Université de Montréal

Analyse des mécanismes cellulaires et moléculaires du guidage axonal sérotoninergique in vitro

par
Bahram Sharif Askari

Département de pathologie et biologie cellulaire
Faculté de médecine

Mémoire présenté à la Faculté des études supérieures
en vue de l'obtention du grade de maîtrise
en pathologie et biologie cellulaires
option système nerveux

Mai 2006

Université de Montréal
Faculté des études supérieures

Ce mémoire intitulé

Analyse des mécanismes cellulaires et moléculaires du guidage axonal sérotoninergique in vitro

présenté par :

Bahram Sharif Askari

a été évalué par un jury composé des personnes suivantes :

Dr Louis-Éric TRUDEAU
président-rapporteur

Dr Guy DOUCET
directeur de recherche

Dr Timothy KENNEDY
membre du jury

Résumé français

Des travaux antérieurs du laboratoire avaient démontré la capacité des neurones sérotoninergiques (5-HT) à reconnaître des signaux de guidage spécifiques dans des extraits de membranes de diverses régions cibles du cerveau. Les objectifs du présent travail étaient de déterminer la réponse axonale de neurones 5-HT individuels exposés à un choix de substrats appliqués en bandes alternantes d'extraits de membranes du cortex cérébral (Ctx), du striatum (Str) ou du mésencéphale ventral (VM) néonatals et de caractériser ou identifier les molécules contribuant à ce guidage axonal.

Nous avons observé que les axones 5-HT formaient des branchements axonaux de préférence sur des bandes de membranes de la même origine cérébrale que celles où étaient localisés leurs corps cellulaires. Cette observation indique que les axones reconnaissent des signaux de guidage distincts, selon les régions cérébrales. De plus, il semble qu'il y ait une induction par contact de la spécificité de reconnaissance axonale, au niveau du corps cellulaire ou de la croissance initiale de l'axone, puisque l'adhérence des corps cellulaires 5-HT sur les membranes extraites de régions cérébrales normalement dénuées de neurones 5-HT, se faisait présumément au hazard. Cette activité a été abolie des membranes du VM par un traitement avec la phospholipase-C spécifique du phosphatidylinositol (PI-PLC), qui défait les ancrages glycosylphosphatidylinositols (GPI), de même que des membranes du Ctx traitées avec une protéine de fusion EphA3-Fc, qui bloque les éphrines-A. Les éphrines-A étant les ligands membranaires à ancrage GPI des récepteurs EphA, elle constituent de bons candidats moléculaires pour cette activité de guidage. La poursuite de ces travaux permettra de déterminer si les membranes des autres régions testées perdront aussi leur activité de guidage avec les mêmes traitements et si on pourra modifier la réponse des

axones 5-HT en jouant avec l'expression des récepteurs EphA ou des éphrines-A dans ces neurones.

Mots clés :

Rat, neurobiologie, développement, guidage axonal, raphé dorsal, sérotonine, culture cellulaire neurale, protéines à ancre GPI, éphrines, récepteurs Eph

Résumé anglais:

Previous work in our laboratory has shown that serotonergic (5-HT) neurones had the capacity to recognize specific guidance signals in cellular membranes extracted from various target brain regions. The objectives of the present work were to determine the axonal response of individual 5-HT neurones challenged with a choice of substrates applied as alternating stripes made of membrane extracts from the neonatal cerebral cortex (Ctx), striatum (Str), or ventral midbrain, and to characterize or identify the molecules involved in this axon guidance activity. We observed that 5-HT axons branched preferentially on the membrane stripes of the same cerebral origin as those on which their parent cell bodies were lying. This observation indicates that the axons recognize guidance signals that are brain region-specific. Moreover, it appears that there is a contact induction of the specificity of axonal recognition, at the level of the perikarya or intitial axon growth, since perikaryal adherence to any stripes occurred presumably at random on membranes extracted from target brain regions that are normally devoid of 5-HT neurons.

This axon guidance activity was abolished from VM membranes treated with the phosphatidylinositol-specific phospholipase-C (PI-PLC), which removes the GPI anchors, as well as from Ctx membranes treated with the fusion protein EphA3-Fc, which blocks ephrin-As. Ephrin-As being the GPI-anchored membrane ligands of EphA receptors, they constitute good candidates for this axon guidance activity. Further experiments will tell if the membranes from the other tested brain regions will also lose their axon branching inducing activity following similar treatments and whether one will be able to modify the response of 5-HT axons by changing the expression of EphAs or ephrin-As in these neurons.

Key words:

Rat, neurobiology, development, axon guidance, dorsal raphe, serotonin neurons, neural cell culture, GPI-anchored proteins, ephrins, Eph receptors

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List of Abbreviations :

5-HT: 5-hydroxytryptamine, (sérotonine)

CAM: cell adhesion molecule (molécule d'adhérence cellulaire)

CNS : central nervous system (système nerveux central)

Ctx : cerebral cortex (cortex cérébral)

ECM : extracellular matrix (matrice extracellulaire)

Efn : ephrin (éphrine)

Eph : Eph receptor (récepteur Eph)

IgCAMs : immunoglobulin CAMs (CAM de la superfamille des immunoglobulines)

NCAM : neural CAM (CAM neurale)

Str : striatum

VM: ventral midbrain (mésencéphale ventral)

PI-PLC : phosphatidylinositol-specific phospholipase C

GPI : glycosylphosphatidylinositol

GDNF : Glial cell line-derived neurotrophic factor

TGF-β : transforming growth factor-β

FGF : fibroblast growth factors

Wnt : wingless

Shh : sonic hedgehog

Robo : Roundabout

Dedication:

To my wife, Manzar

and my daughter, Bahar

Acknowledgements

I wish to thank Pr. Guy Doucet for his supervision of this thesis, Audrey Petit for teaching me the cell culture and stripe assay techniques, David Bouvier for his help in preparing figures, Moogeh Baharnoori for continuing this project, Miguel Chagnon for statistical analyses, and Michel Lauzon for his help with fluorescence microscopy, confocal microscopy and image analysis. I am also grateful to the Département de pathologie et biologie cellulaire for its financial support and to the Canadian Institutes for Health Research which supported this project.

1. Introduction

The main objective of our laboratory is to understand the cellular and molecular factors that control the guidance of serotonergic (5-HT, for 5-hydroxytryptamine, serotonin, or serotonergic) axons projecting from the dorsal raphe to the forebrain and midbrain of the developing and adult rodent.

The laboratory had shown previously that neural grafts from the fetal striatum (Str), implanted into the striatum were better innervated by the neonatal or adult host 5-HT axons than those from the ventral midbrain (VM) (Mounir et al., 1994; Pierret et al., 1998; Doucet & Petit, 2002). Furthermore, it was demonstrated that astrocytes present in the graft, could influence the host-derived 5-HT innervation. Indeed, astrocytes derived from the neonatal cortex (Ctx) or Str could promote the innervation of VM grafts, whereas astrocytes prepared from the VM had no effect on the 5-HT innervation of VM or Str grafts (Petit et al., 2001).

To further define the molecules involved in the guidance of these 5-HT axons, experiments were done in vitro, using neural explant cultures, either in collagen gel or onto substrates made of cellular membrane from various brain regions (Petit et al., 2005). This experimentation disclosed several activities associated with cell membranes and influencing 5-HT axon growth. First, an axon growth promoting activity was detected in membranes from all tested CNS regions, including spinal cord, alternating with PLL in the alternating stripe assay. Second, an axon growth inhibitory activity was found by culturing dorsal raphe explants onto a small carpet of membranes from the Str or VM, adjacent to a second carpet from a different target brain region: 5-HT axons then stopped at the border between the two membrane carpets. Third, it appeared that 5-HT axons are induced to recognize the latter inhibitory factors during their transit on the VM or Str membranes. Indeed, these axons were

not stopped at the border between a Ctx membrane carpet and another, distal carpet from any other brain region. Moreover, treating VM or Str membranes in the proximal carpet, with PI-PLC or high KCl, removed this inducing activity in such a way that 5-HT axons were no longer inhibited to grow onto a distal carpet of membranes from another brain region. This suggested that the inducing activity was associated with glycosylphosphatydilinositol (GPI) anchored membrane proteins in complex with peripheral proteins bound to the extracellular surface (Petit et al., 2005).

In the present study, we developed another in vitro assay that allowed examining the reaction of individual 5-HT neurones, using dissociated neuronal cultures of the foetal dorsal raphe onto alternating stripes of substrates made of membranes from various brain regions, or of poly-L-lysine.

In the following, I will first describe the context of this work by briefly reviewing the functions, organization and development of the 5-HT neuronal systems, I will then review briefly the current knowledge about the major axon guidance molecules.

1.1. Functional roles of the 5-HT systems

Serotonin is found in central nervous system (CNS) neurons as well as in several types of peripheral cells. In neurons, 5-HT acts mainly as a neurotransmitter, or neuromodulator, but is also known to exert various effects at different developmental stages and in different brain regions, such that it is sometimes difficult to separate transmitter actions and other types of effect, e.g., neurotrophic. In the CNS, it also influences neural development, as we will see below, including neuronal proliferation, neurite outgrowth and synapse formation. Moreover, 5-HT and 5-HT receptors have been implicated in neuronal

plasticity and memory formation. (Azmitia *et al.*, 1981; Lauder *et al.*, 1982; Lauder, 1990; Gaspar *et al.*, 2003).

In the mature CNS, 5-HT is involved in several homeostatic functions, such as the central regulation of blood pressure, sodium and glucose balance and body fluid homeostasis (Harding *et al.*, 2004). It also has an important role in various aspects of behaviour including sex, feeding, and affective behaviour, as well as depression and anxiety (Harding *et al.*, 2004). Appetite, the sleep-wake cycle and cognition are all affected by up- or down-regulation of 5-HT secretion (Serrats *et al.*, 2005).

Alterations of the 5-HT systems have been reported in neurodegenerative and psychiatric disorders, including Alzheimer's disease, schizophrenia, autism, depression, as well as in normal aging (Harding et al., 2004). In general, though, the exact contribution of 5-HT, or 5-HT neurons, to pathogenesis remains to be clarified.

The various physiological or pharmacological effects of the 5-HT systems are mediated by multiple receptor subtypes, classified into 7 groups, named 5-HT1 to -7. This classification is based on their amino acid sequence, gene structure (Russo *et al.*, 2005), pharmacological and functional characteristics (Russo *et al.*, 2005). All of the 5-HT receptors belong to the G-protein-coupled receptor family, except 5-HT3 which is unique among 5-HT receptors as a ligand-gated ion channel and ionotropic receptor (Tierney, 2001; Maxwell *et al.*, 2003; Russo *et al.*, 2005). These receptors are differentially scattered in the CNS.

Among the 7 classes of 5-HT receptors, the 5-HT1 class includes 5 subtypes (1A to 1F) (Lanfumey & Hamon, 2004), concentrated in choroid plexus, dentate gyrus, substantia nigra, globus pallidus, reticular formation and entorhinal cortex (Russo *et al.*, 2005).

The 5-HT2 class comprises 3 subtypes: 2A to 2C, with extensive distributions in CNS, except 5-HT2B, which is more restricted in distribution (Leysen, 2004). The 5-HT2B receptors are localized in the olfactory tubercle, layer IV of the neocortex, claustrum, lateral amygdala, nucleus accumbens and hippocampus (Russo *et al.*, 2005).

The 5-HT3 receptors include two subunits, 3A and 3B. They become permeable to Na+ and K+, when activated, and cause membrane depolarisation (Grant, 1995; Tierney, 2001; Maxwell *et al.*, 2003).

The 5-HT4 class, discovered 15 years ago, only has one subtype, that may be a target for the treatment of cognitive deficits, feeding disorders and abdominal pain (Baez *et al.*, 1995; Bockaert *et al.*, 2004).

The 5-HT5 receptors include subtypes 5A and 5B. The 5-HT5A receptor was detected in rat , mouse and human, but 5-HT5B appears to be expressed only in mouse and rat (Nelson, 2004). The expression of the 5-HT5 receptors is essentially restricted to the CNS, with 5-HT5A receptors showing an extensive distribution and 5-HT5B being more restricted (Nelson, 2004). Expression of 5-HT5 in the suprachiasmatic nucleus raises the possibility that they are involved in the regulation of blood circulation (Russo *et al.*, 2005).

The 5-HT6 receptors are newly discovered G-protein-coupled receptors that have a high affinity for a broad spectrum of psychiatric drugs (Woolley *et al.*, 2004; Russo *et al.*, 2005). They are expressed in the CNS and involved in the regulation of glutamatergic and cholinergic neuronal activity, as well as in cognition (Woolley *et al.*, 2004).

The 5-HT7 receptors are expressed mainly in thalamus, hypothalamus and hippocampus, with low expression in the amygdala and cerebral cortex (Russo *et al.*, 2005). There are 4 5-HT7 subtypes, 7A to 7D, found in rat tissues (Russo *et al.*, 2005). They have

known functions in peripheral tissue, such as smooth muscle relaxation, alimentary tractus and cardiovascular system (Thomas & Hagan, 2004). They are implicated in regulation of cicardian pacemaker function, in the suprachiasmatic nucleus, and may play a role in CNS disorders such as cognitive disturbances, anxiety and migraine (Thomas & Hagan, 2004; Russo *et al.*, 2005).

