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Serotonin neurotransmission in 5-HT_{1A} and 5-HT_{1B} receptor knockout mice

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TABLE OF CONTENT

Sommaire

Au cours des dernières années, le nombre de récepteurs de la sérotonine (5-hydroxytryptamine, 5-HT) s'est accru jusqu'à 14 sous-types identifiés à ce jour dans le SNC des mammifères. Parmi ceux-ci, les sous-types 5-HT_{1A} et 5-HT_{1B} ont particulièrement retenu l'attention en raison de leur implication dans les désordres psychoaffectifs comme l'anxiété, la dépression, l'aggressivité et l'addiction. Les souris mutants nuls pour le sous-type réceptoriel 5-HT_{1A} ou 5-HT_{1B} présentent un grand intérêt dans l'étude du rôle de ces récepteurs dans le comportement, ainsi que de leur participation aux mécanismes neurobiologiques sous-jacents. Le phénotype des souris knockout (KO) 5-HT_{1A} est de type anxieux dans divers tests comportementaux, alors que les KO 5-HT_{1B} montrent une aggressivité exagérée et une tendance accrue à l'auto-administration de cocaine. Nous nous sommes fixés comme but principal de mieux définir et caractériser la contribution de chacun de ces récepteurs au comportement, par la mise en évidence et l'analyse des conséquences de leur délétion sur la neurotransmission à 5-HT, et des compensations ou adaptations mutuelles résultant de cette absence constitutive.

Dans un premier temps, nous avons mesuré par chromatographie liquide à haute performance la teneur en monoamines et leurs métabolites de 16 régions cérébrales et la moelle épinière chez les souris de type sauvage (« wild-type » ou WT) et knockout (KO) pour les récepteurs 5-HT_{1A} ou 5-HT_{1B}. Les rapports [métabolites]/[monoamine] ont été calculés pour déterminer les index de renouvellement, ou *turnover*. Chez le KO 5-HT_{1A}, nous avons ainsi

décelé un accroissement du turnover de la 5-HT dans les noyaux raphé dorsal et raphé médian, principaux groupes de corps cellulaires 5-HT du cerveau, de même que dans plusieurs territoires d'innervation 5-HT. Ce résultat a été interprété comme traduisant une augmentation d'activité basale des neurones 5-HT, expliquant ainsi le comportement de type anxieux observé chez le mutant nul 5-HT_{1A}. Nous avons également mesuré une augmentation concomitante de la dopamine et/ou de son turnover, qui pouvaient être le résultat d'une désinhibition des neurones de ce type, ainsi qu'une augmentation de la concentration en noradrénaline dans plusieurs territoires de projection du locus coeruleus, reflétant probablement une diminution d'activité de ses neurones. Chez les KO 5-HT_{1B}, les concentrations en 5-HT sont apparues significativement plus faibles que chez les WT dans le noyau accumbens, le locus coeruleus et la moelle épinière, et l'étaient aussi probablement dans quelques autres territoires d'innervation 5-HT. De plus, nous avons constaté une diminution de la teneur en dopamine, associée à un turnover accru, dans le noyau accumbens. Ces changements de métabolisme de la 5-HT et de la dopamine pourraient sous-tendre l'aggressivité accrue et la propension pour la cocaine des mutants nuls 5-HT_{1B}.

Dans un deuxième temps, nous avons visualisé et mesuré dans le cerveau des souris WT et KO la radioliaison aux récepteurs 5 HT_{1A} et 5-HT_{1B}, de même qu'au transporteur membranaire de la 5-HT (5-HTT), au moyen de l'autoradiographie du [^{3}H]8-OH-DPAT, [^{125}I]cyanopindolol et [^{3}H]citalopram, respectivement. Aucune des nombreuses régions examinées n'a montré de différence significative de radioliaison au récepteur conservé chez les

