telencephalon (Dufour *et al.*, 2003).

During CNS development, EphA8 guides commissural axons from superior colliculus to pass the midline and project to their targets in the contralateral inferior colliculus. In absence of EphA8, these neurons continue their projections through the ipsilateral inferior colliculus and reach the spinal cord (Park *et al.*, 1997).

The entorhino-hippocampal projections are regulated by ephrin-A/EphA. The entorhinal axons and their target hippocampal regions express EphA receptors and ephrin-A ligands, respectively. Ephrin-As are localized in the hippocampal stratum radiatum and the dentate inner molecular layer, where commisural/associative, but not entorhinal projections, terminate. *In vitro* studies showed that outgrowth of entorhinal neurites was inhibited by ephrin-A3. These results, together with the expression of EphA5 by entorhinal-projecting cells, suggest that EphA5-expressing entorhinal axons are repelled by their interaction with ephrin-A expressing dendrites in inappropriate target layers. In contrast to ephrin-A3, ephrin-A5 exerts minor effects on outgrowth and guidance of entorhinal axons and entorhinal axons are not disrupted in ephrin-A5 knockouts (Martinez *et al.*, 2005).

In rodents, corticospinal tract axons originate from layer V in neocortex, and project to forebrain, midbrain, hindbrain and at different levels of spinal cord. In EphA4 knockout mice, projections of corticospinal axons show several defects at medullar and spinal cord levels, and these animals show loss of coordination in hindlimb movements with a kangaroo-like gait (Dottori *et al.*, 1998).

Ephrin/Eph interactions have active roles in guiding topographic projections from hippocampus to lateral septum. In late embryonic and early postnatal days, EphA3-A7 receptors show graded expression from lateral to medial regions of hippocampus and ephrinA2, -A3 and -A5 expression increases from dorso-medial to ventro-lateral area of

lateral septum. This hippocampo-septal topographic map is disrupted in mutant mice expressing a dominant negative form of EphA5 (Yue *et al.*, 2002; Martinez *et al.*, 2005).

1.7 Objectives of present work

In the present study, we worked on the hypothesis that ephrin-As take part in axonal guidance of 5-HT axons in forebrain and midbrain, during development, based on recent cell culture observation in our laboratory (Sharif Askari, 2006). Indeed, this work showed that cell membranes extracted from the neonatal Ctx, Str or VM could influence the growth of 5-HT axons from the dissociated DR. This axon guidance activity of the cell membranes was disrupted by a prior treatment with the enzyme PI-PLC, which removes GPI-anchored cell membrane proteins, and reduced by treatment with EphA3-Fc, a fusion protein comprising the extracellular domain of EphA3 and the Fc domain of human immunoglobulins and known to block specifically the binding of ephrin-As to EphA receptors. We thus hypothesize that 5-HT axons bear EphA receptors that can be activated by ephrin-A ligands present at the surface of the cell membranes in the target brain regions, Ctx, Str or VM. The specificity of the projections is most likely dependant on the combinatorial expression of different sets of EphA receptors by individual 5-HT axons and expression of complementary sets of ephrin-As in the different target regions.

For the current work, we followed 2 objectives. The first one was to confirm the presence of ephrin-As in the target brain regions (Ctx, Str and VM), particularly in membrane extracts from these regions, that were used as substrates for 5-HT neuronal primary cultures. This part of the experimentation was done using Western Blotting techniques. The second objective was to determine the expression of EphA receptors in the DR, where 5-HT neurons projecting to the Ctx, Str and VM are located. For this, we used

in situ hybridization with DiG-labelled riboprobes. We already had plasmids containing constructs for the synthesis of EphA3, EphA4 and EphA5. However, we had to re-insert the EphA4 construct in another plasmid, to be able to synthesise a suitable control sense probe for this molecule. We also prepared a new construct for the EphA7 mRNA.

2. Materials and methods

2.1 Animals

All experimental procedures were approved by the Ethics Committee on the use of animals of the Université de Montréal (*Comité de déontologie de l'expérimentation sur les animaux* - or CDEA).

Embryonic day 15 (E15) and newborn (P0) Sprague-Dawley rats, and P60 C57BL/6 mice were purchased from Charles-River (Montréal, QC, Canada).

For Western Blotting, 15 newborn rats were quickly decapitated with sharp scissors.

For *in situ* hybridization on embryonic brain sections, a pregnant rat was sacrificed by CO_2 inhalation, the heads of the fetuses were dissected and immersed into 4% paraformaldehyde (in 0.1M sodium phosphate buffer, pH7.2, or PB). For newborn and adult cases, 1 P0 rat and 1 adult mouse were deeply anaesthetized with 0.3 ml/100g, or 0.5 ml/100g, respectively, of a mixture containing one part fentanyl citrate (Hypnorm, Janssen pharmaceutica, Beerse, Belgium), one part midazolam (Versed, Roche Biotechnology), and 2 parts water. They were perfused transcardially with 4% paraformaldehyde in PB, after rinsing the vasculature with sodium phosphate-buffered saline (PBS, pH 7.2). Brains were extracted and kept in the same fixative solution overnight at 4°C and then embedded in paraffin.

2.2 Detection of EphA4, EphA5 and EphA7 mRNA by in situ hybridization

2.2.1 Preparation of constructs and insertion into plasmids

2.2.1.1 EphA4 construct

The *EphA4* construct was available in the laboratory (generous gift of Dr. Elena Pasquale, Burnham Institute, LaJolla, CA), but we had to insert it into a different plasmid to be able to efficiently synthesize both sense and antisense probes. This construct, a 313 bp fragment from nucleotides 97 to 409 of the gene, had been originally inserted into the pCR2.1-TOPO plasmid, but with only the T7 promoter controlling the transcription of the antisense riboprobe. To obtain both sense and antisense riboprobes with equal efficiency, we inserted this cDNA fragment in either directions into 2 different pGEM-7Z (+) plasmids, so both sense and antisense probes could be transcribed under the control of the T7 promoter.

For the antisense probe, the *EphA4* fragment was extracted from the original plasmid, pCR2.1-TOPO, by digestion with SpeI and XhoI restriction enzymes (Fermentas Life Science) in their respective specific buffers, for 2 h at 37°C. In parallel, the recipient pGEM plasmid was prepared by digestion with XbaI (Fermentas) and XhoI restriction enzymes (to open the site for EphA4 insertion).

For the sense probe, both the donor pCR2.1-TOPO and the recipient pGEM plasmids were digested with HindIII (Fermentas) and XhoI.

To prevent reverse binding of the 2 free sites of the open pGEM plasmid, following digestion, these sites were de-phosphorylated by incubation with shrimp alkaline phosphatase (Fermentas) for 30 min, at 37°C. After gel electrophoresis of the digested products (digested pGEM and *EphA4* fragment, Fig. 1), the DNA concentration of each

band was measured with the Gel Doc 2000 image analysis system (a CCD camera and *Quantity One* image analysis software measuring the density and size of the spots on the gels; from BioRad). The bands were then removed with a scalpel and purified with the QIAquick Gel extraction kit (QIAGEN Inc., Mississauga, ON).

To ligate the *EphA4* fragment into the pGEM plasmid, 2.2 µl of plasmid (0.37 µg/ µl), and 42 µl of EphA4 fragment (2.11 µg/ µl) were mixed with 1 µl of T4 DNA ligase (1 U/µl; Fermentas), 5.5 µl of 10X ligation buffer (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP; pH 7.8; provided with the enzyme) and 4.3 µl H_2O, and incubated for 1 h at room temperature.

Then, *E.coli* DH10 competent cells were transformed by mixing 0.1 ml of the bacteria (3.75×10^9 bacteria/ml) with 30 µl of the plasmid (0.31 µg/ µl) containing the *EphA4* construct, and submitting them to a thermic shock: i.e. sequential incubation at 4°C for 30 min, at 42°C for 2 min, and at 4°C for 2 min. Then 0.5 ml of sterile Luria-Bertani medium (LB medium, 10 µg tryptophan, 5 µg yeast extract, 10 µg NaCl, and 200 µl 10 M NaOH) was added to the mixture and incubated for 1 h at 37°C. Then, 100 or 200 µl of the cell suspension were sown into 2 separate gelatine-coated Petri dishes, which were incubated overnight under gentle agitation, at 37°C. The next day, 10-20 colonies were randomly chosen and scratched with pipette tips, under sterile conditions (near a flame), and each transferred into a sterile 1.5 ml Eppendorf tube containing 1.0 ml of LB medium. The DNA was then extracted. For this, the tubes were centrifuged twice at 17 000 x g (Thermo IEC, Microlite) for 1 min and the supernatant was discarded. The final pellets were re-suspended in 100 µl of lysing solution, or STET (8% Sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris-HCl, pH 8), boiled during 1 min, and centrifuged again (same

speed) for 1 min. The pellets were adjusted with 5 µl of 5 M NaCl and 105 µl isopropanol, to precipitate the DNA. Eppendorf tubes were placed at -80°C for 10 min, and centrifuged again for 10 min. DNA in the pellet was washed with 500 µl of 70% ethanol, and centrifuged for 2 min. The pellet was re-suspended in 50 µl of TE buffer (Tris/EDTA; 10 mM Tris-HCL pH 8, 1 mM EDTA) containing RNaseA (10 mg/ml). The final products were pGEM plasmids with the T7 promoter controlling the synthesis of either sense or antisense *EphA4* inserts (Fig. 1, A, B and C).