1.2. Organisation of the 5-HT systems

1.2.1 The raphe nuclei

Serotonergic neurons are located in the raphe nuclei, which constitute a collection of cell groups along the midline region of the brainstem tegmentum, from the rostral midbrain to the medulla-spinal cord transition, at the level of the pyramidal tract decussation (Steinbusch & Nieuwenhuys, 1983; Harding et al., 2004). The raphe nuclei comprise the caudal linear nucleus, nucleus raphe dorsalis, median raphe nucleus, nucleus raphe magnus, nucleus raphe obscurus and nucleus raphe pallidus (Steinbusch & Nieuwenhuys, 1983; Harding et al., 2004).

Serotonergic neurons have also been classified into nine cell groups: B1-B9, in a caudal to rostral order (Steinbusch & Nieuwenhuys, 1983; Harding et al., 2004). Group B1 is located in raphe pallidus, in the caudal medulla; B2, in nucleus raphe obscurus; B3, in raphe magnus; B4, in the central grey of the medulla oblongata; B5, in the pontine median raphe; B6 in dorsal raphe; and the B7 and B8 groups in the midbrain dorsal and median raphe nuclei, respectively; B8 also extends into the caudal linear nucleus, while B9 cells are located in the medial lemniscus (Steinbusch & Nieuwenhuys, 1983). The caudal linear nucleus is a subnucleus of the ventral mesencephalic tegmentum, located dorsal to nucleus

interpeduncularis, on the midline, between the two red nuclei. It is the most rostral raphe nucleus.

Among the above nuclei, nucleus raphe dorsalis is the largest one. It is situated in the ventral part of the central grey matter, overlapping the mesencephalon and rostral part of the tegmentum pontis, just ventral to the aquaductus cerebri and rostral part of the fourth ventricle and also dorsal to the fasciculus longitudinalis medialis (Steinbusch & Nieuwenhuys, 1983; Hornung, 2003). It includes four areas with particularly high cell density: one in the caudal rhombencephalic part (caudal part) and the other three in the mesencephalic region, designated as dorsomedian, ventromedian and lateral parts. The dorsomedian part has the highest cell density (Steinbusch & Nieuwenhuys, 1983).

1.2.3 Rostral 5-HT projections

The raphe nuclei may be roughly divided into rostral (oral) and caudal raphe nuclei, according to their projections. The rostral raphe nuclei have ascending projections to the forebrain, while the caudal raphe nuclei send projections primarily to the lower brainstem and spinal cord (Hornung, 2003; Harding et al., 2004).

The rostral groups, B7-B8, include the median raphe, the caudal linear and the dorsal raphe nuclei (Hornung, 2003; Harding et al., 2004). These nuclei send their efferent projections essentially to the forebrain and upper brainstem (Hornung, 2003; Harding et al., 2004). They grow as a fascicle in the marginal zone, within the medial forebrain bundle (Wallace & Lauder, 1992; Rubenstein, 1998). The olfactory bulbs, thalamus, hypothalamus, striatum, septal area, cerebral cortex and hippocampus are the major target structures of these fibers (Harding et al., 2004).

In the rat brain, 5-HT neurons are present in large numbers within the dorsal raphe. These neurons give rise to the majority of the ascending 5-HT projections to the forebrain. The caudal extension of the raphe dorsalis, in the pons, constitutes the B6 group of 5-HT neurons, while the larger B7 group is situated in the midbrain periaqueductal gray. Dorsal raphe 5-HT axons project to most regions of forebrain and midbrain (Vertes, 1991; Vertes & Kocsis, 1994). The rostral part of the dorsal raphe nucleus sends significantly denser projections than its caudal part to all neocortical regions (Vertes, 1991; Vertes & Kocsis, 1994). On the other hand, the caudal part of the nucleus provides moderately dense projections to the hippocampal formation, which receives no projection from the rostral part (Vertes, 1991; Vertes & Kocsis, 1994). Most midbrain structures, including the ventral midbrain, ventral tegmental area, and the substantia nigra are innervated by the B7 group of 5-HT neurons, which originate from the dorsal and median raphe (Vertes, 1991; Harding *et al.*, 2004). The striatum is also innervated by the dorsal and median raphe 5-HT neurons (Vertes, 1991). Many of the 5-HT neurons which project to the striatum send axon collaterals to the substantia nigra in the ventral midbrain (van der Kooy & Hattori, 1980).

The dorsal and median raphe nuclei provide dense parallel 5-HT projections to the cerebral cortex (Hornung, 2003). Most of these fibers enter the cerebral cortex after passing through the diagonal bands of Broca and the septal area, while only a fraction of 5-HT fibers enter the telencephalon via the ganglionic eminence (Rubenstein, 1998).

1.2.3.2 Projections of the caudal raphe nuclei

Raphe magnus, raphe obscurus and raphe pallidus form the caudal group of 5-HT cells (B1-B3). These cells, with a predominance from group B3, provide the main source of 5-HT innervation to the spinal cord (Harding et al., 2004). All the grey matter of the spinal

cord contains 5-HT fibres, but higher densities are found in the intermediolateral column, layer 10 (central), layers 1 and 2 of the dorsal horn, and motor nuclei (Harding et al., 2004).

Most parts of the brainstem and cerebellum receive dense 5-HT projections, mainly from group B3, but also from groups B6, B7, B8 and B9 (Harding et al., 2004).

In addition to the caudal brainstem and spinal cord, several hypothalamic nuclei, the central nucleus of amygdala, and the dorsolateral periaqueductal grey are innervated by 5-HT neurons of the caudal groups (Harding et al., 2004).

1.3. Development of the 5-HT systems

1.3.1. Time of appearance of 5-HT neurons

The development of the 5-HT system was initially examined in 1972 by Olson and Seiger in the rat (Wallace & Lauder, 1992). They provided information regarding the time of appearance (embryonic day, or E; postnatal day, or P) and subsequent development of the dorsal raphe 5-HT neurons and their axonal projections.

In most species, including rat and mouse, the development of the 5-HT neuronal system begins very early, with 5-HT immunoreactive neurons being visible by E13 in the raphe (Wallace *et al.*, 1982; Wallace & Lauder, 1983; Wallace, 1992; Gaspar *et al.*, 2003). Serotonin neurons first differentiate as two separate clusters: a rostral group oriented just caudal to the mesencephalic flexure and giving rise to most ascending 5-HT fibres, and a caudal group in the medulla oblongata giving rise to most of the descending 5-HT fibres (Wallace *et al.*, 1982; Wallace & Lauder, 1983; Wallace & Lauder, 1992; Gaspar *et al.*, 2003).

1.3.2. Development of 5-HT axonal projections

The ascending projections of the raphe nuclei can be detected as early as E12-E13 in mouse and E13 in rat, as soon as 5-HT cell bodies become visible in the rostral rhombencephalon (B4-B9) (Wallace & Lauder, 1983; Lauder, 1990; Wallace, 1992; Gaspar *et al.*, 2003). These fibres branch as they reach the caudal diencephalon, by E14-E15. At this time, they spread out in dorsoventral and mediolateral directions and they enter into the intermediate zone (Lauder, 1990). The number of 5-HT–immunoreactive (5-HT-IR) fibres increases in the lateral hypothalamus by late E15 and early E16. These fibres can be visualised at the border of the diencephalon and telencephalon at E16 and, by E17, this projection has reached the frontal pole of the telencephalon. A dense plexus of 5-HT-IR fibres exists in the septal area by E18 and many of them pass through this region to reach the neocortex medially, while other 5-HT fibres traverse the ventral portion of the ganglionic eminence to enter the lateral neocortex (Wallace, 1992).

The first 5-HT fibers appear in developing neocortex by E16-17 in rat and mouse (Janusonis *et al.*, 2004). Such early 5-HT projections to the marginal zone raised the possibility that they may influence cortical development. The 5-HT innervation of cerebral cortex originates from the dorsal and median raphe (Dori *et al.*, 1996; Hornung, 2003; Morgane *et al.*, 2005). The dorsal raphe was said to provide fine axons with small varicosities while the median raphe would provide beaded axons with large varicosities. Fine 5-HT terminals abound in various cortical areas, but beaded 5-HT axon terminals reach mainly the outer cortical layers (Morgane *et al.*, 2005). After arriving in the cortex, 5-HT axons divide in two tangential sheets, above and below the cortical plate. Then they arborize gradually and send branches to all cortical layers (Dori *et al.*, 1996; Janusonis *et al.*, 2004).

These projections reach the different regions of the neocortex by the end of embryonic life, the occipital cortex being reached just shortly after birth (Dori *et al.*, 1996). The adult pattern of 5-HT innervation in neocortex is completed by the end of the 3rd postnatal week (Dori *et al.*, 1996).

1.3.3. Molecules affecting the growth of 5-HT axons

The growth of axons occurs at their tip, involving the growth cone, a structure specialised in sensing the local chemical environment for directed orientation (Tessier-Lavigne & Goodman, 1996). In many instances, the axonal growth cone needs to navigate over long distances, along specific pathways to find the correct innervation targets. The growth cone is thus a highly motile structure, with a core filled with microtubules, and peripheral appendices, known as lamellipodia and filopodia, which are filled with actin microfilaments. The latter appendices are responsible for sensing and integrating the multiple signals present in its environment. Then, growth cones translate these signals into cytoskeletal changes, which determine the direction of extension and rate of growth. The growth cone must therefore express receptors for appropriate axon guidance molecules present in its microenvironment.

Growth cone guidance depends on a least 4 different types of extracellular signals: contact attraction (short range, non diffusible molecules), chemo-attraction (long range, diffusible molecules), contact repulsion (short range, non diffusible) and chemo-repulsion (long range, diffusible) (Tessier-Lavigne & Goodman, 1996). These mechanisms act in a coordinated manner to direct pathfinding. Axons also fasciculate to constitute white matter tracts. Such fasciculation also appears to depend on a balance between attraction and repulsion among axons and with the surrounding environment.

The above four types of guidance cues have been associated with several families of molecules identified over the past 20 years. Interestingly, any given family of molecules may be categorised in more than one type of signal (Tessier-Lavigne & Goodman, 1996). Semaphorins, for example may be released into the extracellular space and diffuse to form concentration gradients for long range effects. But other members of this family are transmembrane or glycosylphosphatidylinositol- (GPI-) anchored proteins. Netrins are similarly considered as diffusible proteins, but some GPI-anchored members have been identified recently (Nakashiba et al., 2000). They must then be considered as short-range cues. Moreover, the diffusible semaphorins and netrins have been shown to bind to extracellular matrix proteins or proteoglycans (Manitt & Kennedy, 2002; Kantor et al., 2004).

These mechanisms are mediated by ligand-receptor interactions and depend on the intracellular signalling machinery of the axon (Tessier-Lavigne & Goodman, 1996; Barton et al., 2004). Thus, the same guidance molecules may elicit diverse actions, such as attraction, repulsion, STOP signals, or collateral branch formation (e.g., netrins, ephrins/Eph), depending on the sets of receptors that are expressed at the surface of the recipient axon, as well as on the signalling pathways that are activated. Furthermore some guidance molecules, or their receptors, interact with one another to control axonal outgrowth (e.g.: DCC and Robo). For example, the commissural axons of the vertebrate spinal cord are guided by the coordinated action of members of netrin and Shh families (acting as chemoattractants) and members of the slit and semaphorin families (acting as chemorepellents) (Tessier-Lavigne & Goodman, 1996; Barton et al., 2004).

Axon guidance molecules comprise molecules of the extracellular matrix; cell adhesion molecules (CAM) of the immunoglobulin (Ig) or cadherin types; other types of transmembrane or GPI-anchored proteins, such as ephrins (Efn) and Ephs, semaphorins, or netrins; and diffusible proteins such as netrins, semaphorins, slits, Shh, Wnt, and their respective receptors (integrins, CAM, Eph, Efn, neuropillins/plexins, Robos, etc). Trophic factors, such as fibroblast growth factor (FGF), or neurotrophins may also influence axonal branching (Tessier-Lavigne & Goodman, 1996; Barton et al., 2004).

Currently, little is known about the factors that determine the projections or branching of the 5-HT axons in any specific part of the CNS.

The last decade has seen the discovery of several molecules affecting axon growth, including secreted proteins such as trophic factors (glial cell line-derived trophic factor or GDNF; neurotrophins, fibroblast growth factors, or FGFs; S-100β); morphogens (Shh, wingless/integrated or Wnt), and cytokines (ciliary neurotrophic factor, or CNTF), as well as members of so-called axon guidance molecules, which may be secreted (netrins, slits, semaphorins) or membrane anchored (ephrins, semaphorins, cell adhesion molecules, cadherins) (Tessier-Lavigne & Goodman, 1996).

Even 5-HT itself has been demonstrated to have trophic effects on 5-HT axons, perhaps via an action on glia (Azmitia *et al.*, 1981; Lauder *et al.*, 1983; Lauder, 1990; Gaspar *et al.*, 2003). The receptor subtypes underlying these effects have not yet been identified, although some reports have suggested that 5-HT1A receptors might be involved (Whitaker-Azmitia et al., 1990).

1.3.4. Extracellular matrix proteins and integrins

Laminins, tenascins, collagens, fibronectin, vitronectin, thrombospondin and several types of proteoglycans are extracellular matrix (ECM) molecules that may promote or inhibit axon growth (Tessier-Lavigne & Goodman, 1996). Their receptors are predominantly members of the integrin family, but may also include Ig CAMs, or proteoglycans.