mutants. Par contre, des diminutions significatives de la densité de liaison au 5-HTT ont été détectées dans quelques régions du cerveau des deux KO. De plus, la densité de liaison au 5-HTT est apparue significativement augmentée dans le noyau amygdalo-hippocampique et l'hippocampe ventral du KO 5-HT_{1B}. Des mesures immunocytochimiques ont révélé un accroissement proportionnel de la densité d'innervation 5-HT dans ces deux régions, tandis qu'aucune des diminutions n'était associée à ce type de changement. Il en a été conclu que (1) les récepteurs 5-HT_{1B} ne s'adaptent pas à l'absence des récepteurs 5-HT_{1A}, et vice versa; (2) les 5-HTT font l'objet d'une régulation à la baisse dans plusieurs région du cerveau chez les deux KO; (3) cette régulation à la baisse pourrait contribuer au comportement de type anxieux des souris KO 5-HT_{1A}, en réduisant la disparition (clearance) de la 5-HT dans plusieurs territoires d'innervation 5-HT; (4) l'hyperinnervation 5-HT du noyau amygdalo-hippocampique et de l'hippocampe ventral de la souris KO 5-HT_{1B} pourrait jouer un rôle dans le comportement hyperaggressif de ces mutants et leur meilleure performance lors de certains tests cognitifs; (5) ces innervations 5-HT en excès représentent la première indication d'un contrôle négatif de la croissance des neurones 5-HT exercé par l'entremise des récepteurs 5-HT_{1B}.

Dans un troisième temps, nous avons voulu détecter et caractériser des capacités adaptatives éventuelles des récepteurs 5-HT_{1A} et 5-HT_{1B} l'un envers l'autre, en termes de couplage aux protéines G, sachant que la densité des récepteurs conservés était inchangée chez les deux mutants. Pour ce faire, nous avons eu recours à une nouvelle méthode qui permet de mesurer au moyen de l'autoradiographie l'incorporation d'un analogue non-

hydrolisable de la guanosine triphosphate, le [^{35}S]GTPγS, lors de son activation par un agoniste. Tel que prévu, chez les WT, la 5-carboxyamidotryptamine (5-CT), un agoniste 5-HT$_{1A/1B}$ non sélectif, a stimulé l'incorporation de [^{35}S]GTPγS dans plusieurs régions cérébrales munies de l'un et/ou l'autre récepteur, tandis qu'aucune activation n'a été mesurée dans les régions connues pour exprimer surtout ou exclusivement le récepteur 5-HT$_{1A}$ ou le récepteur 5-HT$_{1B}$ chez les KO correspondants. L'activation de la protéine G par la 5-CT se comparait à celle du WT dans toutes les régions contenant le récepteur 5-HT$_{1B}$ du KO 5-HT$_{1A}$, mais s'est avérée significativement plus faible que chez le WT dans l'amygdale des KO 5-HT$_{1B}$, malgré l'existence d'une forte stimulation via le récepteur 5-HT$_{1A}$ chez les WT. Des résultats similaires ont été obtenus dans l'amygdale des KO 5-HT$_{1B}$ par suite d'activation avec le 8-OH-DPAT, un agoniste sélectif des récepteurs 5-HT$_{1A}$. En outre, dans ces conditions, l'incorporation de [^{35}S]GTPγS est apparue significativement plus faible dans la plupart des régions contenant le récepteur 5-HT$_{1A}$, incluant le noyau raphé dorsalis. Cette diminution de l'efficacité de couplage aux protéines G des récepteurs 5-HT$_{1A}$ en l'absence de changement de leur nombre pourrait fournir le premier indice d'une interaction locale, ou cross-talk, entre ces deux sous-types réceptoriels, du moins dans les régions cérébrales ou ils co-existent dans les mêmes neurones.

Qu'il s'agisse de conséquences directes ou de changements compensatoires ou adaptatifs, de telles altérations de la structure et du fonctionnement des neurones à sérotonine du cerveau, résultant de l'absence constitutive des récepteurs 5-HT$_{1A}$ ou 5-HT$_{1B}$, doivent possiblement

contribuer au phénotype comportemental observé chez les mutants nuls respectifs.