2.2.1.2 EphA5 construct

The construct for *EphA5* had already been prepared by Cyrinne Ben Mamou, a former student in our laboratory, and Damien Barbas in the laboratory of Dr. L. DesGroseillers, and inserted into the pZAPA-TA plasmid, derived from the pBluescript SK+ plasmid (Barbas *et al.*, 2002). A 0.91 kb mouse cDNA fragment, extending from nucleotides 3297 to 4206 of the *EphA5* gene (Genbank access U07357), had been extracted from *Mus musculus* Balb/c DNA. Synthesis of the sense and antisense probes was under the control of the T3 and T7 promoters, respectively (Fig.1 B, D).

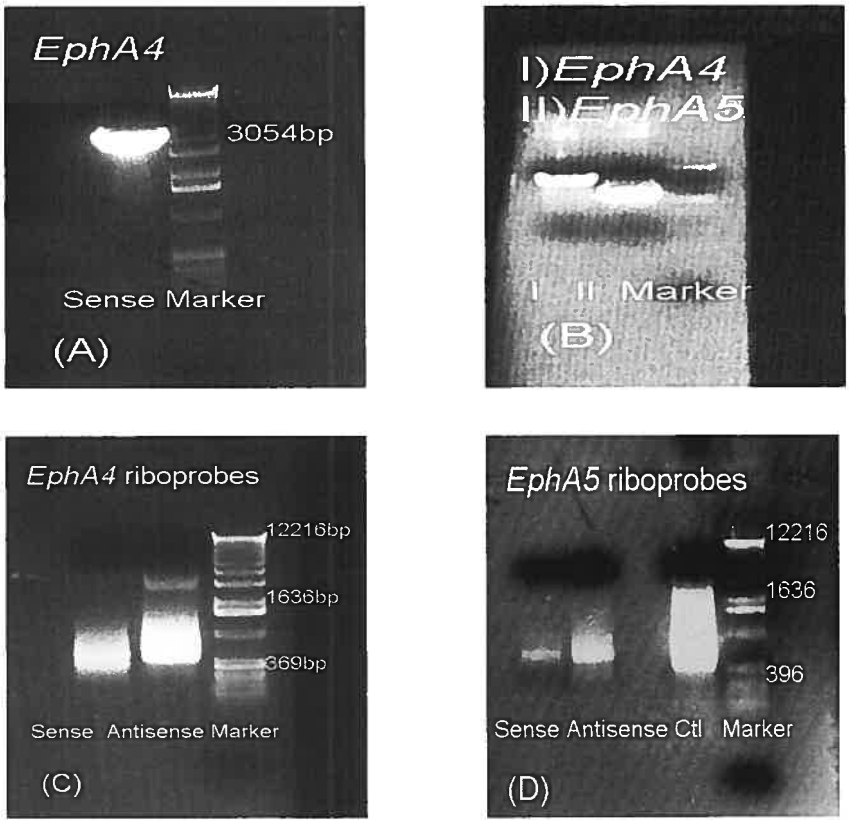


Figure 1. Production of the EphA4 and EphA5 riboprobes

A) Linearized pGEM-*EphA4-Reversed* plasmid following digestion with HindIII restriction enzyme for production of the *EphA4* sense probe. B) Linearized pGEM-*EphA4* plasmid (lane 1), following digestion with HindIII restriction enzyme for synthesis of the *EphA4* antisense probe; and linearized pZapata-*EphA5* plasmid, digested with NotI restriction enzyme for the synthesis of the *EphA5* antisense probe. C) DiG-labelled *EphA4* sense and

antisense riboprobes. D) DiG-labelled *EphA5* sense and antisense riboprobes ("Ctl" is control DNA from the Roche RNA DiG labelling kit).

2.2.1.3 EphA7 construct

The *EphA7* construct was not available. In collaboration with Frédérique Badeaux, in Dr. L. DesGroseillers's laboratory, we prepared a construct by generating a 499 bp DNA fragment of the *EphA7* gene by a polymerase chain reaction (PCR) from genomic DNA extracted from a culture of Neuro2A cell line. Sequence-specific sense and antisense primers (EphA7 sense, 5′-AAG AAT TCG TGG GAA GAA ATT AGT GGT TTG-3′; and EphA7 antisense, 5′-TTG AAT TCG GAA AGA CAG CTA AGT TCT CAA-3′; purchased from Integrated DNA Technologies, Coralville, IA) were used to amplify the DNA fragment. The PCR was done with the Phusion kit (High-Fidelity DNA Polymerase, Finnzymes, OY, Finland), following the manufacturer's instructions. Briefly, 5 µl of Neuro2A DNA (2 ng/µl), 10 µl of Phusion buffer, 1 µl dNTP (nucleotide triphosphate, final concentration: 200 µM), 31.5 µl H_2O, 1 µl sense primer (30.2 µg/OD260), 1 µl antisense primer (30.6 µg/OD260) and 0.1 µl Phusion DNA polymerase (2 U/µl) were mixed together and incubated in a T3 Thermocycler (Biometra, France), again according to the manufacturer's parameters. The day after, the PCR product was electrophoresed in a 1% agarose gel containing ethidium bromide, in presence of 2 DNA markers, respectively 100 bp (Invitrogen) and 1 Kbp (New England Biolab). The band containing the PCR product was cut out from the gel and the DNA was extracted and purified using the QIAquick Gel Extraction Kit (Fig. 2A). Next, phosphorylation of *EphA7* PCR product was performed on 50 µl PCR product, 1 µl T4 polynucleotide kinase (10 U/µl; Fermentas),

6.5 µl 10X reaction buffer A (provided with the enzyme), 6.5 µl ATP (10 mM), 1 µl H_2O for 20 min at 37°C.

EphA7 was inserted into the pBluscript II KS (+/-) plasmid, with T7 and T3 promoters. For this, the plasmid was digested with EcoRV restriction enzyme (Fermentas; 7 µl mini pBluescript KS, 10.5 µl H_2O, 2 µl Buffer Red, 0.5 µl EcoRV) during 2 h at 37°C. Dephosphorylation of the digested plasmid was done in the presence of 0.5 µl of shrimp alkaline phosphatase (1 U/µl), 5 µl 10X Reaction buffer (provided with the enzyme) and 24.5 µl H_2O, for 30 min at 37°C. After the respective gel electrophoresis of the digested pBluescript and *EphA7* fragment, the DNA concentration of each band was measured in the Gel Doc 2000 image analysis system. The bands were then removed with a scalpel and purified with the QIAquick Gel extraction kit.

For the ligation of the *EphA7* fragment into the pBluescript plasmid, 8.5 µl of the plasmid (8.8 ng/µl), and 43 µl of the fragment (2.9 ng/µl) (to obtain 75 ng of plasmid and 125 ng of *EphA7* fragment, or a ratio of 10 *EphA7* fragments for each plasmid) were mixed with 1 µl of the T4 DNA ligase (1 U/µl), 6 µl of 10X ligation buffer (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP pH 7.8) and 1.5 µl H_2O, and incubated for 1 h

2.2.2 Synthesis of antisense and sense riboprobes

To synthesize the antisense and sense riboprobes, 15 µl of the respective pGEM plasmids (0.3 µg/µl) containing the *EphA4* fragment in one or the other orientation were linearized with 2 µl of HindIII restriction enzyme (10U/µl), 2 µl of the 10X REact 2 buffer provided with the enzyme, and 1 µl H_2O, for 2 h at 37°C. Gel electrophoresis (1% agarose gel with ethidium bromide) was done in the presence of 1 Kb DNA ladder marker (Invitrogen) and the digested plasmids. After migration at 120V for 45 min the gel was visualized under UV light. The band corresponding to each plasmid was cut with a scalpel. The DNA was extracted and purified from the gel by using the QIAEX II Gel Extraction Kit (QIAGEN), according to the manufacturer's instructions. Antisense and sense riboprobes were produced by *in vitro* transcription of the linearized plasmids, using the T7 polymerase (DiG RNA labelling kit; Roche). Following the manufacturer's instructions, 1 µl of linearized plasmid was incubated with 2 µl 10x NTP labelling mixture, 2 µl 10x transcription buffer, 1 µl RNase inhibitor (20 U/µl), 2 µl RNA T7 polymerase (20 U/µl) and 3.5 µl of H_2O, at 37°C for 2 h. Two microliters of DNase-I (10 U/µl) were then added to the mixture, to remove template DNA. The RNA transcripts were analysed by agarose gel electrophoresis with ethidium bromide staining (Fig. 1).

For *EphA5*, 15 µl of pZAPA-TA plasmid (0.7 µg/µl) was linearized with 2 µl of either Not-I (8 U/µl, Amersham Pharmacia Biotech) or Kpn-I (10 U/µl Invitrogen) restriction enzymes, 2 µl of their respective buffers (10X H buffer, 10X REact 4 buffer), and 1 µl H_2O, for 2 h at 37°C. Gel electrophoresis and DNA extraction was performed as above. Antisense and sense riboprobes were respectively produced by *in vitro* transcription

of the NotI-linearized plasmid, using the T7 polymerase, or the KpnI-linearized plasmid using T3 polymerase.

For *EphA7*, 15 µl of either pBluescript antisense plasmid (0.36 µg/µl) or pBluescript sense probe (0.47 µg/µl) were digested with 2 µl of either HindIII or Not-I restriction enzymes, 2 µl of their respective buffer, and 1 µl H_2O, as above. Gel electrophoresis and DNA extraction was done as above. The antisense and sense riboprobes were respectively produced by *in vitro* transcription, using T7 polymerase (on HindIII-linearized plasmid), or T3 polymerase (on Not-I-linearized plasmid) from the Roche DIG RNA labelling kit (Fig. 2).