Integrins belong to a large family of transmembrane glycoproteins, with different heterodimeric α/β subunits, with the β1 integrins representing the largest group, expressed in the nervous tissue by neurones and glial cells (Tate et al., 2004; Andressen et al., 2005). Most integrins are connected to bundles of actin filaments via molecules such as talin, α-actinin and filamin. The most important function of integrins is indeed to mediate cytoskeleton - extracellular matrix interactions. There is currently no data about the contribution of ECM and their receptors in 5-HT axon guidance or growth.

1.3.5. Cell adhesion molecules

Cadherin and immunoglobulin CAMs (IgCAMs) are two large families of cell adhesion molecules (CAMs) that are involved in axon pathfinding. Both families include over 100 members (Walsh & Doherty, 1997). Most of them serve as ligands as well as receptors (often by homophilic interactions), but some of them act as heterophilic ligands or receptors for other cell-surface or extracellular matrix (ECM) molecules (Tessier-Lavigne & Goodman, 1996). Most of these proteins are transmembrane molecules, but neural CAM (NCAM) exists in 3 forms, including a GPI-anchored version that may also function as a diffusible guidance cue (Walsh & Doherty, 1997).

Several IgCAMs contain a cytoplasmic region with protein tyrosine kinase or protein tyrosine phosphatase domains but most of them do not. Cell adhesion molecules not only can

mediate cell-cell adhesion in vitro, but also have been implicated in axon fasciculation in vivo. Indeed, IgCAMs such as Fasciclin II or L1/NgCAM can function to pull axons together. It has been proposed that fasciculation results from the relative balance between attractive and inhibitory forces, involving CAMs, among others (Tessier-Lavigne & Goodman, 1996). The expression of polysialylated NCAM has been shown to be influenced by 5-HT in adult rat brain (Brezun & Daszuta, 1999; 2000a; b).

1.3.6 Trophic factors

Glial cell line-derived neurotrophic factor (GDNF) and neurturin are members of the TGF-β gene family, with known patterning roles in early stages of development and promotion of neuronal survival (Hynes & Rosenthal, 1999; Zihlmann *et al.*, 2005). These neurotrophic factors act through receptor tyrosine kinase stimulation –GFRα and Ret (Mehlen, 2005). GDNF is expressed at high levels in the developing rat striatum and low levels in the ventral midbrain (Zihlmann et al., 2005). Several studies have shown that GDNF or neurturin influence dopaminergic and serotonergic neuron survival and neurite growth in culture (Beck et al., 1996; Schaller et al., 2005; Zihlmann et al., 2005; Ducray et al., 2006). Furthermore, GDNF increases motor neuron survival during development and following lesion in vitro and in vivo (Beck et al., 1996).

BDNF is a member of the neurotrophin family, which also includes NGF, NT-3 and NT4/5. It interacts with high affinity with the receptor tyrosine kinase, TrkB, which is expressed in 5-HT neurons, notably (Rumajogee et al., 2002). Studies have also demonstrated that BDNF can induce sprouting of 5-HT axons in the adult rodent cerebral cortex (Mamounas et al., 1995; Mamounas et al., 2000), or on fetal 5-HT neurons in culture (Nishi et al., 2000; Djalali et al., 2005). Observations in BDNF knockout mice suggest that

BDNF may also influence the development of 5-HT neurones through indirect action onto astrocytes and oligodendrocytes and their respective expression of S100β and myelin basic protein (Djalali et al., 2005).

The fibroblast growth factor (FGF) family contains at least 23 members, found in different multicellular organisms which signal through tyrosine kinase receptors (Cayuso & Marti, 2005; Sanchez-Camacho et al., 2005). The FGFs are implicated in the induction of posterior neural tissue, and their signals can control 5-HT cell fate in the anterior neural plate (Sanchez-Camacho *et al.*, 2005). FGF8 has midbrain-inducing and polarizing abilities (Sanchez-Camacho et al., 2005). It is expressed in the midbrain-hindbrain boundary and anterior neural ridge, as early as E9 in rat embryo and has inductive actions for the differentiation of 5-HT neurons by E14 in rat embryo (Ye et al., 1998; Hynes & Rosenthal, 1999). In vitro and in vivo experiments have also revealed that FGF8 influences the guidance of trochlear axons out of the neural tube (Sanchez-Camacho et al., 2005).

S100 proteins are members of calcium-binding proteins which play major roles in cellular processes such as inflammation, exocytosis, transcription, proliferation and differentiation (Adami et al., 2001; Donato, 2001; Marenholz et al., 2004). The S100 family includes more than 20 different proteins, which act in tissue-specific or cell-type-specific manners. Among these, S100β, which is expressed in astrocytes has been shown to induce axon outgrowth (Donato, 2001), including in 5-HT neurons (Whitaker-Azmitia et al., 1990; Nishi et al., 2000; Nishiyama et al., 2002; Djalali et al., 2005).

1.3.7. Morphogens in axon guidance

Morphogens are secreted extracellular signalling molecules which diffuse away from their source, and can induce various cellular responses including important mechanisms

underlying the progressive patterning of the embryo (Cayuso & Marti, 2005; Mehlen, 2005; Sanchez-Camacho et al., 2005). The best known morphogens are the hedgehogs (hh), wingless/integrated (Wnt), fibroblast growth factors (FGFs, see Trophic factors) and transforming growth factor-βs (TGF- β).

Sonic hedgehog (Shh) is expressed by the notochord and neural floor plate, and is involved in the differentiation of the floor plate, motoneurons, and ventral interneurons (Cayuso & Marti, 2005; Mehlen, 2005; Sanchez-Camacho et al., 2005). It is also involved in the differentiation of dorsal brain structures such as the cerebellum, neocortex, and tectum (Cayuso & Marti, 2005) as well as in the proximo-distal and dorso-ventral patterning of the optic vesicle during eye formation (Sanchez-Camacho et al., 2005). Serotonergic neurons differentiation is also regulated by Shh (Hendricks et al., 1999; Hynes & Rosenthal, 1999; Ding et al., 2003). Recently, floor plate-derived Shh has been involved in the guidance of spinal cord commissural axons towards the ventral midline (Charron et al., 2003).

Wnts are a large family of secreted glycoproteins implicated in tissue patterning, cell proliferation, and differentiation in several tissues, including nervous tissue (Cayuso & Marti, 2005; Mehlen, 2005; Sanchez-Camacho et al., 2005). The Wnts are related to drosophila wingless protein, which regulates cell-to-cell interactions. They influence survival of vertebrate neural crest cells and CNS progenitors, during embryogenesis (Cayuso & Marti, 2005; Mehlen, 2005; Sanchez-Camacho et al., 2005). Two different signalling pathways ("Wnt canonical" and Wnt/Ca^{2+} pathway) are activated following binding of Wnts to their frizzled receptors, which are G-protein-coupled receptors (Sanchez-Camacho et al., 2005). The growth of commissural axons, in fly as well as in rodents also involves Wnt signalling (Sanchez-Camacho et al., 2005). In particular, Wnt-5 has a direct role as a

repellent axon guidance cue for anterior commissural axons, whereas Wnt-4 is a guidance cue required for the anterior growth of commissural axons (Sanchez-Camacho et al., 2005).

1.3.8. Netrins and their receptors

Netrins constitute a small family of 6 members first identified as axon guidance cues in vertebrates. The netrins gene family includes four members in mammals: netrin-1, -3, -G1 and -4 or ß-netrin (Nakashiba et al., 2000). They are small proteins (~600 amino acids), related to the much larger laminins (Manitt & Kennedy, 2002). Most netrins are secreted proteins, which direct cell migration and axonal growth cone guidance, during neural development. Recently, however, new members with a GPI-anchor, netrin-G1, with 6 isoforms (netrin-G1a-f) and netrin–G2 with 3 isoforms (netrin-G2a-c), have been identified (Nakashiba et al., 2000; Lin et al., 2003; Barallobre et al., 2005). These netrin-G proteins, expressed as early as E12 in several areas of the CNS, reach their highest level of expression at the perinatal stage (Barallobre et al., 2005).

Netrin-1 and -2 were first identified in chicken as proteins expressed by floor plate cells and having an important role in the guidance of spinal cord commissural axons (Kennedy et al., 1994). Netrin-3 has different properties, and it expressed in motor, sensory and sympathetic neurons during peripheral nervous system development. It is expressed also in mesenchymal and muscle cells (Barallobre et al., 2005). Members of this family were later found to be multifunctional in axon guidance, attracting some axons and repelling others (Tessier-Lavigne & Goodman, 1996; Manitt & Kennedy, 2002).

Netrins have a homologue, UNC-6, in the nematode C. elegans, for which receptors had already been identified: UNC-40 and UNC-5. UNC-40 turned out to have an already known homologue in mammals, Deleted in Colorectal Cancer (DCC), and another that was

recently identified, neogenin, which are the receptors mediating the attraction elicited by netrins. UNC-5 homologues, UNC5H-1 and -2 have also been found in vertebrates and are mediating the repulsive effects of netrins. However, mediation of this repulsive action depends on the co-expression of UNC-40 (Tessier-Lavigne & Goodman, 1996; Dickson, 2002).

There is currently no available information on the expression of netrins in 5-HT neurons. Preliminary work by A. Petit did not detect DCC in 5-HT neuron (Petit, Kennedy, and Doucet, unpublished).

1.3.9. Slits and Robos

Roundabout (Robo) was first identified through genetic screening in drosophila as the receptor for a midline repellent suspected to be responsible for midline axon guidance defects (Dickson, 2002). The repellent was found to be Slit, a large secreted protein. Several Slit and Robo homologues (Slit-1 to -3; Robo-1 to -3) have later been identified in vertebrates (Dickson, 2002). Slits are involved in several functions, such as stimulating sensory axon branching and elongation, but midline guidance in drosophila and formation of the optic chiasm in vertebrates are their best known functions (Dickson, 2002). Slits are expressed by midline floor plate cells, whereas Robos are expressed by longitudinal axons, preventing ipsilateral axons from crossing, and commissural axons from re-crossing, the midline.

Inactivation of Slits was shown to affect major 5-HT projections in Slit-1 and Slit-2 mutant animals where a significant percentage of 5-HT fibres abnormally crossed the midline in the basal telencephalon (Bagri et al., 2002). Up to now, Slit-1 and Slit-2 are the

only axon guidance molecules to have been involved in the guidance of ascending 5-HT axons (Bagri et al., 2002; McIness & Michaud, 2005).

1.3.10. Semaphorins and their receptors

Semaphorins are a large family of cell surface and secreted guidance molecules which function as axon chemorepellent or growth inhibitors. The family is defined by the presence of a conserved ~420 amino acid sema domain at the NH2 terminal. They are divided into 8 classes, including secreted and cell surface –transmembrane or GPI-anchored - members (Barton et al., 2004). Some of these classes are found in invertebrates (classes 1 & 2) while others are found in vertebrates (classes 3-7), and one (class V) is encoded by viruses (Tessier-Lavigne & Goodman, 1996; Dickson, 2002). Approximately 20 members of the semaphorin family have been identified in higher vertebrates, among which one third are secreted molecules, one is attached to the cell surface via a GPI-anchor, and the remaining ones are trans-membrane molecules (Tamagnone & Comoglio, 2004). One of the trans-membrane and most of the secreted semaphorins function in repelling specific subset of axons in culture, while two of the secreted ones can act either as axon repellents or attractants (Tessier-Lavigne & Goodman, 1996; Dickson, 2002).

Plexins are the primary receptors of semaphorins. Many, and perhaps all, semaphorin receptor complexes include a plexin. Many transmembrane semaphorins bind directly to plexins, but secreted semaphorins (class 3), in vertebrates, bind to a second class of receptor component named neuropilins (Dickson, 2002; Tamagnone & Comoglio, 2004). Plexins are a large family of trans-membrane proteins, grouped in 4 subfamilies, plexin-A to -D, on the basis of sequence similarity (Dickson, 2002; Swiercz et al., 2002).

There is currently no available information on the expression of semaphorins or their receptors in 5-HT neurons or on their possible role in the guidance of 5-HT axons during development.

1.3.11. Ephrins and Eph receptors

Ephrins (Efn) were discovered, in 1994 as important regulators of the retinotopic projections onto the vertebrate visual tectum (Cheng & Flanagan, 1994; Drescher et al., 1995). This family of membrane-bound guidance molecules was classified in two major classes according to the type of insertion in the cell membrane, a GPI- anchor for EfnAs and a transmembrane domain for EfnBs (Eph Nomenclature Committee, 1997). Ephrin-As and EfnBs respectively interact with EphA and EphB tyrosine kinase receptors. Currently, 9 Efns and 15 Eph receptors have been identified in mammals (Murai & Pasquale, 2003; Martinez & Soriano, 2005). The 9 mammalian EphA receptors bind to 5 EfnAs, with a variable affinity, while the 6 EphBs do similarly with the 3 EfnBs. However, this general rule has a few exceptions: EphA4 being able to bind both EfnAs and EfnBs, and EfnA5 recognizing EphB2 in addition to EphAs (Huot, 2004; Surawska et al., 2004).

Ephrins and Ephs are involved in several neural developmental processes, including topographic mapping, brain commissure formation and axon guidance (Martinez & Soriano, 2005). EphA5 and EfnA5 are expressed in dopaminergic neurons in the ventral midbrain, suggesting their involvement in the regulation of substantia nigra-striatum interactions (Halladay et al., 2004).