Mots clés: 5-HT_{1A} et 5-HT_{1B} mutants nuls — cerveau — monoamines — métabolites — index de renouvellement — transporteur 5-HT — récepteurs 5-HT_{1A} et 5-HT_{1B} — hyperinnervation — couplage aux protéines G — autoradiographie [^{35}S]GTPγS — 5-carboxyamidotryptamine, (5-CT) — 8-hydroxy-2-(di-*n*-propylamino) tetralin, (8-OH-DPAT)

Summary

In recent years, the number of serotonin (5-hydroxytryptamine, 5-HT) receptors has increased to 14 subtypes thus far identified in the mammalian central nervous system (CNS). The 5-HT_{1A} and 5-HT_{1B} subtypes have received the most attention, because of their implication in some psychiatric disorders, particularly in anxiety, depression, aggressiveness and drug abuse. Mice lacking the 5-HT_{1A} or 5-HT_{1B} receptor subtypes provide a strategy to investigate the role of these 5-HT receptors in behavior, and of their implication in the underlying neurobiological mechanisms. 5-HT_{1A} knockout (KO) mice display an anxious-like behavioral phenotype in several paradigms, whereas 5-HT_{1B} KOs are over aggressive and show greater propensity to self-administer cocaine. Our main objectives were to further define and characterize the contribution of each of these receptors to behavior, by detecting and analyzing the consequences of their absence on 5-HT neurotransmission and eventual mutual compensations or adaptations resulting from their constitutive absence.

In a first step, tissue levels of monoamines and their main metabolites were measured by high performance liquid chromatography in 16 brain regions and the spinal cord of wild type (WT), 5-HT_{1A} and 5-HT_{1B} KO mice. Ratios of [metabolites]/[monoamine] were used as an index of *turnover*. In the 5-HT_{1A} KO, an increased 5-HT turnover was thus demonstrated in the dorsal and medial raphe nuclei, the main 5-HT cell body groups in brain, as

well as in several of their territories of projection. This was taken as an indication of increased basal activity of the 5-HT neurons, and provided a likely explanation for the increased anxiety-like behavior observed in the 5-HT_{1A} knockout mice. Concomitant increases in dopamine content and/or turnover were interpreted as the result of a disinhibition of these neurons, whereas increases in noradrenaline concentration in several territories of projection of the locus coeruleus were likely to reflect a diminished activity of its neurons. In 5-HT_{1B} knockouts, 5-HT concentrations were significantly lower than WT in nucleus accumbens, locus coeruleus, and the spinal cord, and probably also in several other territories of 5-HT innervation. Moreover, a decrease in dopamine level, associated with increased turnover, was measured in the nucleus accumbens. These changes in 5-HT and dopamine metabolism were consistent with the increased aggressiveness and supersensitivity to cocaine reported in the 5-HT_{1B} knockouts.

In a second step, binding sites for 5 HT_{1A} and 5-HT_{1B} receptors, as well as for 5-HT uptake sites or transporters (5-HTTs) were visualized and measured throughout the brain of WT and KO mice, by autoradiography with [^{3}H]8-OH-DPAT, [^{125}I]cyanopindolol and [^{3}H]citalopram, respectively. None of the numerous regions examined showed any significant difference in binding to the conserved receptor in the mutants. In contrast, there were significant decreases in 5-HTT binding densities in several brain regions of both null mutants, but in the amygdalo-hippocampal nucleus and ventral hippocampus of 5-HT_{1B} KOs 5-HTT levels were significantly increased.

Quantitative immunocytochemical measurements revealed proportional increases in 5-HT innervation in these two regions, whereas none of the decreases in 5-HTT binding sites could be associated with any such changes. It was therefore concluded that (1) 5-HT_{1B} receptors do not adapt in 5-HT_{1A} KOs, nor 5-HT_{1A} receptors in 5-HT_{1B} Kos; (2) 5-HTT are down-regulated in several brain regions of the two KOs; (3) this down-regulation could contribute to the anxious-like phenotype of the 5-HT_{1A} Kos, by reducing 5-HT clearance in several territories of 5-HT innervation; (4) the 5-HT hyperinnervation in the amygdalo-hippocampal nucleus and ventral hippocampus of 5-HT_{1B} KO mice could play a role in the increased aggressiveness displayed by these mutants, and might also explain their better performance in some cognitive tests; (5) these increases in 5-HT innervation provided the first evidence for a negative control of 5-HT neuron growth that could be mediated by 5-HT_{1B} receptors.