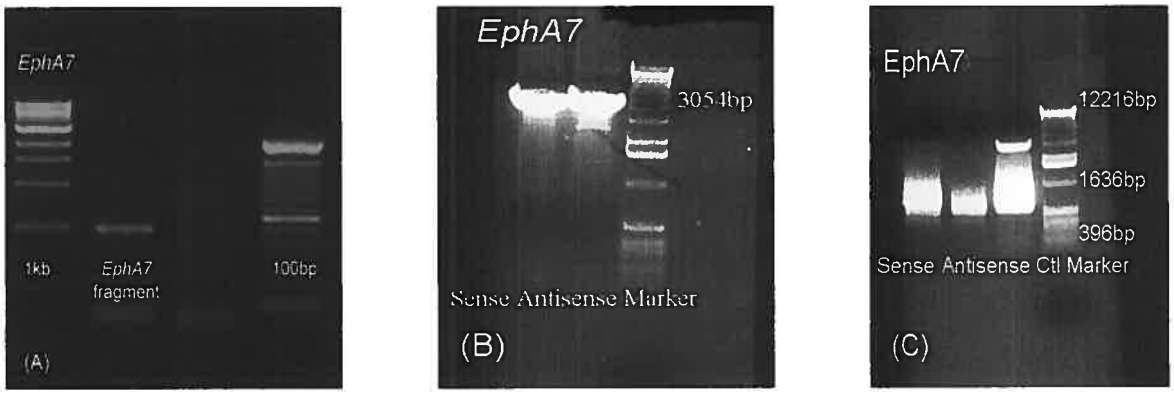


Figure 2. Production of EphA7 riboprobes

A) *EphA7* fragment extracted from Neuro2A cell line and amplified by PCR. B) SK-*EphA7* plasmid linearized by digestion with HindIII (for antisense riboprobe) or with NotI (for sense riboprobe) restriction enzymes. C) DiG-labelled *EphA7* sense and antisense riboprobes, and control DNA (Ctl).

2.2.3 In situ *hybridization*

In situ hybridization was performed on 6 µm-thick coronal brain sections (embryonic and neonatal rat, and adult mouse). Sections were de-paraffinated in 3 baths of xylenes (10 min) and gradually re-hydrated by successive baths of ethanol 100% (2x5 min), 95%, 70%, 50% and 30% (3 min each), and finally in SSC (Sodium chloride 300 mM; sodium citrate 30 mM) 2X, pH 7.5 for at least 5 minutes. The hybridization solution contained 40% formamide, SSC 4X, Denhardt solution 5X (Sigma-Aldrich), 150 µg/ml salmon sperm DNA (Invitrogen), 250 µg/ml yeast tRNA (Invitrogen), dextran sulfate 10% (Sigma-Aldrich) and RNA probe 1 µg/ml. Two-hundred microliters of hybridization solution were applied onto each section, covered with a piece of Parafilm, and the slides were incubated in a humid chamber at 52°C for 16-20 h. After hybridization, the slides were rinsed twice in SSC 2X pH 7.5, and twice in SSC 0.1X pH 7.5 (52°C; 15 min each).

For the immunohistochemical detection of digoxigenin, the sections were first incubated at room temperature with Tris-buffered saline (TBS; Tris-HCl 100mM, NaCl 100mM, $MgCl_2$ 2 mM, pH 7.5) containing 1% bovine serum albumin (BSA), for 30 min, to block nonspecific sites, and then incubated for 1 h at room temperature in TBS-BSA 1% containing alkaline phosphatase-conjugated anti-digoxigenin antibody (1:500, Dig Nucleic Acid Detection Kit, Roche). Slides were washed 3 times in TBS pH 7.5 (15 min each), and once in TBS pH 9.5 (Tris-HCL 100 mM, NaCl 150 mM, $MgCl_2$ 5mM). Riboprobes were visualized by the alkaline phosphatase reaction in the presence of nitro blue tetrazolium chloride (NBT) and 4-bromo-4-chloro-3 indolyl phosphate (BCIP). The reaction was stopped by rinsing in water for 10 min and the sections were air dried, and coverslipped with DPX (Fluka, Buchs, Switzerland).

2.3 Detection of 5-HT neurons by SERT immunohistochemistry

To detect 5-HT neurons in the DR, SERT immunohistochemistry was performed on paraffin-embedded sections from the same animals. De-paraffinization and hydratation steps were performed as above. Then, the slides were pre-incubated with penetrating and blocking agents (0.3% Triton X100, and 5% normal goat serum) for 2-3 h at room temperature. The slides were incubated overnight at room temperature in a humid chamber, under 200 µl of PBS containing 5% normal rabbit serum, 0.3% Triton X100 and the anti-SERT primary antibody (1:500, sc-1458; Santa Cruz Biotechnology). The following day, slides were washed 3 times in PBS (5 min each), and incubated under 200 µl of a biotin-conjugated rabbit secondary antibody anti-goat immunoglobulins (1:500 in PBS; Jackson ImmunoResearch) for 2-3 h. They were washed 3 times with PBS, and then incubated under 200 µl of a streptavidin-horseradish peroxidase (HRP) complex (1:1000 in PBS; Jackson) for 1 h at room temperature. After several washes in PBS and then in 50 mM Tris–HCl, they were reacted with 400 µl of 3-3'diaminobenzidine tetrachloride (Sigma-aldrich), dried and coverslipped with DPX.

2.4 Cortical, striatal, and ventral midbrain cell membrane extraction and Western blotting

To test for the presence of ephrin-As in the membranes extracted from the 3 potential target brain regions examined in the laboratory (Sharif Askari, 2006), pieces from each of the three regions (Ctx, Str, and VM) were dissected from neonatal rat brain (Fig. 3). The dissected pieces were transferred to 1.5 ml centrifuge tubes containing Buffer H (10 mM Tris-HCl, pH7.4, 1.5 mM $MgCl_2$ and 1 mM spermidine with 40 µl protease inhibitor; Roche). The tissue was homogenized by several strokes with P1000 and P200

pipettes (Pipetman), followed by an ultrasonic bath (Branson 2200) for 5-10 min. The homogenate was put on top of a sucrose gradient (300 µl sucrose 5% and 700 µl sucrose 50%) and ultracentrifuged (Sorval Ultra pro 80; rotor AH650) for 20 min at 50 000 x g at 4°C. The band containing the purified membranes was collected, washed in PBS, and centrifuged for 10 min at 16 000 g (Eppendorf centrifuge 5415C), at 4°C (twice). The pellet was re-suspended in 1 ml PBS and the protein concentration was determined by using the Lowry assay with ovalbumine as standard. Equal amounts of purified membranes (20 µg/ml) were resolved in 12.5% SDS and transfered to nitrocellulose membrane in a transfer apparatus (Mini protein II, Bio-Rad). After transfer, the membrane was blocked with 5% non fat milk in TBS 1X, pH 7.5 plus 0.05% Tween 20 (Bio-Rad) for 1h at room temperature. Antibodies used included purified polyclonal rabbit anti-ephrin-A4 (1:500; Zymed Laboratories, South San Francisco, CA), anti-ephrin-A5 (1:250; Zymed) and a monoclonal mouse anti-actin (1:10 000; Sigma-Aldrich). Membranes were incubated with either primary antibody in TBS-BSA 3% overnight. The next day, blots were rinsed 3 times 15 min with TBS containing 0.05% Tween-20 on a horizontal shaker. Secondary antibodies used were HRP-conjugated Affinipure goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG (1:5000; Jackson) and membranes were incubated for 1h at room temperature, followed by 6 washes of 5 min in TBS containing 0.05% Tween-20, on a horizontal shaker. Immunoreactive bands were detected on X-ray films (X-OMAT, Kodak diagnostic films) using the chemiluminescent ECL Western Blotting Detection system (ECL Western Blotting System, Amersham Biosciences).

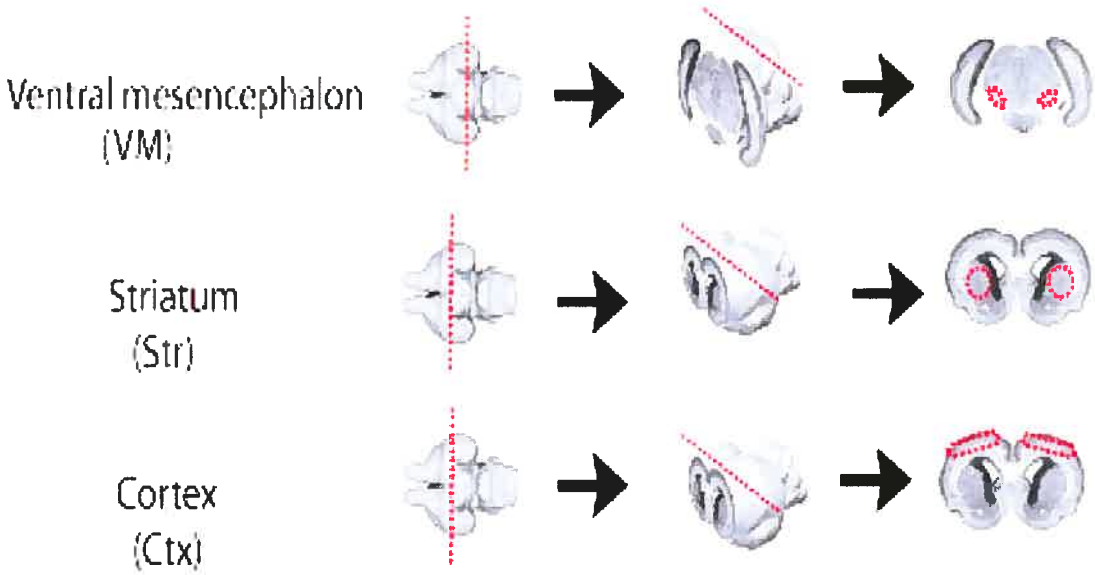


Figure 3. Dissection of VM, Str and Ctx tissues from neonatal rat brain (modified from Petit *et al.*, 2005).