Analyses of dominant negative EphA5 transgenic mice suggested that this receptor might be involved in 5-HT axon growth, since 5-HT levels were decreased in these mice (Halladay et al, 2004). The latter observations are interesting in the context of our study,

which suggests that EfnAs influence 5-HT axon branching in vitro, on membranes from the cerebral cortex, striatum or ventral midbrain. At least some EphA receptors (EphA5, EphA7) are expressed in the dorsal raphe (Maisonpierre et al., 1993) (see www.genepaint.org).

1.4. Objectives of the present work

Our hypothesis is that 5-HT axon guidance molecules are associated with cell membranes in 5-HT innervation target fields. Our objectives were to test the capacity of individual 5-HT neurons to recognize guidance cues present in distinct target brain regions and to identify molecules involved in these processes. As will be described, our work has fulfilled all 3 objectives, at least partially: we have found that 5-HT axons can discriminate membranes extracted from the neonatal cerebral cortex (Ctx), striatum (Str) and ventral midbrain (VM). Moreover, we observed that the choice of a membrane substrate by 5-HT axons is determined by the position of the cell body on the same type of membranes; strongly suggesting an induction of guidance cue recognition. Lastly, our experiments indicate that ephrin-As are involved in 5-HT axon branching, at least on membranes extracted from the cerebral cortex.

2. Materials and Methods

2.1. Cell membrane preparation

Cell membranes were prepared as described by (Petit et al., 2005), and modified from (Walter et al., 1987). Newborn (P0) female Sprague-Dawley rats were quickly decapitated with sharp scissors, and blocks of Ctx, VM or Str (Figure1) were dissected in Gey's Balanced Salt Solution (GBSS; Sigma G-9779) supplemented with glucose (6.5mg/ml; Sigma G-7021).

The pia matter was removed and slices were cut with a sharpened tungsten needle (unsheathed, tungsten-rhenium, Omega Engineering Inc, WW 26-020). Tungsten needles were sharpened by immersing the wire in NaOH (1N) and applying a voltage of 4-5 V). The dissected tissues were transferred into 1.5 ml centrifuge tubes (Corning) containing 1 ml H buffer which includes 10mM tris-HCL (Sigma T3253) pH 7.4, 1.5 mM CaCl2 and 1 mM Spermidin plus 40 µl protease inhibitor (Protease inhibitor, complete EDTA-free; Roche no. 1873580).

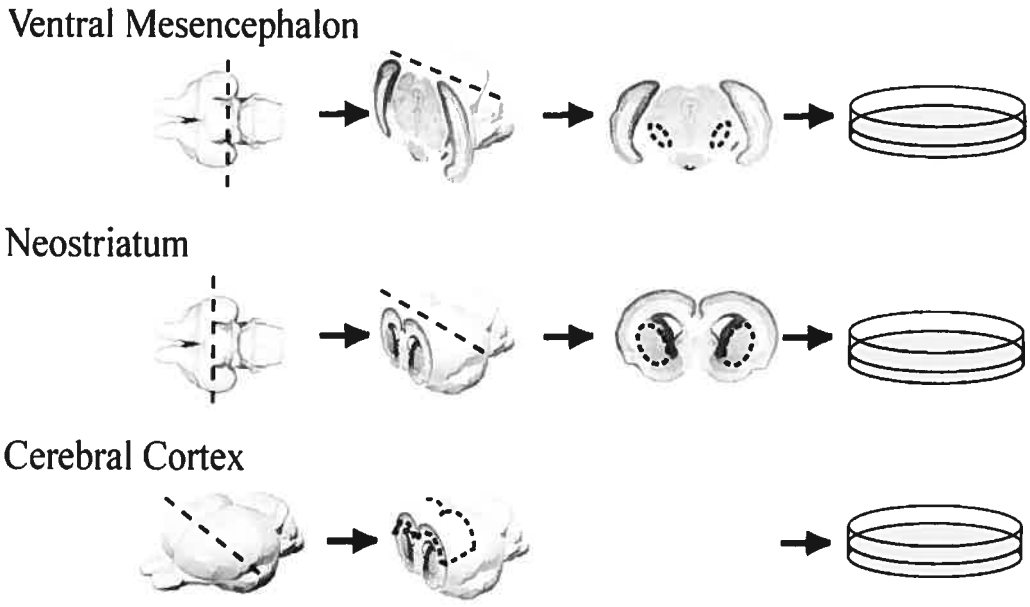

FIGURE 1. DISSECTION OF CTX, STR AND VM TISSUE PIECES FROM NEONATAL RAT BRAIN. AFTER PETIT ET AL., 2005 (WITH PERMISSION).

Tissues were homogenized by several strokes through P1000 and P200 pipettes and then a 1 ml syringe, until all tissue debris had disappeared. Homogenization was continued in an ultrasound bath (Branson 2200) during 5-10 min. The homogenate was centrifuged during 20 min at 50 000 g, at 4° C, in a sucrose step gradient (300 µl sucrose 5 % in upper phase and 700 µl sucrose 50 % in lower phase; Sigma no. S-5390, in 5 ml centrifuge tubes; Beckman) using an ultracentrifuge (Sorval Ultra pro 80; rotor AH650). The inter-band, containing the cell membrane fraction was collected and washed twice in 1 ml sodium phosphate buffered saline (PBS, 1X, pH 7.2) and centrifuged for 10 min at 16 000 g (Eppendorf Model 4515C centrifuge), at 4°C. The pellet was re-suspended in 1 ml PBS and the concentration of purified membrane was determined by optical density (OD) at 220 nm in a spectrophotometer (Ultraspec 2100 pro). The concentration was then adjusted with PBS-

glycerol (50 % PBS- 50 % glycerol; Fisher no. G33-1) to a standard level yielding an OD of 1.0.

At this point, membranes could be treated with PI-PLC or EphA3-Fc. For PI-PLC, 10 µL of PI-PLC (1 U/mL, Sigma-Aldrich) were added to 500 µL of membrane preparation from neonatal VM during 1 h, at 37°C (Petit et al., 2005). The membranes were then rinsed and re-suspended at the same OD. Neurons were cultured on stripes of PI-PLC-treated VM membranes alternating with stripes of untreated VM or Str membranes.

For EphA3-Fc treatment (Janis *et al.*, 1999), 200 ng of the protein (R&D) were added to 500 µL of membrane suspension from neonatal Ctx. As controls, other Ctx membranes were treated with Fc alone (Jackson ImmunoResearch Laboratories; 420 ng in 500 µl of membrane suspension). The membranes were rinsed and re-suspended, as above. Neurons were then cultured onto stripes of EphA3-Fc, or Fc, alternating with stripes of untreated membranes from Ctx.

2.2. Alternating membrane stripe preparation

Alternating stripes of membrane substrates were prepared as described (Carlson et al., 1987; Petit et al., 2005). A 100 µl drop of purified membrane was first mixed with 3 µl of a suspension of blue fluorescent polymer micro spheres (1:825; B500; Duke Scientific, Palo Alto, CA). The labelled membrane suspension was applied to a polycarbonate nucleopore filter (pore size 0.1 µm; Whatman), deposited on top of a silicone matrix with 90 µm-wide parallel channels (Jurgen Jung, Max-Plank Institute, Tuebingen, Germany). The labelled membrane was aspirated across the filter by applying a negative pressure (-0.8 mbar) in the underlying channels with a vacuum pump (model DAA- V175-EB; GAST, Benton Harbor, MI), until the filter stripes above the channels were saturated.

For preparation of membrane stripes alternating with poly-L-lysine (PLL), these stripes were printed onto coverslips pre-coated with poly-L-lysine (PLL; 20µg/ml, Sigma; P.2636). For alternating stripes of different membranes preparations, the filter with saturated stripes was transferred onto a nylon grid matrix (fine mesh, provided by J. Jung) and then a 100 µl droplet of a second type of membrane suspension was applied and aspirated as above, to fill the non-saturated stripes of the filter. These alternating membrane stripes were printed onto PLL pre-coated coverslips. These coverslips were kept at 37 °C until used, on the same day.

2.3. Preparation of dissociated cells from the foetal dorsal raphe

Rat fœtuses (E14-E15) were dissected in HBSS 1X (Gibco; 14180-053 or 14170-112) supplemented with 1% HEPES solution (Gibco; 156030-80 or 156030-106). Explants of the dorsal raphe were transferred onto a 15 ml centrifuge tube containing 4.5 ml HBSS/HEPES; (1.0 ml HEPES in 100 ml Hank's solution), supplemented with 500 µl trypsin (2.5 %) and incubated at 37°C for 15-20 min. The tissue was re-suspended every 5 min, with a Pasteur pipette (under the culture hood).

After dissociation, the suspension was supplemented with 5 ml Neurobasal Complete Medium and 1.0 ml Heat-Inactivated Horse Serum (HIHS), and was centrifuged during 10 min at 2 000 g (Centra CL2, 15 ml tube). The supernatant was discarded and the pellet re-suspended 500 µl of Neurobasal Complete medium. Cells were counted in a 10 µl sample using a hæmocytometer. The dissociated dorsal raphe neurons were plated (~18 000/cm²) onto the membrane stripe substrates and incubated in Neurobasal Complete medium for 72 h at 37°C under an atmosphere of 5 % CO2.

2.4. Immunocytochemistry

Cultures were fixed in 4 % paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.4) for 1 h at room temperature. They were pre-incubated in PBS containing 10 % bovine serum albumin (Sigma), 1 % normal goat serum (Jackson), 0,05 % -1 % Triton X100, for 3-4 h, at room temperature. They were incubated during 16 h with a polyclonal antiserum against 5-HT (1:5 000, Diasorin, Stillwater, MN) and a monoclonal antibody against β_{III}-tubulin (1:200; Sigma), to visualize, respectively, 5-HT and all neurons in the cultures. After primary incubation, coverslips were rinsed in PBS and incubated with a rhodamine–conjugated goat anti-rabbit IgG and a fluorescein (FITC)-conjugated, affinity-purified Fab fragment of goat anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA).

2.5. Image analysis and quantification

The immuno-labelling was examined and photographed under a Zeiss Axiophot, fluorescence microscope connected to a digital camera (Retiga 1300; Q-imaging; Canada) and analysed with the Northern Eclipse 6.0 software (Empix Imaging, Toronto, ON).

The following measurements were performed on the cultures. First, the number of neurons immunopositive for 5-HT or for β_{III}-tubulin was counted in each type of stripe. The number of neurites emanating from the cell bodies was also registered. Lastly, for each neuron, the length of axon and the number of collateral branches in each type of stripe was scored, according to the position of the parent 5-HT or β_{III}-tubulin cell body in a specific type of stripe. All 5-HT neurons in every culture were analysed in this way. Since the number of β_{III}-tubulin-labelled neurons was much higher than 5-HT neurons, 20 β_{III}-tubulin-positive; 5-HT-negative neurons were chosen at random in every culture for analysis [every

neuron out of x ÷ 20; "x" being the total number of such neurons in the culture]. For this thesis, only the results on 5-HT axon branching will be presented.

Experiments were repeated at least 3 times. For each type of alternating substrates, the data comparing the neurons between the two sets of stripes were analysed using an ANOVA with repeated measures (SPSS 13.0; done by Dr. Miguel Chagnon, Département de mathématiques et statistiques, Université de Montréal).

3. Results

3.1. Experimental framework

We tested the response of foetal rat dorsal raphe neurons (E14-E15) challenged with stripes of cell membranes prepared from the neonatal cerebral cortex (Ctx), striatum (Str), or ventral midbrain (VM), alternating with stripes of poly-L-lysine (PLL), or of membranes prepared from a different neonatal brain region. Since these experiments demonstrated that 5-HT axons were able to discriminate among the different types of stripes, we submitted the membrane preparations to treatments with phosphatidylinositol-specific phospholipase C (PI-PLC) or with a fusion protein comprising the extracellular domain of EphA3 and the Fc fragment of human immunoglobulin-G (EphA3-Fc), in order to respectively remove all GPI-anchored membrane proteins, or to block EfnAs.

The results presented below represent the number of axon branches (or collaterals) and the total length of axon (including all branches) in each type of membrane or PLL stripes for every individual neuron located in a given type of stripe. These two measurements of axonal innervation gave very similar results.

3.2. Serotonergic axons *prefer* membrane to PLL stripes

Examples of non 5-HT and 5-HT neurons cultured onto alternating stripes of membranes and PLL are illustrated in Figure 2A,A'. In general, both 5-HT and non-5-HT perikarya were more numerous on membrane than PLL stripes.

Non 5-HT neurons (β_{III}-tubulin+; 5 HT$^-$) made few branches and had relatively short axons that tended to remain in the same stripe as the parent cell bodies (Fig. 2A). In contrast, 5-HT neurons had long axons that could cross several stripes and made several branches, usually in a different stripe than that of the parent cell bodies (Fig. 2A').

The results also indicated that 5-HT axons *preferred* membrane to PLL stripes. Indeed, these axons branched clearly more often in Ctx, Str or VM membrane stripes than PLL stripes, even when the cell bodies were located in PLL stripes (Fig. 4). When the perikarya were in Ctx stripes (n = 18 5-HT neurons), the number of branches in the Ctx stripes was nearly 7 times larger than in PLL stripes (Fig.4A, left side), and the total length of individual axons 4 times larger than in PLL stripes (Fig.4B, left side). When the 5-HT perikarya were in a PLL stripe (n = 9), their axons still *preferred* the Ctx membrane stripes, but produced only 2.6 times more branches in the membrane than PLL stripes (Fig. 4A,B, right side). However, the interaction between the location of the cell body and the axon growth onto a specific type of stripe was not significant ($p=0.054$).