3. Results

3.1. *In situ* hybridization in hippocampus

We used the hippocampus to test the efficacy of our riboprobes (EphA4, EphA5 and EphA7), since most previous reports have shown strong expression of these receptors in this specific brain region. In our experiments, the 3 receptors, *EphA4*, *EphA5* and *EphA7* were strongly expressed in the adult mouse hippocampus, as detected with the respective antisense riboprobes. In the 3 cases, there was only very faint or no staining on sections exposed to the sense riboprobes (Figs. 4-6).

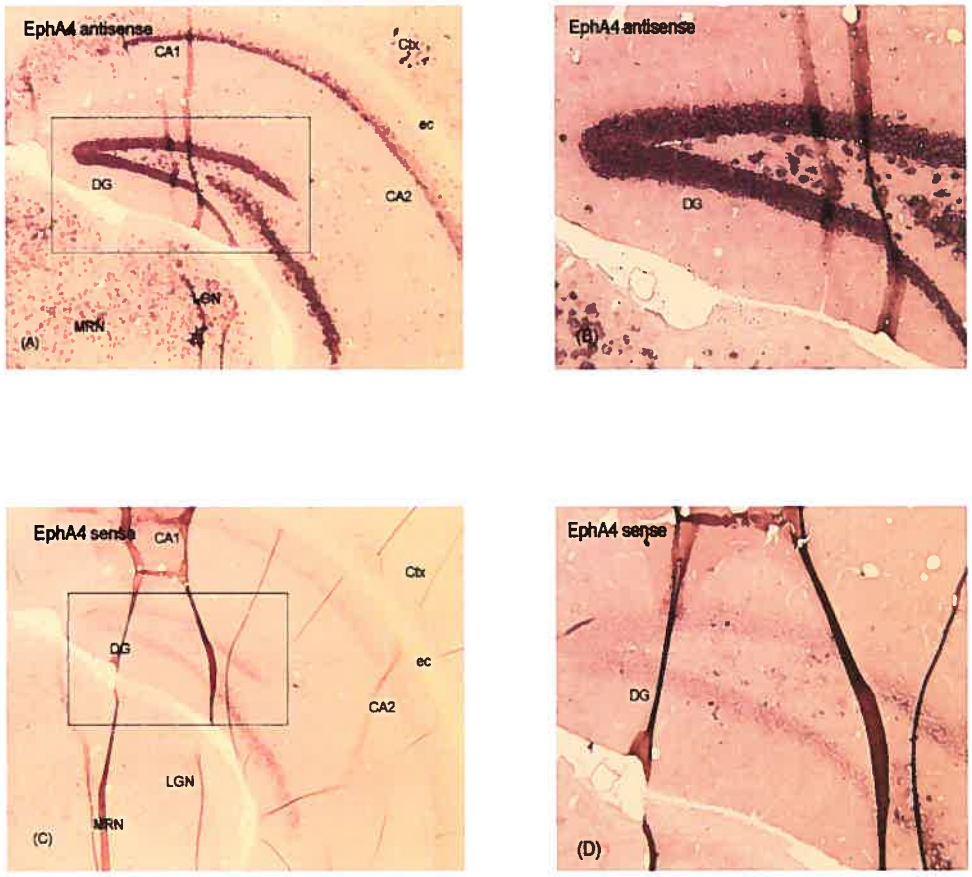


Figure 4. EphA4 *in situ* hybridization in adult mouse hippocampal tissue.

A) *EphA4* expression in the cerebral cortex, hippocampus and mesencephalon, as detected with the *EphA4* antisense probe. The area outlined is shown at higher magnification in B. C) Control *in situ* hybridization in an adjacent section, with the *EphA4* sense riboprobe. The outlined area is represented in D, at higher magnification. CA1: CA1 field of Ammon's horn, CA2: CA2 field of Ammon's horn, Ctx: cortex, ec: external capsule, DG: dentate gyrus, LGN: lateral geniculate nucleus, MRN: median raphe nucleus.

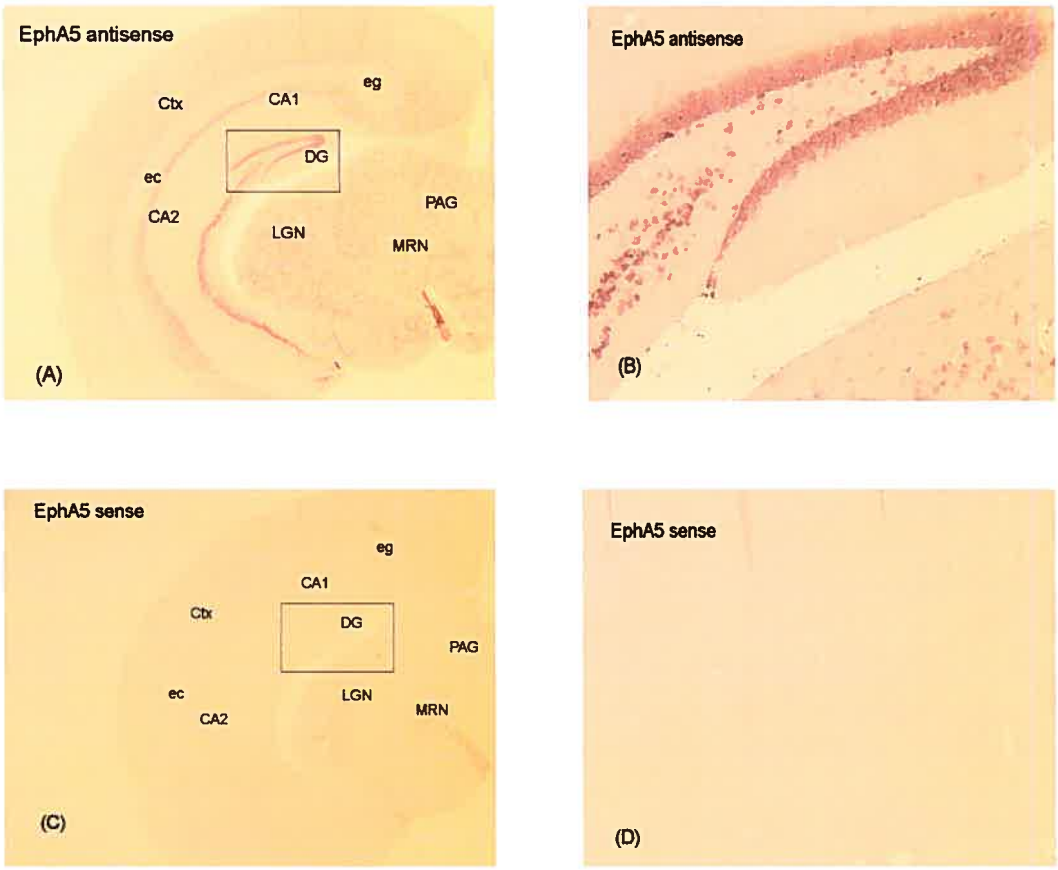


Figure 5. EphA5 *in situ* hybridization in adult mouse hippocampal tissue.

A) *EphA5* expression in the cerebral cortex, hippocampus and mesencephalon, as detected with the *EphA5* antisense riboprobe. The area outlined is shown at higher magnification in B. C) Control in situ hybridization in an adjacent section, with the *EphA5* sense riboprobe. The outlined area is represented in D, at higher magnification. CA1: CA1 field of Ammon's horn, CA2: CA2 field of Ammon's horn, cg: cingulum bundle, Ctx: cortex, ec: external capsule, DG: dentate gyrus, PAG: preaqueductal gray matter LGN: lateral geniculate nucleus, MRN: median raphe nucleus

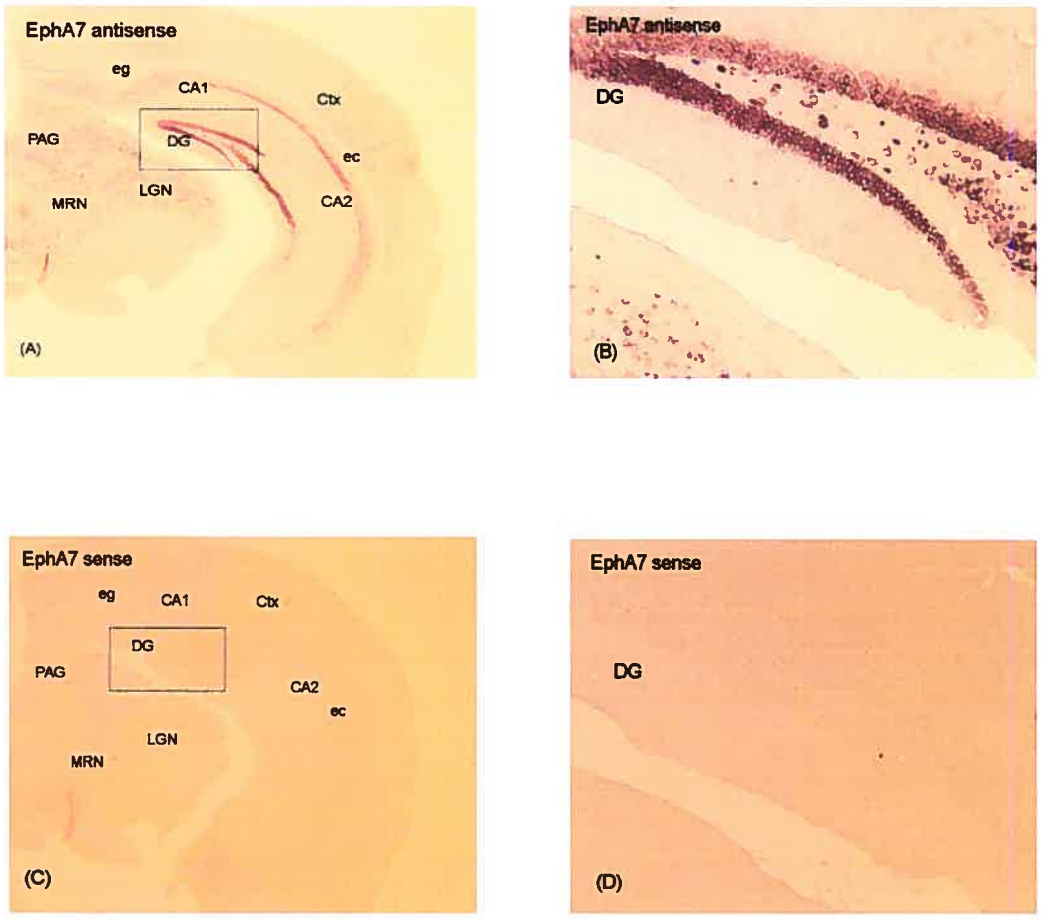


Figure 6. EphA7 in situ hybridization in adult mouse hippocampal tissue.

A) *EphA7* expression in the cerebral cortex, hippocampus and mesencephalon, as detected with the *EphA7* antisense riboprobe. The area outlined is shown at higher magnification in B. C) Control *in situ* hybridization in an adjacent section, with the *EphA7* sense riboprobe. The outlined area is represented in D, at higher magnification. PAG: periaqueductal gray; LGN: lateral geniculate nucleus, MRN: median raphe nucleus

3.2. Expression of *EphA4*, *EphA5* and *EphA7* in the DR region of rat embryos

We used immunohistochemistry with anti-SERT and anti-5HT antibodies to localize 5-HT neurons in the DR. Unfortunately, this approach dud not work on embryonic tissue, despite the fact that previous *in vivo* as well as *in vitro* studies had shown that 5-HT neurons of the DR are already differentiated at E15 and immunoreactive for both markers, sending projections to some regions of the CNS (Liu & Lauder, 1991; Rajaofetra *et al.*, 1992). Therefore, we had to rely on the Atlas of the Developing Rat Nervous System (Paxinos *et al.*, 1994) and the Chemoarchitectonic Atlas of the Developing Mouse brain (Jacobowitz & Abbott, 1998) to identify the DR in the sections of embryonic brain.

In situ hybridization with the 3 antisense riboprobes, showed mRNA expression of the 3 receptors in the DR of E15 rats (Figs. 7-9). All 3 receptors were also extensively expressed in the surrounding regions of the brainstem, including the superior and inferior colliculi. There was no staining in control sections exposed to the sense probes.

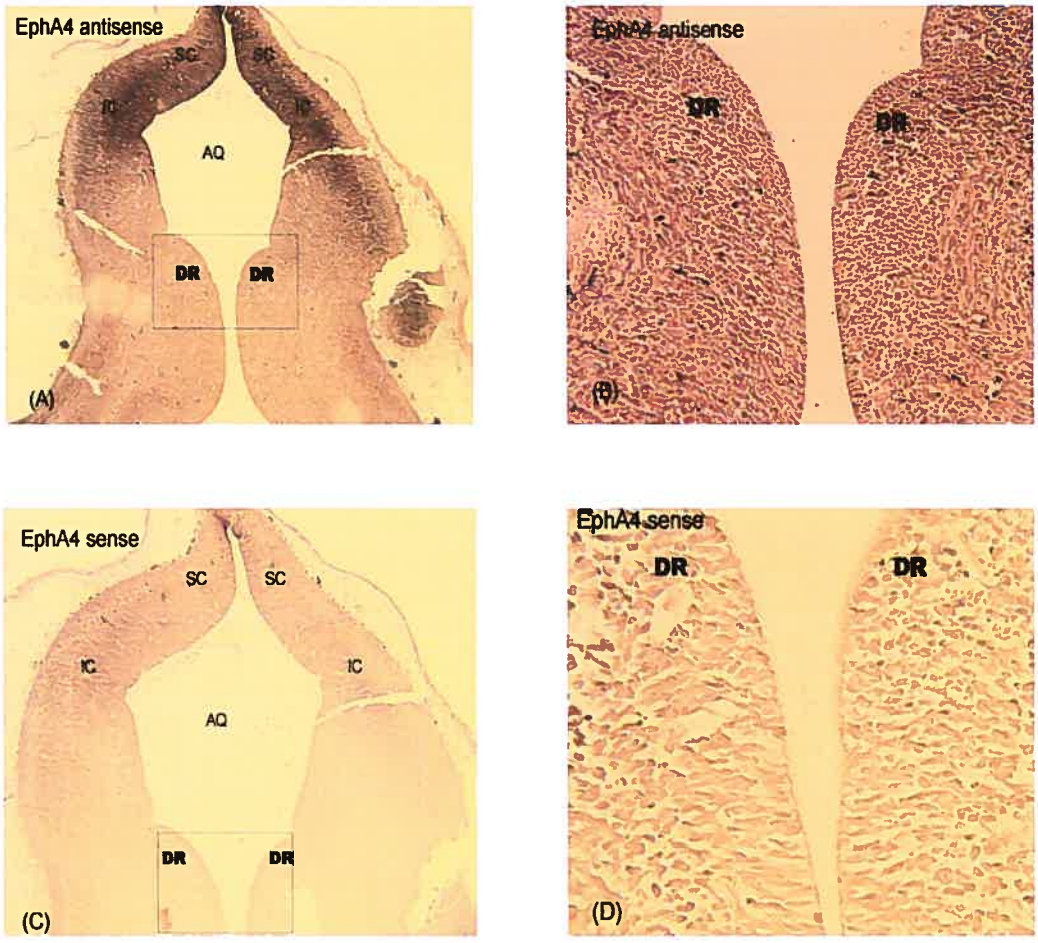


Figure 7. Expression of EphA4 in the DR of E15 rat brainstem. A) *In situ* hybridization with the *EphA4* antisense riboprobe, showing expression in the DR, as well as in many cells in the surrounding regions, such as the superior colliculi (SC), and inferior colliculi (IC). B) Area outlined in A, at higher magnification. C and D) Adjacent section at comparable magnifications, exposed to the control *EphA4* sense riboprobe. AQ: aqueduct, DR: dorsal raphe

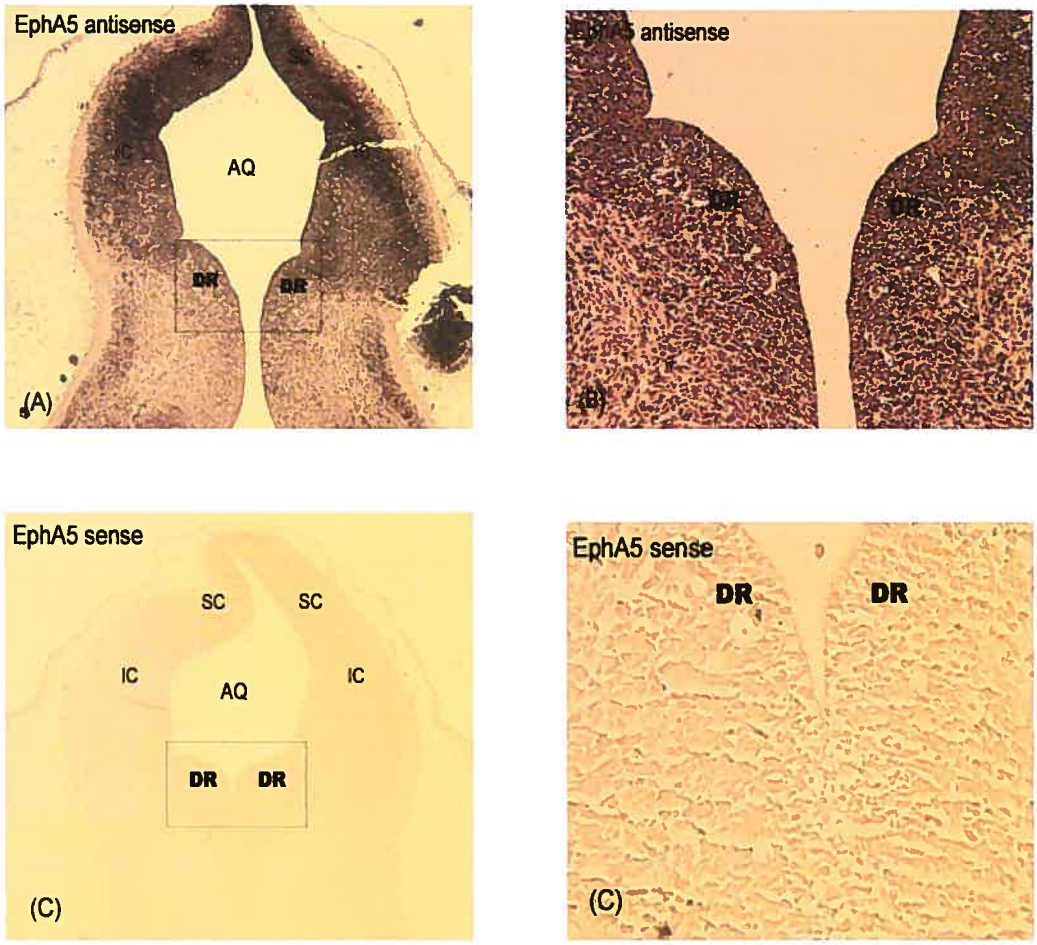


Figure 8. Expression of EphA5 in the DR of E15 rat brainstem. A) *In situ* hybridization with the *EphA5* antisense probe, showing strong expression in the DR, superior colliculi (SC), and inferior colliculi (IC). B) Higher magnification of area outlined in A. C and D) Adjacent section at comparable magnifications, exposed to the control *EphA5* sense riboprobe.