Similar results were obtained with Str or VM membrane vs PLL stripes (Fig.4C-F). For 5-HT perikarya located in Str stripes (n = 14), there were nearly 8 times more 5-HT axon branches in Str than PLL stripes, whereas those located in PLL stripes (n = 10) gave 2.5 times more branches in Str than PLL stripes. For 5-HT perikarya located in VM stripes (n = 17), the number of branches was nearly 8 times higher in VM than PLL stripes, compared to 6 times higher for those located in PLL stripes (n = 7). In both cases, the total length of axon branches showed the same trend.

Thus, whatever the location of the 5-HT perikarya, their axons clearly *preferred* to grow onto a cell membrane substrate than onto PLL, which is recognized as an axon growth permissive substrate.

3.3. Axon growth on membranes from different brain regions

When challenged with alternating stripes of membranes from either Ctx, Str or VM, 5-HT axons branched and grew preferentially in other stripes made with the same type of

membranes as those where their parent cell body was located (Fig. 2B,B'). Non 5-HT neurons still made few branches and had short axons.

In alternating membrane stripes of Ctx and Str, when the cell body was located in Ctx stripes (n = 36), the number and total length of 5-HT axon branches were, respectively, 5 and 3 times higher in Ctx than Str stripes (Fig. 5A,B, left side). However, 5-HT axons showed no stripe preference when their cell bodies were located in Str membrane stripes (n = 29) (Fig. 5A,B, right side). There is a significant interaction between the location of the perikarya in Ctx or Str membrane stripes, and 5-HT axon branching in specific stripes ($p<0.001$).

With dorsal raphe neurons cultured on alternating VM and Str stripes (Fig. 5C,D), there was also a significant interaction between the location of the perikarya and the number and length of axon branches in any given type of stripes ($p<0.001$). When the 5-HT cell bodies were located in VM stripes (n = 41), the number and total length of axon branches were 6 and 3 times larger, respectively in VM than Str stripes (Fig. 5C,D, left side). When the perikarya were in Str stripes (n = 22), the number and length of 5-HT axonal branches were 3 and 1.7 time larger in Str than VM stripes (Fig.5C,D, right side).

With alternating stripes of VM and Ctx membrane, there was, again a significant interaction between cell body location and number/length of 5-HT axonal branches in each type of stripes (Fig. 5 E,F). With 5-HT perikarya in Ctx stripes (n = 25), axons had 2.3 times more branches that were 1.6 times longer in Ctx than VM stripes; whereas those in VM stripes (n = 33) had 4 times more branches, 2.3 time longer in VM than Ctx stripes.

Thus, except for 5-HT neurons in Str stripes alternating with Ctx stripes, 5-HT axons always branched preferentially on the same type of membranes as those bearing their parent

perikarya. It is noteworthy that the large majority of these axons branched in the same **type** of membranes, but in different **stripes** than their cell body. They therefore had the choice to branch in similar or different types of membrane stripes.

3.4. Membranes treated with PI-PLC no longer induce 5-HT axon branching

The molecules involved in the guidance or branching of 5-HT axons are presumed to be proteins (Petit *et al.*, 2005). Three major modes of protein association exist with cell membranes: transmembrane, GPI-anchored and peripheral proteins. The enzyme PI-PLC is known to remove the GPI-anchored proteins (Walter *et al.*, 1987). To gain some insight into the molecules involved in the above phenomena, we first examined the effects of membranes treated with PI-PLC on axon branching.

For the present thesis, we could only test this treatment with VM membranes. Our results show that, with alternating stripes of VM membranes that were either treated or untreated with PI-PLC, 5-HT axons branched almost exclusively onto the untreated membrane stripes, whatever the location of their parent perikarya on treated or untreated membrane stripes (Fig. 6A,B, n = 17 and 14 5-HT neurons in VM and $VM_{PI\text{-}PLC}$ stripes, respectively.). Similar results were obtained with stripes of membranes from Str alternating with VM membranes treated with PI-PLC, where branching occurred almost exclusively in untreated Str stripes (Figs. 3A,A', 6C,D, n = 4 in Str and 6 in $VM_{PI\text{-}PLC}$ stripes).

The effect of PI-PLC was probably stronger than it appears here, since it also affected branching in untreated VM stripes. Indeed, there was a significant difference in the number and length of branches in untreated VM stripes in these experiments compared to all other experiments with VM stripes (2.59 axon branches and a length of 142 μm in VM alternating with $VM_{PI\text{-}PLC}$ compared to 4.01-4.58 and 177-192 μm in VM stripes alternating with Ctx,

Str or PLL). Perhaps some of the enzyme remained in the treated membranes when the stripes were prepared, and diffused to "untreated" stripes. A more likely explanation might be that axons growing on treated stripes were affected in some way by PI-PLC-treated membranes.

In the experiments where $VM_{PI\text{-}PLC}$ altered with Str stripes, branching in the latter was not affected: the number of branches in Str stripes, in other experiments, was 2.48-2.95 and their length varied between 96 and 139 μm, with no significant difference between experiments.

3.5. Treatment with EphA3-Fc attenuates 5-HT axon branching

Since ephrin-As are GPI-anchored proteins involved in axon branching (Gao *et al.*, 1999; Mann *et al.*, 2002), we next tested membranes treated with EphA3-Fc, a blocker of ephrin-As. Up to now, we have only tested this treatment on membranes extracted from Ctx (Fig. 3B.B'). With EphA3-Fc-treated Ctx membranes alternating with stripes of untreated Ctx membranes 5-HT axons made 1.8 times more branches in untreated than EphA3-Fc-treated membrane stripes, whatever the location of their parent perikarya in untreated (n = 34) or treated (n = 26) stripes (Fig. 7A). However, the number of branches made in untreated membrane stripes tended to be lower than in untreated Ctx membrane stripes in all other experiments (2.79 vs 3.56-4.0; p=0.078); which might indicate that the fusion protein, EphA3-Fc diffused to "untreated" stripes, or that 5-HT axons were affected by their contact with EphA3-Fc-treated membranes, even when they finally branched in untreated stripes.

On the other hand, the total length of 5-HT branches per axon, in treated versus untreated membrane stripes, showed an interaction with cell body location ($p = 0.021$). When 5-HT perikarya were in untreated Ctx stripes, the length of their axon branches in Ctx

stripes was twice that in EphA3-Fc-treated Ctx stripes (Fig. 7B). The length of these axon branches was also 1.4 times that of those in Ctx stripes, when the perikarya were in $Ctx_{EphA3-Fc}$ stripes. The length of branches in EphA3-Fc-treated stripes was comparable, whatever the location of the perikarya. When the perikarya were in $Ctx_{EphA3-Fc}$ stripes, the length of their axon branches showed no significant difference in Ctx vs $Ctx_{EphA3-Fc}$ stripes.

In summary, the reduction in 5-HT axon branching in membranes treated with EphA3-Fc was not as strong as with the PI-PLC treatment, but it was significant.

Control treatment of Ctx membranes with Fc alone had no effect on the number or length of 5-HT axon branches. Indeed, the number of branches and their total length was comparable (3.85 in Ctx_{Fc} vs 4.00 in Ctx stripes) to that found in Ctx stripes in all other experiments (Figs. 3C,C' and 7C,D). However, there was an interaction between the number and length of branches and the stripe location of the parent perikarya ($p<0.001$). Indeed, axons from perikarya located in Ctx stripes branched in Ctx stripes, while those from perikarya located in Ctx_{Fc} stripes branched in this type of stripes. However, this interaction is due to a phenomenon different from experiments with untreated membranes stripes from different brain regions: the large majority of 5-HT axons branched in the **same** stripe as that of their parent cell body, when stripes were treated with Fc. Thus, the growth of the axon out of both Fc-treated and untreated was apparently hindered, but the axon branching was not affected, even in Fc-treated stripes, at variance with EphA3-Fc-treated membranes. Future experiments with membranes from other brain regions treated with EphA3-Fc (or other EphA-Fc fusion proteins) or with Fc alone will tell whether this effect of Fc will be confirmed.

Nevertheless, we conclude that 5-HT axons branching is at least partly regulated by ephrin-As, in membranes extracted from the cerebral cortex.

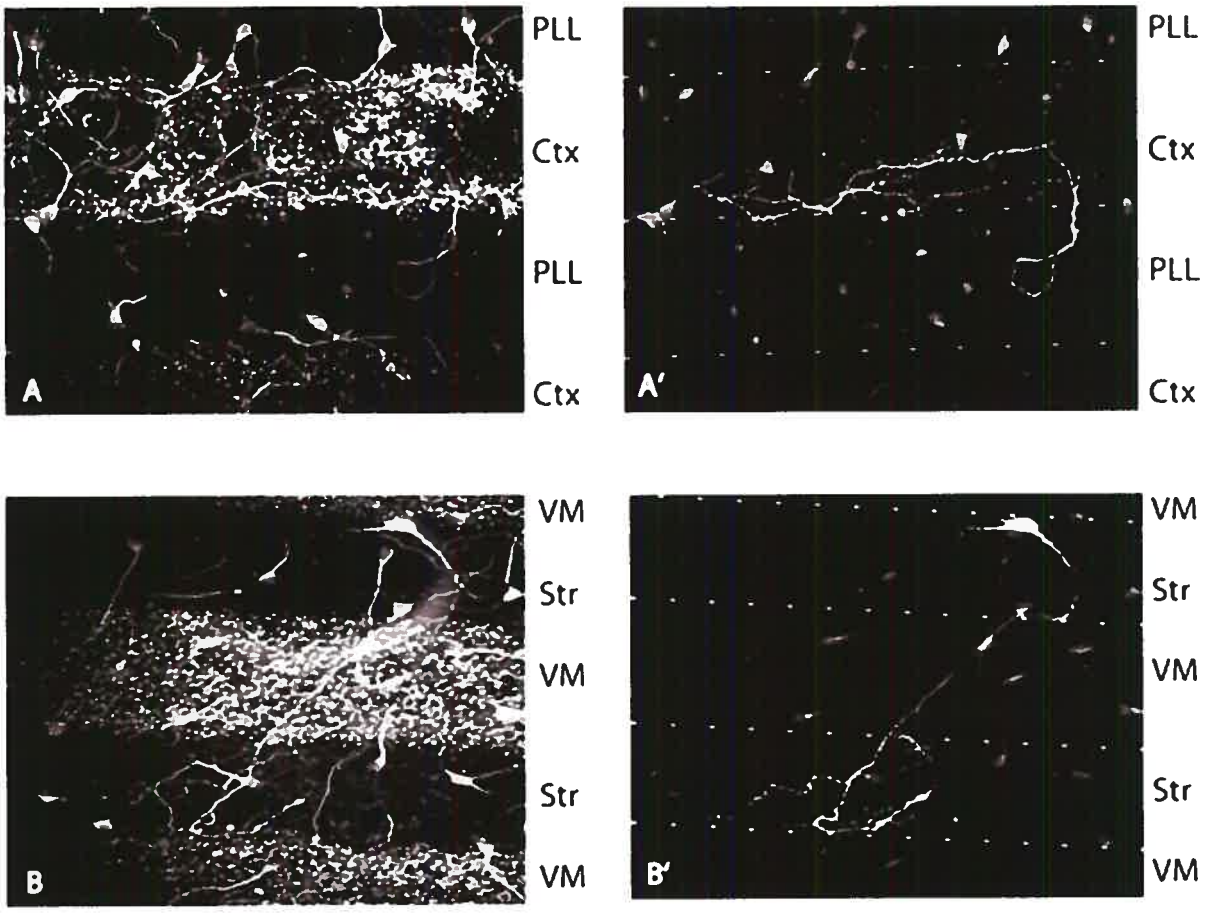

FIGURE 2. DORSAL RAPHE NEURONS CULTURED ON STRIPES OF PLL OR CELL MEMBRANES PREPARED FROM DIFFERENT BRAIN REGIONS

A,A') Alternating stripes of PLL and membranes prepared from the neonatal cerebral cortex. The membranes were labelled with fluorescent microbeads, which can be seen in A, together with β_{III}-tubulin immunostaining. In A', only 5-HT neurons are visualized. B,B') Alternating stripes of membrane preparations from the striatum (Str) and ventral midbrain (VM), with microbeads and β_{III}-tubulin immunostaining being visible in B and 5-HT immunostaining in B'. Note that the 5-HT neuron, in B' branches in a different Str stripe than the parent cell body, after crossing a VM stripe without branching.