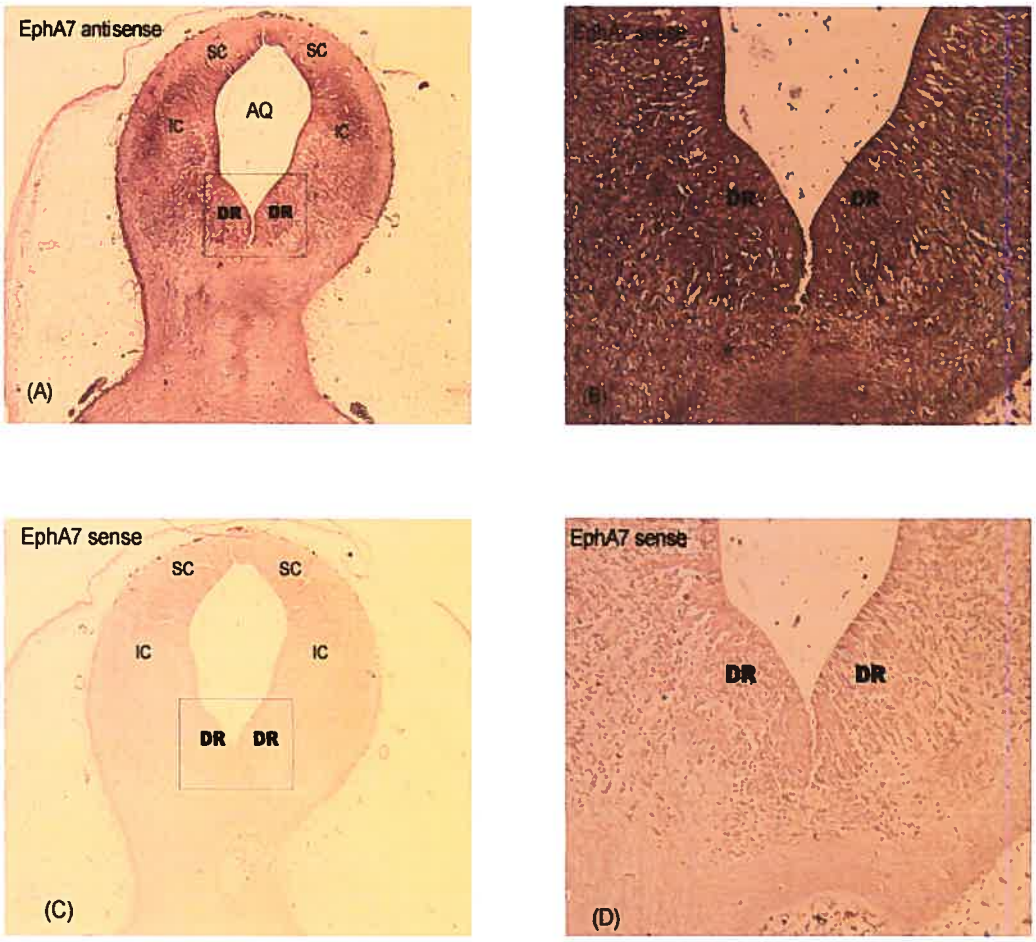


Figure 9. Expression of EphA7 in the DR of E15 rat brainstem. A) *In situ* hybridization with the *EphA7* antisense riboprobe. B) Higher magnification of area outlined in A. C and D) Adjacent section at comparable magnifications, exposed to the control *EphA7* sense probe.

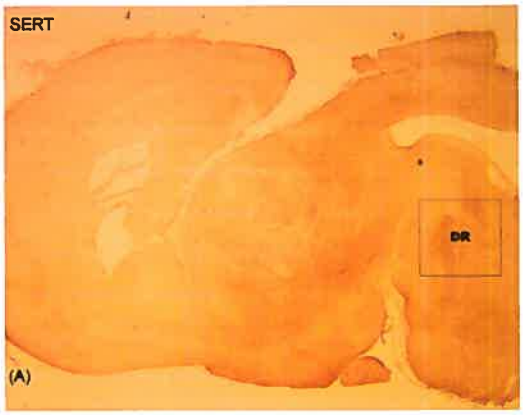
SC: superior colliculi, IC: inferior colliculi, AQ: aqueduct, DR: dorsal raphe

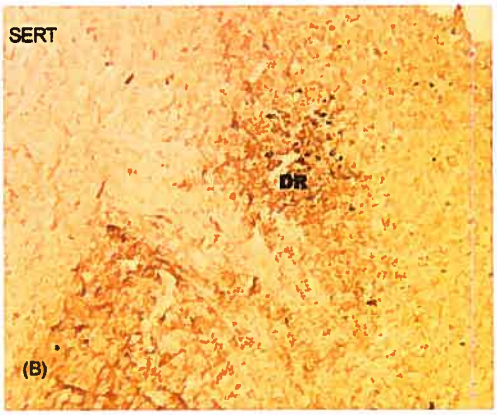
3.3. Expression of *EphA5* in the DR of neonatal rat brain

We used anti-SERT immunohistochemistry to locate the 5-HT neurons of the dorsal raphe in the neonatal brain, on sections adjacent to those used for *in situ* hybridization (Fig. 10A, B). The *in situ* hybridization shows that *EphA5* is still expressed in the DR at this stage, as well as in adjacent brainstem regions, including the ventral tegmental area (VTA) or central gray (CG) (Fig. 10C-F). Technical difficulties made it impossible to obtain data for the other two molecules in time for this thesis.

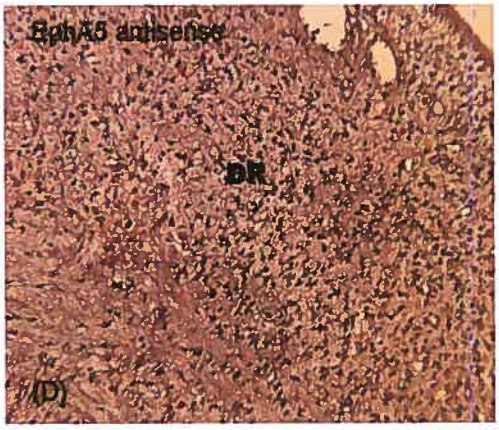
3.4. Expression of *EphA4, EphA5 and EphA7* in the DR of adult mouse brain

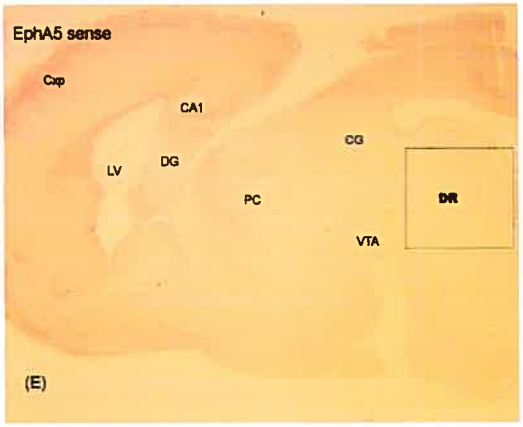
Anti-SERT immunohistochemistry was also used to locate the 5-HT neurons in the DR of the adult mouse in sections adjacent to those used for *in situ* hybridization (Fig. 11A,B). Compared to embryonic (and neonatal, for *EphA5*) brain, the number of labelled cells was markedly decreased in the adult brainstem and DR. Nevertheless, *EphA4*, *EphA5*, and *EphA7* were still expressed in many cells, in the DR as well as in other areas such as the inferior colliculi and periaqueductal gray (Figs. 11C-F, 12, 13).


SERT
DR
(A)


SERT
DR
(B)


EphA5 antisense
Cxp
CA1
CG
DG
LV
PC
VTA
DR
(C)


EphA5 antisense
DR
(D)


EphA5 sense
Cxp
CA1
CG
LV
DG
PC
DR
VTA
(E)

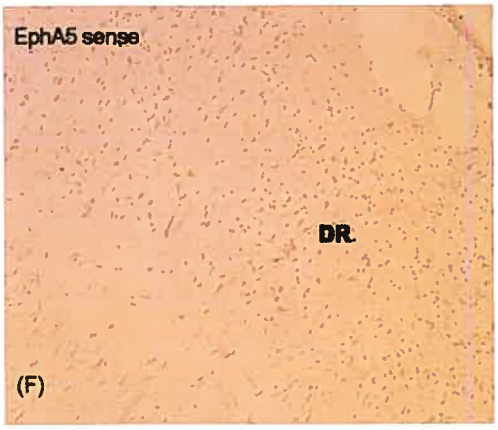

EphA5 sense
DR
(F)

Figure 10. Expression of EphA5 in the DR of P0 rat brainstem. A) *In situ* hybridization with the *EphA5* antisense riboprobe, showing expression in the DR and surrounding regions. B) Area outlined in A, at higher magnification. C and D) Adjacent section at comparable magnifications, exposed to the control *EphA5* sense probe. LV: lateal venricle, Cxp : cortical plate, PC : paracentral thalami nucleus.