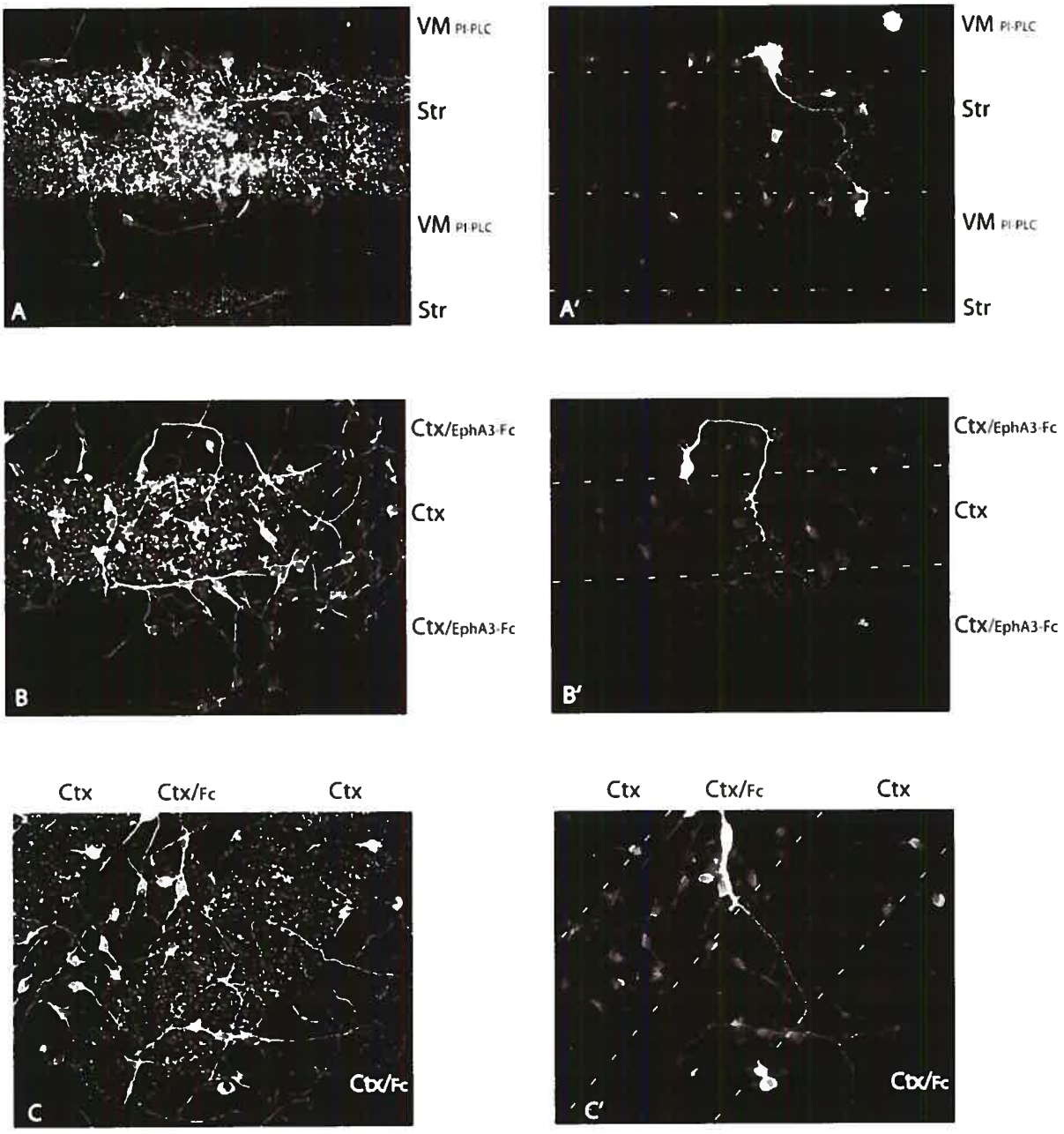

FIGURE 3. DORSAL RAPHE NEURONS ONTO MEMBRANES TREATED WITH PI-PLC OR EPHA3-FC

A,A') Alternating stripes of VM membranes treated with PI-PLC, and untreated Str membranes (labelled with fluorescent beads (A, beads and β_{III}-tubulin immunostaining are shown; A', 5-HT neurons). B,B') Stripes of cerebral cortex (Ctx) membranes treated or not with EphA3-Fc. Note that a 5-HT neuron located in a treated Ctx membrane stripe branches

in an untreated Ctx membrane stripe. C,C') Stripes of Ctx membranes treated or not with Fc, as control for experiment illustrated in B,B'. Treatment with Fc alone had no effect on the branching choices of 5-HT axons.

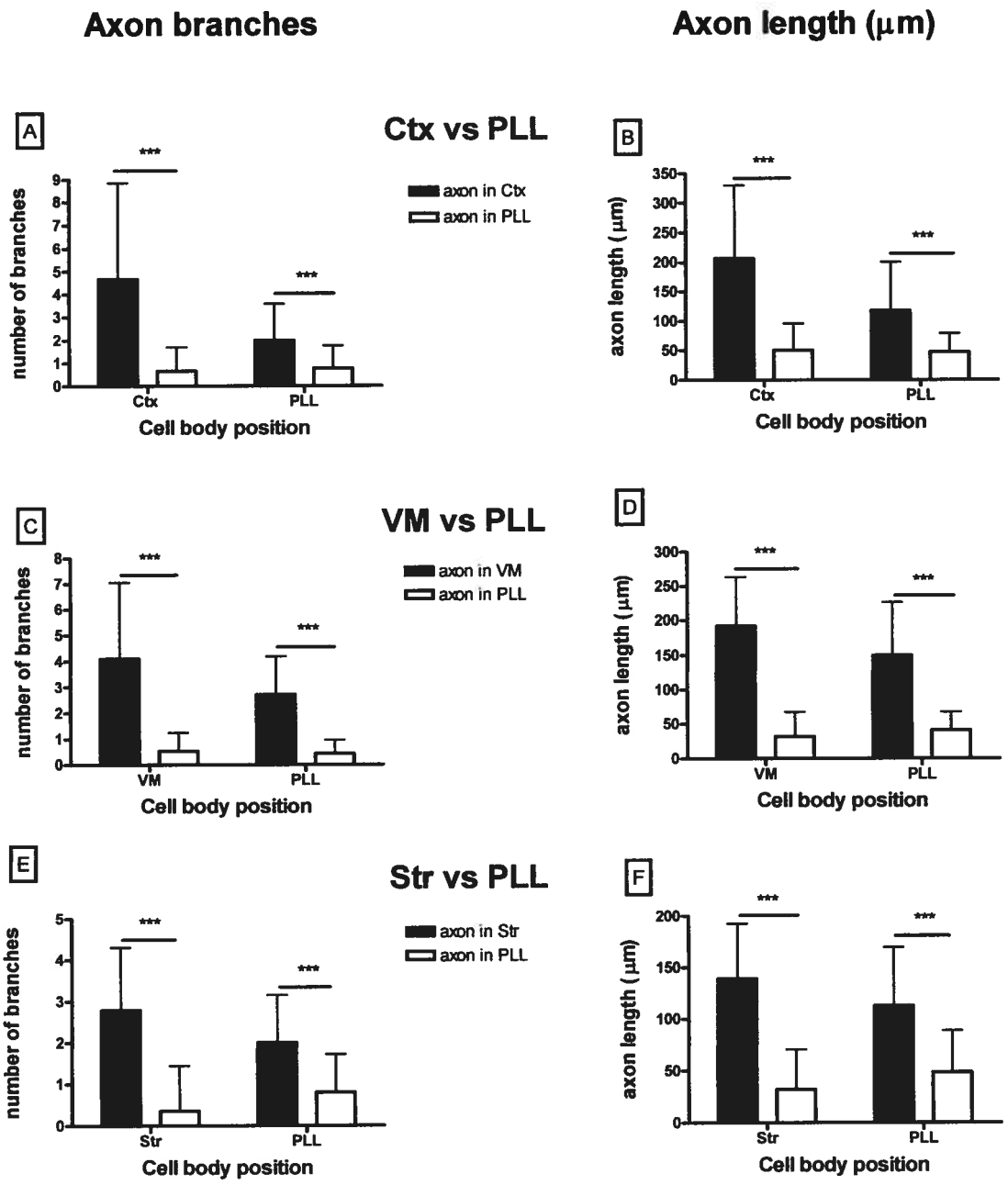

FIGURE 4. SEROTONERGIC AXON BRANCHING (NUMBER OF "AXON BRANCHES" AND "AXON LENGTH"), IN STRIPES OF NEONATAL BRAIN CELL MEMBRANE, ALTERNATING WITH STRIPES OF POLY-L-LYSINE (PLL), ACCORDING TO PARENT CELL BODY LOCATION IN MEMBRANE OR PLL STRIPES ("CELL BODY POSITION", ON THE X AXIS).

Stripes of membranes from the frontal cerebral cortex (Ctx, A,B), from the ventral midbrain (VM, C,D), or from the striatum (Str, E,F), alternating with stripes of PLL, according cell body location. Filled bars indicate branching in respective membrane stripes and white bars indicate branching in PLL stripes. ANOVA with repeated measures; * $p<0.05$; ** $p<0.01$; ***$p<0.001$.

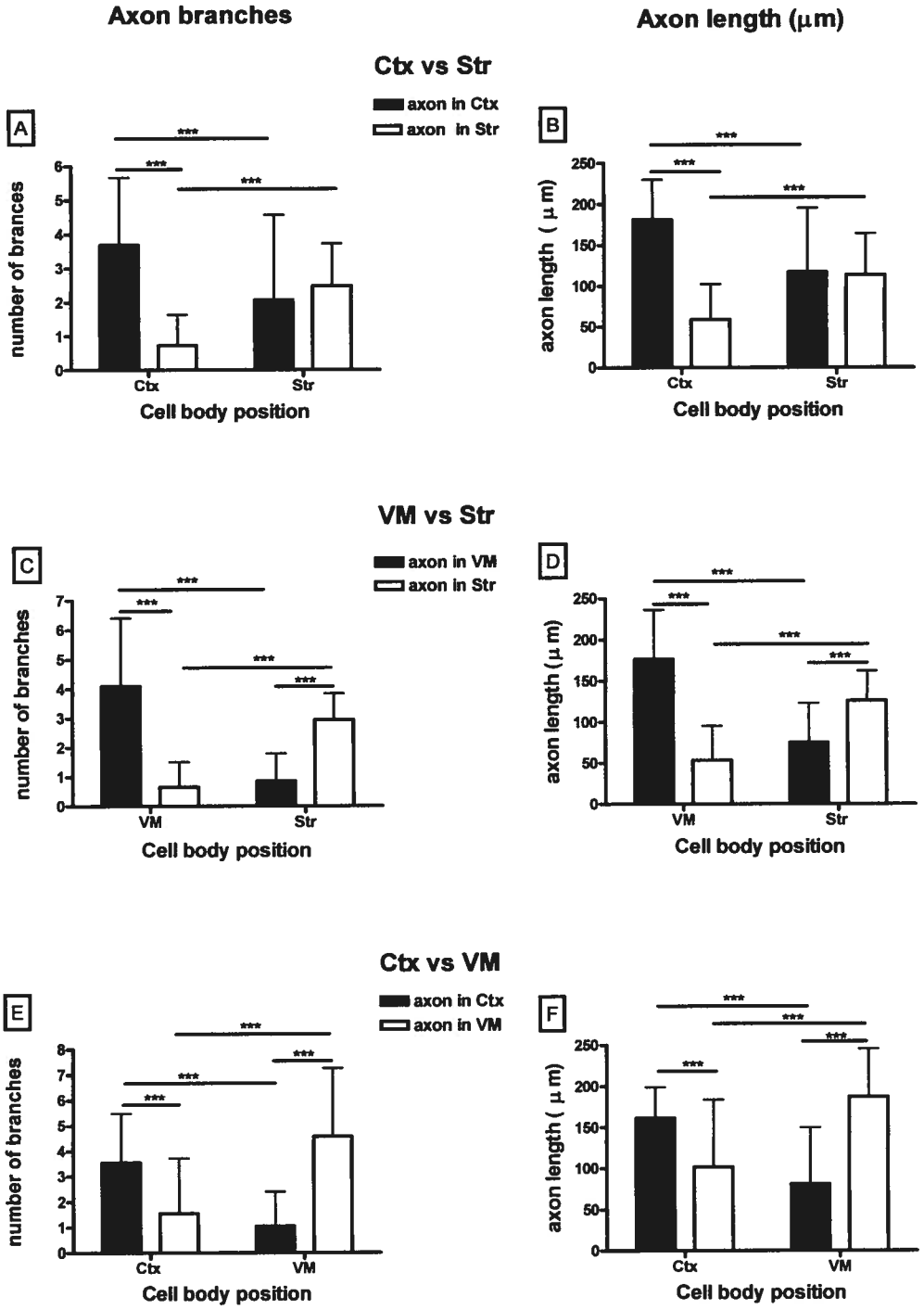

FIGURE 5. SEROTONERGIC AXON BRANCHING (NUMBER OF "AXON BRANCHES" AND "AXON LENGTH"), IN ALTERNATING STRIPES OF CELL MEMBRANE PREPARED FROM THE NEONATAL

CTX, STR OR VM, ACCORDING TO PARENT CELL BODY LOCATION IN SPECIFIC MEMBRANE STRIPES ("CELL BODY POSITION", ON THE X AXIS).

A,B) Ctx and Str membrane stripes: filled bars indicate axon branching in Ctx membrane stripes and white bars branching in Str membrane stripes. C,D) VM and Str membrane stripes: filled bars for branching in VM membrane stripes; white bars in Str membrane stripes. E,F) Ctx and VM membrane stripes: filled bars, branching in Ctx membrane stripes; white bars in VM membrane stripes. ANOVA with repeated measures; * $p<0.05$; ** $p<0.01$; ***$p<0.001$.

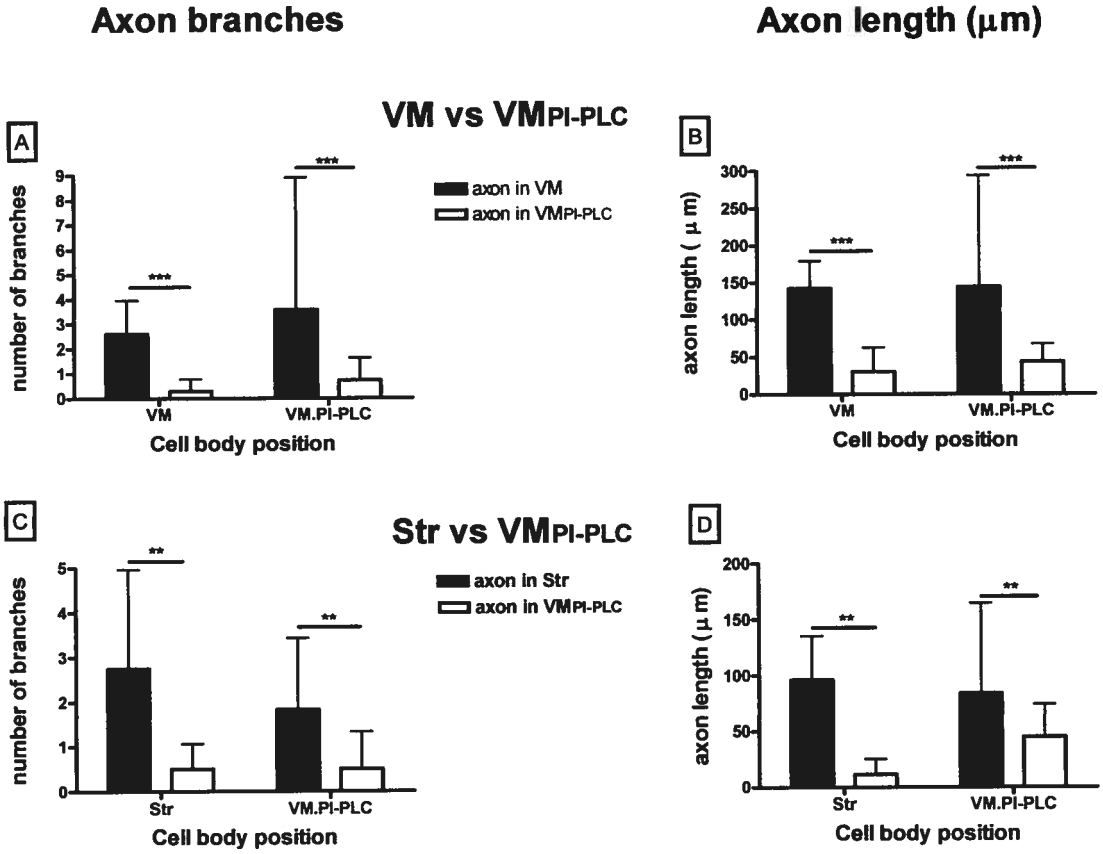

FIGURE 6. SEROTONERGIC AXON BRANCHING (NUMBER OF "AXON BRANCHES" AND "AXON LENGTH"), IN ALTERNATING STRIPES OF CELL MEMBRANE PREPARED FROM THE NEONATAL STR OR VM, UNTREATED (VM OR STR) OR TREATED WITH THE ENZYME PI-PLC (VMPI-PLC).

Results presented according to location of the parent cell body in treated or untreated membrane stripes ("cell body position", on the X axis). A,B) Untreated (VM) and treated (VM_{PI-PLC}) VM membrane stripes: filled bars indicate axon branching in untreated VM membrane stripes and white bars, branching in stripes of PI-PLC-treated VM membranes. C,D) Stripes of untreated Str membranes and PI-PLC-treated VM membranes: filled bars for branching in Str membrane stripes; white bars in stripes of VM_{PI-PLC}-treated membranes. ANOVA with repeated measures; * $p<0.05$; ** $p<0.01$; ***$p<0.001$.

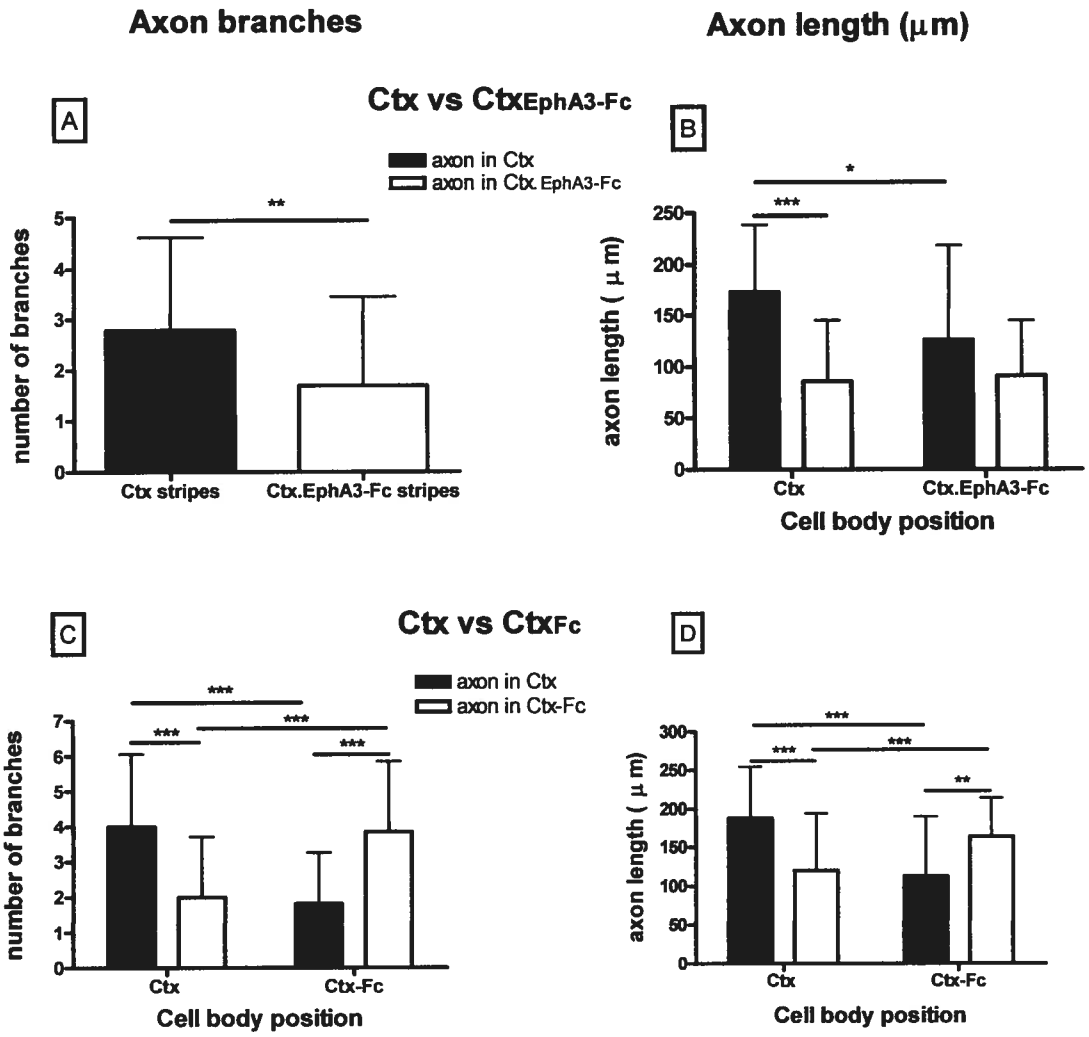

FIGURE 7. SEROTONERGIC AXON BRANCHING (NUMBER OF "AXON BRANCHES" AND "AXON LENGTH"), IN ALTERNATING STRIPES OF CELL MEMBRANE PREPARED FROM THE NEONATAL CTX, UNTREATED (CTX) OR TREATED WITH EITHER THE FUSION PROTEIN EPHA3-FC ($CTX_{EphA3\text{-}Fc}$), OR THE CONTROL FC PROTEIN (CTX_{Fc}).

Results presented according to location of the parent cell body in treated or untreated membrane stripes ("cell body position", on the X axis). A,B) Untreated (Ctx) and treated ($Ctx_{EphA3\ Fc}$) membrane stripes: filled bars indicate axon branching in untreated Ctx membrane stripes and white bars, branching in EphA3-Fc-treated Ctx membranes. C,D)

Control experiment: stripes of untreated Ctx membranes and Fc-treated Ctx membranes: filled bars for branching in untreated Ctx membrane stripes; white bars in stripes of Ctx_{Fc}-treated membranes. ANOVA with repeated measures; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

4. Discussion

Individual dorsal raphe 5-HT neurons project axon collaterals to multiple regions, including substantia nigra in VM, the striatum, and the cerebral cortex. It is unknown how 5-HT axon collaterals select between multiple target fields, or even whether 5-HT axons require specific guidance cues to innervate their targets. Our main objective was to identify molecules present in the membrane preparations from different brain regions that influence the growth of 5-HT axons.

Our current results indicate that 5-HT axons are able to discriminate among cell membranes extracted from the neonatal Ctx, Str and VM. Cues present in the membranes induced the branching of 5-HT axons, selectively in the same type of membrane stripes as those on which the parent cell bodies were located. Interestingly, the latter observation that the choice of a membrane substrate by 5-HT axons is determined by the location of the cell body strongly suggests the induction or up- / down-regulation of receptors for the guidance cue in 5-HT neurons. We demonstrate that the branch-inducing activity disappeared following PI-PLC treatment of the VM membranes, suggesting that the guidance cue was a GPI-anchored membrane protein. The observation that treatment of the Ctx membranes with EphA3-Fc also attenuated the branching of 5-HT axons provides evidence for a role for ephrin-As in this process. The latter observations must now be repeated with cellular membranes from the other two brain regions.

4.1. Selective branching of 5-HT axons in distinct substrate stripes

With stripes of PLL alternating with membrane stripes, 5-HT as well as non-5-HT neuronal cell bodies attached preferentially to the membrane stripes. Their axons also grew

and branched preferentially in membrane stripes, rather than onto PLL, as previously reported (Petit et al., 2005).

With alternating membrane stripes, 5-HT perikarya distributed rather randomly on the different types of substrates. There was maybe some tendency for 5-HT neurons to *prefer* Ctx or VM stripes to Str ones, but the experiments were not designed to test this point statistically (which would require a much larger number of separate experiments).

The density of 5-HT axons also appeared rather uniform among membrane stripes. It was only by following individual axons, from their parent perikarya that we found that they clearly branched preferentially in stripes made with the same type of membranes as those where their parent cell body was located. Interestingly, the mean number of branches emitted by individual 5-HT axons in any given type of untreated membrane stripes was remarkably similar between experiments, whatever the alternative substrate (PLL or membranes from another brain region). Thus, the number of 5-HT axon branches was near 4 on Ctx or VM stripes and around 3 on Str stripes. Such a preference of 5-HT axons for Ctx or VM, compared to Str membranes was also observed in a different context (Petit et al., 2005).

The total length of individual 5-HT axon was closely correlated with the number of branches, and lead precisely to the same conclusions. Indeed, the distance traveled by the axons did not seem to depend on the type of substrate (apart from PLL); although our experimental design does not allow to draw definitive conclusions on this aspect. But the total length of the axonal arborization obviously depended on the number of branches emitted by the axons.

It is also of interest to note that the majority of the axons did not branch in the same stripe bearing their perikarya. They generally crossed at least one or two stripes before

branching. Moreover, a preliminary evaluation of alternating stripes of untreated VM membranes, labelled or not with fluorescent microbeads showed no difference in axon branching onto labelled or unlabelled stripes for 5-HT neurons located in one or the other type of stripes. Thus, 5-HT axons had the choice between substrates, and the differences in branching truly reflected an underlying specific branching activity that was brain region-specific.

4.2. Nature of the guidance cues involved in 5-HT axon branching

A number of molecules have already been attributed axon branching inducing activity, including growth factors, netrins, semaphorins and ephrins (Roskies & O'Leary, 1994; Bolz *et al.*, 1996; Castellani *et al.*, 1998; Yates *et al.*, 2001; Ng *et al.*, 2002; Gaspar *et al.*, 2003; Dent *et al.*, 2004; Kalil & Dent, 2005; Tang & Kalil, 2005; Liu *et al.*, 2006). Since ephrin-As are GPI-anchored proteins, we tested the effect of the enzyme PI-PLC on the axon branching activity of VM membranes, to evaluate the possibility of GPI-anchored proteins being involved (Brown & Waneck, 1992; Wada *et al.*, 1998).

When untreated VM or Str membranes alternated with VM membranes treated with PI-PLC, 5-HT axons branched preferentially in the untreated VM or Str membrane stripes, even when their parent cell body was located in the PI-PLC-treated membrane stripes. Indeed, the number of 5-HT axon branches was very small in PI-PLC-treated membrane stripes. Thus, PI-PLC has a clear effect on the branching of 5-HT axons, suggesting that some GPI-anchored proteins are involved, directly or indirectly, in this process. It will be interesting to test the effect of such treatment with membranes from the other brain regions.