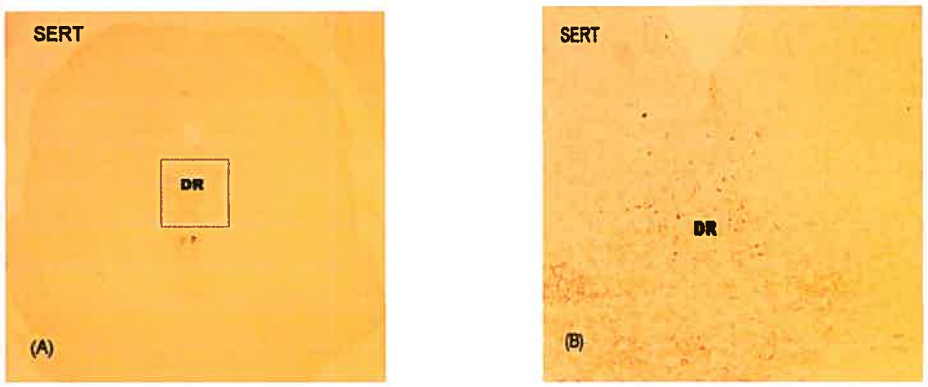


Figure 11. Serotonin neurons in DR of adult mouse. A) Immunohistochemistry anti-SERT to identify 5-HT neurons in DR. B) Area outlined in A, at higher maginification.

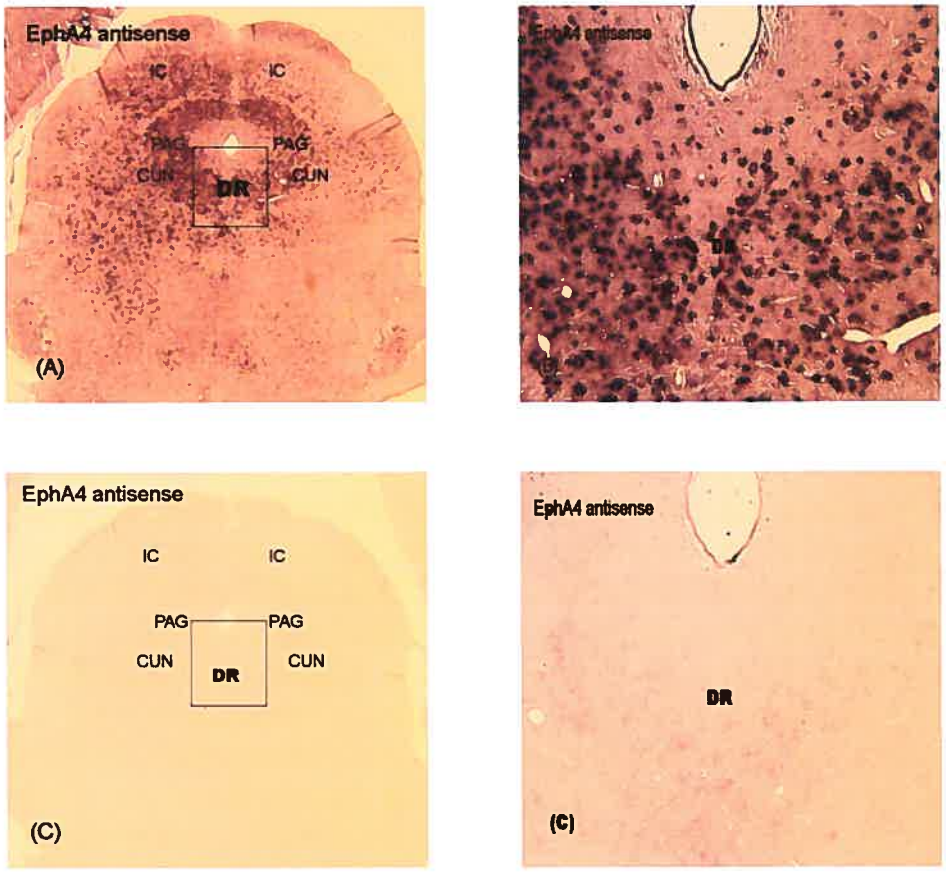


Figure 12. Expression of EphA4 in the DR of P60 adult mouse brainstem. A) *EphA4* antisense riboprobe, showing a relatively strong expression in the DR, as well as in some cells in the surrounding regions, such as peri aqueductal grey and cuneiform nucleus; B) Area outlined in A, at higher magnification. C and D) Adjacent section at comparable magnifications, exposed to the control *EphA4* sense probe. IC: inferior colliculi, DR: dorsal raphe, PAG: peri aqueductal grey matter, CUN: cuneiform nucleus.

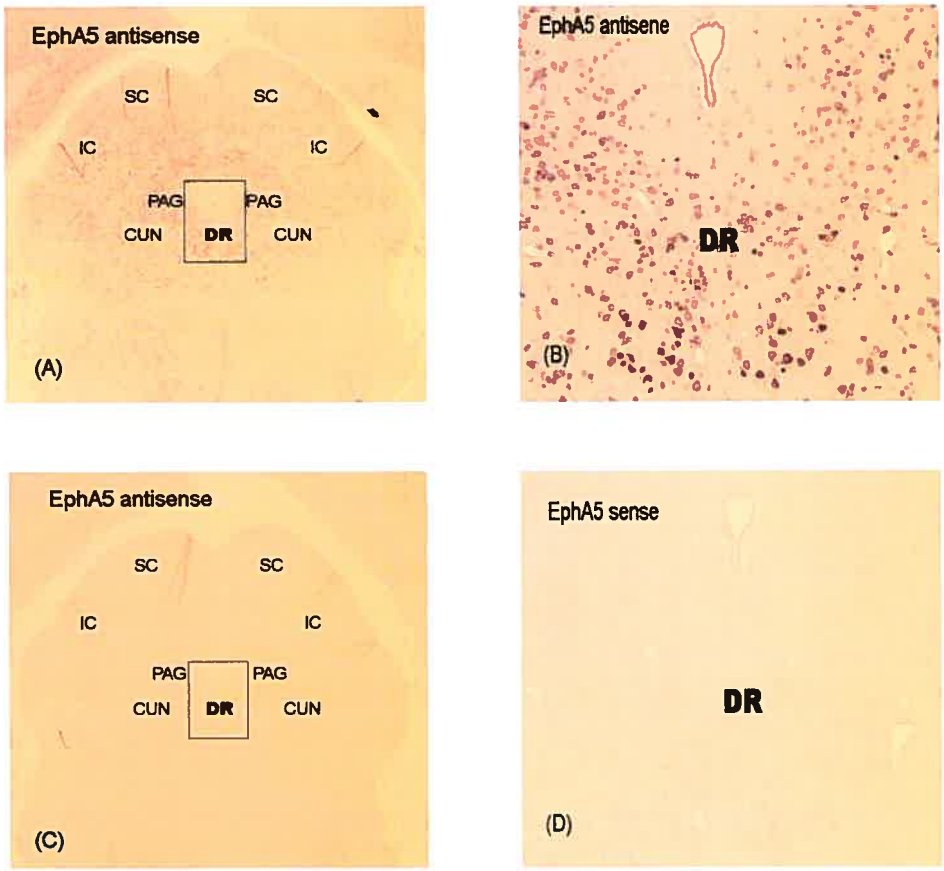


Figure 13. Expression of EphA5 in the DR of P60 adult mouse brainstem. A) *EphA5* antisense riboprobe; B) Higher magnification. C and D) Control *EphA4* sense riboprobe.SC: superior colliculi, IC: inferior colliculi.

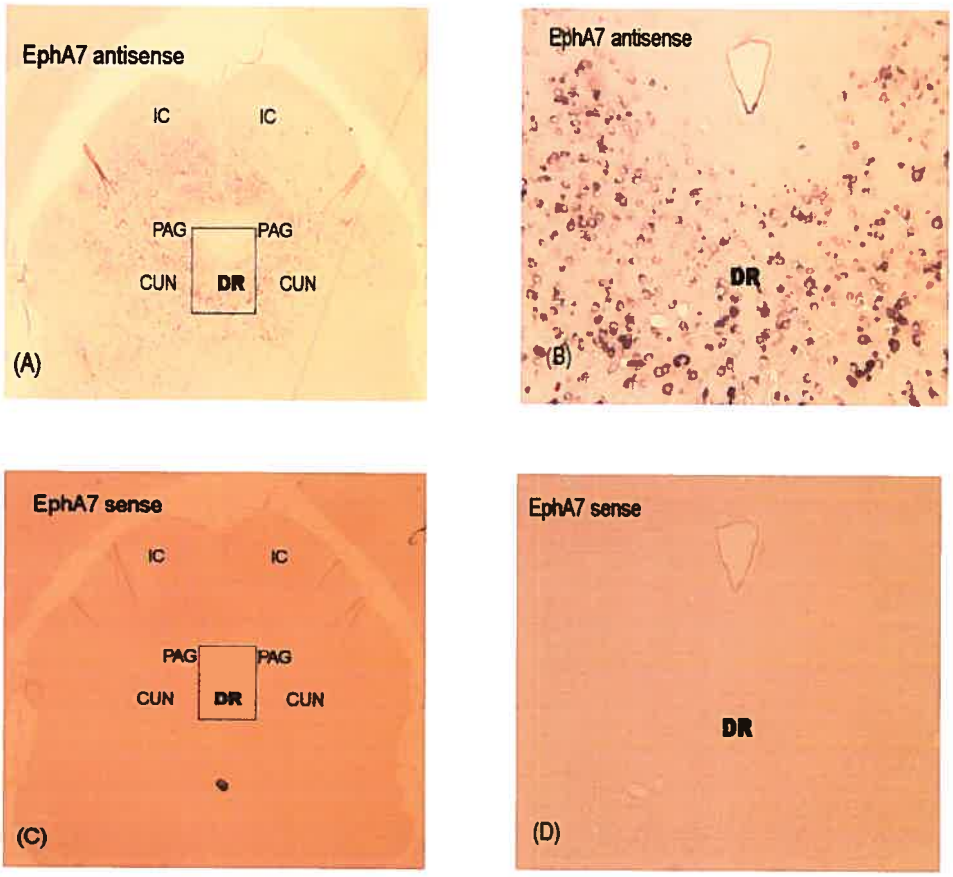


Figure 14. Expression of EphA7 in the DR region of P60 adult mouse brainstem. A) *EphA7* antisense probe; B) Higher magnification. C and D) Control *EphA7* sense riboprobe. Abbreviations as above.