Nevertheless, the number of branches emitted by 5-HT axons in untreated VM or Str membrane stripes was also slightly reduced even for perikarya located in these untreated

stripes, compared to the number of branches produced in the same type of stripes in other experiments. Indeed, the number of 5-HT axon branches was highly reproducible among experiments for a given type of membranes, whatever the alternating membrane stripe, except when the latter had been treated with PI-PLC (or EphA3-Fc. but not Fc alone; see below). One explanation for this reduction might be that most 5-HT axons transited onto treated membranes at some point during their growth, which might have affected the expression of axon guidance receptors at the surface of their growth cones. Another, less likely, explanation would be that some PI-PLC remained in the treated membranes and diffused to the stripes of non-treated membranes. However, the amount of enzyme would then likely be highly diluted.

Thus our results indicate that PI-PLC treatment reduces the number of branches and thus the total length of axons in VM membranes treated with PI-PLC. A similar effect of PI-PLC treatment was previously reported for cortical layer 4 membranes which abolished their branch-promoting effect on layer 6 neurons of cortex (Castellani et al,. 1998).

Among GPI-linked proteins with known action on axon guidance are ephrin-As. The interaction of ephrin-As can be blocked by using soluble "immunoadhesins" such as EphA3-Fc (Gerlai *et al.*, 1999; Gerlai & McNamara, 2000; Yates *et al.*, 2001; Xu *et al.*, 2003). When 5-HT neurons were cultured onto stripes of Ctx membranes pretreated with EphA3-Fc, alternating with untreated Ctx membrane, 5-HT axons branched preferentially in the untreated membrane stripes. This effect was independent of the location of the parent perikarya in stripes of treated or untreated membranes. This result provides evidence that branching of 5-HT axons is influenced by EfnAs present in Ctx membranes. Thus 5-HT axon branching appears to be controlled by EfnAs, at least with membranes extracted from

the cerebral cortex. Here again, further experiments will tell whether the activity present in membranes from other brain regions could also be partly or totally attributed to ephrin-As. Clearly, the effect of EphA3-Fc was weaker than that of PI-PLC. It might be due to a suboptimal concentration of EphA3-Fc. But, it appears more likely that other GPI-anchored proteins participate in this activity, including some EfnAs for which the affinity of EphA3 may be too low to have an effect at the used concentration.

We speculate that 5-HT axons express EphA receptors, which recognize EfnAs, and that these interactions determine the branching of the axons in a given target. The specificity of recognition would then depend on the combination of several EfnAs, recognized by sets of EphAs expressed by the 5-HT axons. Co-expression of EfnAs, by 5-HT axons could also be involved in such combinatorial communication. (Hornberger et al., 1999).

Control treatment of Ctx membranes with Fc alone did not affect 5-HT axon length and branching. However, 5-HT axons then tended to branch in the same stripe bearing their parent cell body, at variance with experiments with alternating stripes of untreated membranes, where 5-HT axons usually branched in other stripes made of similar membranes. This effect may be related to Fc treatment. Further experimentation will tell whether this observation will be reproduced with membranes from other brain regions. Nevertheless, the results clearly show that Fc alone did not reduce the number of 5-HT axon branches, which was comparable to that found for Ctx stripes in experiments with only untreated membranes.

A similar branch-inducing activity has previously been demonstrated for ephrin-A5 on intrinsic cortical axon expressing Eph-A5 (Castellani et al,. 1998). Ephrin-A5 promoted

the branching of layer 6 cortical neurons. In contrast ephrin-A5 has a repulsive effect on axons of cortical layers 2, 3 (Castellani et al,. 1998).

Current Western Blotting assays in our laboratory (Bahar Noori et al, unpublished) show that ephrin-A4 and ephrin-A5 are expressed differentially in membranes extracted from the Ctx, Str and VM of newborn rats. On the other hand, available data in the literature show that at least EphA5 and EphA7 are expressed in the dorsal raphe (Maisonpierre *et al.*, 1993) (see also www.genepaint.org). Further experiments will give a more complete picture of the ephrin-As and EphAs expressed in the dorsal raphe and its target brain regions. Experiments aimed at modifying the expression of specific ephrins or Eph (transfection with constructs of Eph or Efn, or interference with expression using RNAi) should help to test the hypothesis of a combinatorial mode of communication determining the local branching of 5-HT axons.

4.3. Induction of the capacity of 5-HT axons to recognize the guidance cues

Our results demonstrate that 5-HT axons can discriminate among membranes from different neonatal brain regions. In addition, they also suggest that membranes from distinct target brain regions could induce 5-HT axons to *prefer* the same substrate.

Indeed, 5-HT neurons are normally meant to reside in the dorsal raphe environment, rather than the Ctx, Str or VM. We presume that the perikarya adhered to the diverse substrates indiscriminately, although they appeared to have a lower tendency to reside onto Str than Ctx or VM membranes stripes. Normally, individual 5-HT neurons project to several brain regions (van der Kooy & Hattori, 1980). Thus, their adherence to any given stripe of membranes was likely made at random. However, it is possible that the initial contact of their growth cone with membranes from target brain regions could induce the

expression of specific sets of axon guidance receptors (e.g., sets of EphA and ephrin-A molecules) that would specify their preference for a specific target region. Alternatively, this initial contact might affect signal transduction of EphA receptors through phosphorylation of the intracellular domain of intracellular targets of their signalling pathways. Recent work from our laboratory led to a similar conclusion of induction of guidance cue recognition, but then the initial growth onto Str or VM membranes induced an axon growth inhibitory response in 5-HT axons towards some cues present in membranes from other brain regions (Petit et al., 2005).

4.4. Future experiments

To further characterize the roles of ephrins in 5-HT axons branching, it will be necessary to test the effects of EphA3-Fc treatment of membranes from other brain regions (VM or Str). Since 5-HT axons were able to discriminate among membranes from different brain regions, we presume that the effects of EphA3-Fc will be more, or less, efficient with other membranes than with Ctx membranes. Indeed, EphA3-Fc does not have a uniform affinity for different ephrin-As (Janis et al., 1999). In the same line of thinking, we expect that treatment with other immunoadhesins, such as EphA2-Fc, EphA5-Fc, or EphA7-Fc, will have differential effects with membranes from each of the 3 brain regions.

Other approaches, using small inhibitory RNAs, to inhibit the expression of specific EphA or ephrin-A in 5-HT neurons in culture, or transfection of the genes coding for these molecules, will also contribute to testing the hypothesis that specific recognition of target membranes by 5-HT axons is based on a combinatorial code of EphA/ephrin-A in projecting axons and their targets.

The identity of molecules responsible for inducing the 5-HT axon sensitivity towards membrane molecules eliciting axon branching is more obscure, since there is currently no known example of molecules with such an action. Nevertheless, since this activity is associated with membrane preparations, we now have an experimental model which might allow exploration of these molecules.

5. Conclusions

Our results show that 5-HT axon are able to recognize axon guidance cues present in cell membranes, allowing them to discriminate among membranes extracted from various neonatal brain regions. The choice of a membrane substrate by 5-HT axons was determined by the location of the cell body on the same type of membranes; strongly suggesting an induction of guidance cue recognition.

Several molecules have already been attributed axon branching inducing activity, such as growth factors, netrins, semaphorins and ephrins. The current observations with VM membranes treated with PI-PLC and Ctx membranes treated with EphA3-Fc, provide evidence that ephrin-A molecules are involved in this axon branching induction. Further evidence of this is currently being sought in our laboratory, with membranes from the other brain regions treated with PI-PLC or EphA3-Fc, as well as with other immunoadhesins (EphA2-Fc etc).

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Appendix: Curriculum vitae

BAHRAM SHARIF ASKARI

[redacted]

Degrees:

2004-2006 : M.Sc, in Pathology and cell Biology, option Neuroscience, Department of Pathology and Cell Biology, Faculty of Medicine, University of Montreal
1980-1987 : D.V.M, Doctor of Veterinary Medicine, Faculty of Veterinary Medicine, University of Shiraz, Shiraz, Iran

Academic experiences:

1992-2002 : Lecturer, Department of Pathobiology, Faculty of Veterinary Medicine, Azad University, Iran
1996-2002 : Laboratory Supervisor, Histology and Histopathology laboratories, Faculty of Veterinary Medicine, Azad University, Iran
1992-1996 : Research Assistant, Department of Pathobiology, Faculty of Veterinary Medicine, Azad University, Iran

Technical experiences:

- Cell culture
- Cell membrane preparing
- Stripe assay techniques
- Cell isolation and proliferation
- Immunohistochemistry
- Fluorescence microscopy techniques
- Histology and Histopathology slides preparation
- Histotechnique and Tissue processing

Clinical and laboratory experiences:

- Veterinary consultant and advisor of large beef cattle and fattening lamb farms, responsibilities include different services such as clinical examination, vaccination program, performation treatment, prevention and medication as well as necropsies
- Administer anesthesia, performs surgery, clinical laboratory and paracilinic diagnostic procedures
- Adjustment and control of food requirement for beef and dairy cattle and fattening lamb

- Diagnosis and treatment of laboratory animals diseases
- Diagnosed and reported suspected cases, reffered to histopathology lab
- Laboratory animals dissection and minor surgery
- Drug proving experiences in small animals
- Laboratory and small animals drug administration (oral and parenteral)
- Laboratory animals handling and manipulation

Teaching experiences:

- **1992-2002:** Histology and Histopathology of Cell , Connective tissue, Blood and Hematopoietic tissue, Faculty Veterinary Medicine, Azad University, Iran
- **1994-2000:** Cancer Pathology, Faculty of Veterinary Medicine, Azad University, Iran

Awards:

- **2001**, Azad University award for Histology teaching, Shahrekord, Iran
- **1998**, Azad University award for Pathology teaching, Kazeroun, Iran

Contribution to develop and establish laboratories:

- **1998:** Histopathology laboratory, Faculty of Veterinary Medicine, Azad University, Iran
- **1996:** Histotechnique laboratory, Faculty of Veterinary Medicine, Azad University, Iran

Research Activities:

- **2004-2006,** " Cellular and molecular mechanism of Serotonergic axon guidance, extract from fetal dorsal raphe ", Department of Pathology and Cell Biology, Faculty of Medicine, University of Montreal
- **2001-2002,** " Pathological and Histopathological survey on Pulmonary Adenomatosis", Department of Pathobiology, Faculty of Veterinary Medicine, Azad University, Iran
- **1998-2002,** " Histopathology comparative survey of Blood and Bone marrow tissue between different species", Department of Pathobiology, Faculty of Veterinary Medicine, Azad University, Iran

Bahram Sharif Askari

Contribution to D.V.M Student Thesis:

- **2002**, Rashidi.A, " Histopathology survey on Ovine Pulmonary Adenomatosis in Tehran province", **Director**
- **2001**, Abedini. M." Proving of Homoeopathic Belladona", **Director**
- **1999**, Javadi.B, " Histological survey on Salmon fish Blood tissue", **Director**
- **1998**, Maftoon. P, " Histological comparative survey on bone marrow between Human and Carnivores", **Director**
- **1997**, Abedi.A, " Histopathology survey on Bovine Spongy Encephalitis (B.S.E) in Tehran province", **Co-director**

Publications:

- Hekmati.P, Radmehr.B, **Sharif askari.B** and Hoshmand. M, 2000, "Natural health", First edition, Anvare danesh publication, Tehran, Iran
  Second edition: 2001
  Third edition: 2002
- Hekmati .P and **Sharif Askari. B**,1997, "General guide for writing postgraduate thesis", First edition, Azad University, Azad University publication, Iran
- **Sharif Askari. B,** 1997, "Sulphur toxicosis in a flock of sheep", Scientific Journal of Veterinary Faculty, Azad University, Iran, Vol.5, No.14,34-40
- Hekmati. P and **Sharif Askari. B,** 1994, "Application of Naso-Ruminal Instrument in collection of ruminal fluid for paraclinical examination", Journal of Vet. Faculty, University of Tehran, Tehran, Iran, Vol .46, No.3,25-30
- **Sharif Askari .B** and Rezakhani. A, 1994, "Radio epidemiological survey on Splints in horses", World Veterinary Congress Bulletin, Rio de Janeiro, Brazil
- Hekmati .P and **Sharif Askari. B**,1993, "Disorders of cattle and sheep digits", First edition, Azad University, Iran

Seminars:

- **2005**, " Implication of EphA3-Fc in Serotonergic axonal guidance extract from dorsal raphe ", First Retraite of GRSNC, Orford, 23-25 Sep 2005, Oral presentation
- **2005**, " Implication of EphA3-Fc in Serotonergic axonal guidance extract from dorsal raphe ", 22 Scientific Pathology day, Faculty of Medicine, University of Montreal, 2 June 2005, Oral presentation
- **2004**, " Cellular and molecular mechanism of Serotonergic axonal guidance in vitro", 21 Scientific Pathology day, Faculty of Medicine, University of Montreal, 20 May 2004, Poster presentation

Bahram Sharif Askari

Membership:

- Member of Society for Neuroscience (*SFN*), Washington, DC

References:

- **Dr. Guy Doucet**, Professor of department of Pathology and Cell Biology, Faculty of Medicine, University of Montreal, [redacted]
- **Dr. Adriana Di Polo**, Professor of department of Pathology and Cell Biology, Faculty of Medicine, University of Montreal, [redacted]
- **Dr. Nicole Leclerc**, Professor of department of Pathology and Cell Biology, Faculty of Medicine, University of Montreal, [redacted]