3.5. Detection of ephrin-A4 and ephrin-A5 in membranes extracted from the Ctx, Str, and VM

We used Western blotting to assess the presence of ephrin-A4 and ephrin-A5 in the membranes extracted from the neonatal Ctx, Str, and VM, the 3 brain regions that had been used to test the guidance of 5-HT axons in our laboratory (Petit *et al.*, 2005; Sharif Askari, 2006). After the immunodetection of each ephrin, we performed a stripping of the blot membranes to process with an anti-β–actin antibody in order to monitor the amount of proteins in each lane. The immunoblots showed that ephrin-A4 was relatively abundant in Ctx and VM membrane extracts, but somewhat less in Str membranes (Fig.15). Ephrin-A5, was most abundant in VM, somewhat less in Ctx, and barely detectable in Str membranes from neonatal rat brain (Fig.16).

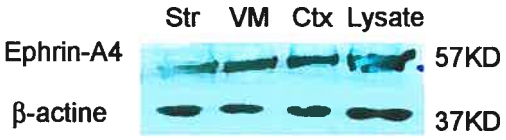


Figure.15. Ephrin-A4 expression in membranes extracted from target regions.

Upper line, Western blot with ephrin-A4 antibody on the membranes extracted from frontal cortex (Ctx), striatum (Str), and ventral midbrain (VM). Whole brain lysate was used as a positive control. In the lower lane, following stripping, Western blot for βactin antibody showing the amounts of protein in each lane.

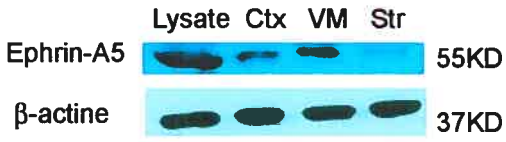


Figure.16. Ephrin-A5 expression in membranes extracted from target regions.

Upper line, Western blot with anti-ephrin-A5 antibody on the membranes extracted from Ctx, VM and Str; whole brain lysate serving as positive control. Bottom lane: anti-β-actin.

4. Discussion:

The 5-HT neurons of the DR, project to several brain regions including the VM, hypothalamus, thalamus, Str, hippocampus and Ctx (Moore *et al.*, 1978; van der Kooy & Hattori, 1980; Fallon & Loughlin, 1982; Imai *et al.*, 1986). Our laboratory is aiming to define the factors that guide 5-HT axons to their target fields in the forebrain and midbrain. Since recent *in vitro* experiments in our laboratory suggested that ephrin-As might be involved in the branching of 5-HT axons on cell membranes extracted from the Ctx, Str or VM, the aim of the present work was to investigate the presence of such molecules in these brain regions, as well as the expression of some of their receptors, EphAs, in the DR.

EphAs and ephrin-As are expressed in several brain regions during development, but their expression has not been examined in detail in the brainstem untill now.

4.1. EphA4, EphA5 and EphA7 are expressed in the DR at all stages examined

We first used adult mouse hippocampal sections as positive controls for our riboprobes, since strong expression of EphA4, EphA5 and EphA7 had previously been reported several times in this brain region of adult rodents (Gao *et al.*, 1998b; Ciossek *et al.*, 1999; Gerlai *et al.*, 1999; Murai *et al.*, 2003). In the 3 cases, the distribution of the labeling with the antisense riboprobes was consistent with the published observations, while there was practically no staining with the control sense riboprobe; demonstrating the specificity of the labeling.

The i*n situ* hybridization showed clear expression of EphA4, EphA5 and EphA7 in E15 rat brainstem, including the DR, as well as surrounding areas, like the superior and inferior colliculi. It was, in fact, rather surprising to find such extensive expression of all 3

molecules in the brainstem, considering that these are presumed to be receptors to axon guidance molecules. Nevertheless, this labeling pattern is consistent with the distributions published in online databases (www.brainatlas.org/aba; www.genepaint.org). Since the 5-HT innervation of forebrain and midbrain target regions develops perinatally, we tried to investigate the expression of the Eph receptors at the time of birth, but succeeded only for EphA5, for technical reasons that extended the deadline for this thesis. Thus, in newborn rat, the *in situ* hybridization for EphA5 also showed an extensive expression of this receptor in the brainstem, including the DR. The DIG approach used here for in situ hybridization did not allow detecting any eventual gradient of expression within the DR itself or among other brainstem cells. The extensive distribution of EphAs in brainstem in E15 and at birth suggests that they have an important function during development. A number of *in vivo* and *in vitro* studies that showed that ephrin-A gradients guide retinal ganglion cell axons to their appropriate topographic locations in the superior colliculi (SC) and therefore EphAs have a demonstrated role in the establishment of the retinotectal mapping and the axonal guidance in visual pathways (Feldheim *et al.*, 2000; Dufour *et al.*, 2003; Feldheim *et al.*, 2004). A more recent report showed that EphA4 also regulates the organization of auditory pathways from the cochlea to the brainstem (Huffman & Cramer, 2007). Eph receptors and ephrins have also been involved in the topographic guidance of other axonal projection systems, including the nigrostriatal and mesolimbic dopamine (DA) pathways (Sieber *et al.*, 2004). But their role has not yet been investigated in 5-HT systems.

The functions of EphAs in the DR has not been invertigated yet. The extensive expression of all 3 receptors, EphA4, EphA5 and EphA7, in the fetal and neonatal brainstem does not necessarily fit with the presumed roles in axon guidance or neuronal migration. To follow up on these studies, it will be necessary to use a more quantitative

technique for in situ hybridization, such as autoradiography, with radiolabelled riboprobes, or fluorescence *in situ* hybridization. In this manner, it might be possible to detect variations in the levels of expression of these molecules among brainstem and DR neurons. Gradients of expression would fit better with roles in axon guidance, in analogy with the visual system.

However, the 3 receptors showed a more restricted expression in the adult DR, suggesting that these molecules may have more specific roles in adult 5-HT, or other types of DR neurons. As in the adult hippocampus, they might be involved dendritic spine maturation, synaptogenesis, or synaptic plasticity (Murai & Pasquale, 2004). It will be important to use double-labeling experiments to positively demonstrate that the expression of these molecules concerns 5-HT neurons. At the moment, we can only presume that, since the expression appeared to interest all neurons in the DR, including large cells similar to 5-HT neurons, 5-HT neurons were indeed among the labeled cells.

In summary, we were successful in developing riboprobes and *in situ* hybridization protocols allowing to detect the expression of EphA4, EphA5 and EphA7 in fetal and neonatal rat, as well as adult mouse brain. Our preliminary observations show that the expression of these molecules is extensive in the developing brainstem. It will thus be necessary to refine the in situ hybridization approach to more finely characterize the topographic expression of these molecules. It will also be useful to examine the expression of these molecules in vitro, in 5-HT neurons that are challenged with membranes extracted from target brain regions, as well as with membranes from cell lines expressing or not any of their ephrin-A ligands.

4.2. Ephrin-A expression in Ctx, Str and VM of neonatal rat brain

We used Western blotting to detect the presence of ephrin-A4 and ephrin-A5 in the cell membranes extracted from the target regions of 5-HT innervations used in our experimentations *in vitro* (neonatal Ctx, Str and VM). The specificity of the antibodies had been tested in cell lines expressing or not these molecules (K. Murai, personal communication). Both ephrin-A4 and ephrin-A5 were present in the membranes extracted from the 3 target regions, but with a lower intensity in Str. This is consistent with previous reports. Ephrin-A5 was reported to be expressed throughout the sensorimotor and auditory cortices. Its mRNA was detectable first at low levels at E16 and increased to peak intensity during the late embryogenesis and early postnatal days (Gao *et al.*, 1998a; Yun *et al.*, 2003). It was also shown that ephrin-A4 and ephrin-A5 mRNAs are present in the striatum, in newborn rats, where they appear to take part in regulating the compartmental organization of striatum (Janis *et al.*, 1999). Little is known about their fine distribution in brainstem.

A finer description of the distribution of these ephrins would benefit from an immunohistochemical approach. Unfortunately, antibodies convenient for immunohistochemistry are difficult to find. Our laboratory has tested several commercial anti-ephrin-A2, anti-ephrin-A3 and anti-ephrin-A5 antibodies (including antibodies from R&D, Zymed, or Santa Cruz) on brain sections from ephrin-A2, ephrin-A3, or ephrin-A5 knockout mice, respectively, which showed that these antibodies were not suitable for immunohistochemistry (although they were correct for Western blotting).

Nevertheless, our current results demonstrate that at least some of the ephrin-As are present in cell membranes from the target brain regions innervated by DR 5-HT neurons.

Together with the results of the in vitro experiments by B. Sharif Askari, these observations are consistent with the hypothesis that ephrin-As and EphAs may serve as guidance cues for 5-HT axons.

5. Conclusion

Ongoing experimentation in our laboratory indicates a possible role for ephrins in the guidance of 5-HT axons that project from DR to the Ctx, Str or VM. Our present preliminary results show that EphA4, EphA5 and EphA7 are expressed in the DR of embryonic, neonatal and adult rodents. The presence of ephrin-A4 and ephrin-A5 proteins has also been demonstrated in cell membranes extracted from the neonatal Ctx, Str and VM. Together, these observations are consistent with the hypothesis of a role of these molecules in the guidance of 5-HT axons to the selected regions. These preliminary results need to be confirmed and refined with more *in vitro* and *in vivo* experiments, notably to better define the distribution of these molecules in the DR and its innervation target domains. It will also be useful to define the eventual co-localization of several ephrin-As and EphAs in the 5-HT neurons, since the guidance of their axons is most likely the result of a combination of signals.

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