In a third step, we meant to detect and characterize eventual adaptive capacities of 5-HT_{1A} and 5-HT_{1B} receptors toward one another in terms of G-protein coupling, knowing that the density of the conserved receptor was unchanged in both mutants. This was carried out by means of a newly developed method for measuring receptor agonist-stimulated [^{35}S]GTPγS incorporation by autoradiography. As expected, in WT, the non selective 5-$HT_{1A/1B}$ receptor agonist 5-carboxyamidotryptamine (5-CT) stimulated [^{35}S]GTPγS incorporation in many brain regions endowed with one and/or the other receptor, whereas in the respective knockouts, no stimulation was

measured in regions known to express mainly, or exclusively, 5-HT_{1A} or 5-HT_{1B} receptors. G-protein activation by 5-CT was of the same magnitude as WT in every 5-HT_{1B} containing region of 5-HT_{1A} KO, but significantly lower than WT in the amygdala, in spite of a strong stimulation, via 5-HT_{1A} receptors, in the WT. Similar results were obtained in the amygdala of the 5-HT_{1B} KO after activation by the selective 5-HT_{1A} receptor agonist 8-OH-DPAT. Moreover, under these conditions, [^{35}S]GTPγS incorporation was significantly reduced in most regions endowed with 5-HT_{1A} receptors, including the dorsal raphe nucleus. Thus, there appeared to be a compensatory decrease in the efficiency of G-protein coupling to 5-HT_{1A} receptors in the 5-HT_{1B} mutant, which could represent the first indication of a cross-talk between these two 5-HT receptor subtypes, at least in brain regions where they are colocalized in the same neurons.

Whether viewed as direct consequences, or as compensatory adaptive changes, these neurobiological alterations of the central 5-HT system, resulting from the absence of its 5-HT_{1A} or 5-HT_{1B} receptors, presumably contribute to the behavioral phenotypes observed in the 5-HT_{1A} and 5-HT_{1B} KO mice.

Key words: 5-HT_{1A} and 5-HT_{1B} knockouts — brain — monoamines — metabolites — turnover index — 5 HT transporter — 5 HT_{1A} and 5 HT_{1B} receptors — hyperinnervation — G-protein coupling — [^{35}S]GTPγS autoradiography — 5-carboxyamidotryptamine, (5-CT) — 8-hydroxy-2-(di-*n*-propylamino) tetralin, (8-OH-DPAT)

List of Tables

List of Figures

List of Abbreviations

1A –/–	5-HT_{1A} receptor knockout mouse
AADC	aromatic aminoacid decarboxylase
Acb	nucleus accumbens
Ag/AgCl	silver/silver chloride
AHiPM	amygdalo-hippocampal posteriomedial nucleus
AHi	amygdalo-hippocampal nuclei, amygdala
Amyg	amygdala
ATP	adenosine triphosphate
B	specific bound
B1	nucleus raphe pallidus
B2	nucleus raphe obscurus
B3	nucleus raphe magnus
B4	nucleus raphe obscurus, dorsolateral part
B5	median raphe nucleus, caudal part
B6	dorsal raphe nucleus, caudal part
B7	dorsal raphe nucleus, rostral part
B8	median raphe nucleus, rostral part; caudal linear nucleus; nucleus pontis oralis
B9	nucleus pontis oralis
1B –/–	5-HT_{1B} receptor knockout mouse
BLP	basolateral posterior nucleus, amygdala
B_{MAX}	maximum binding capacity
^{14}C	carbon 14 isotope
CA1	Ammon's horn, hippocampus
Ca Cl_2	calcium chloride

cAMP	cyclic adenosine monophosphate
CB	cerebellum
Ce	central nucleus, amygdala
Ci	curie
Cin	cingulate cortex
Cl	clastrum
CNS	central nervous system
COMT	catechol-O-methyl-transpherase
CPu	caudate-putamen
5-CT	5-caboxyamidotryptamine
DA	dopamine
5,7-DHT	5,7-dihydroxytryptamine
DOPAC	3,4-dihydroxyphenylacetic acid
DRN	dorsal raphe nucleus
En	endopiriform nucleus
EnPi	entorhinal/pirifrom cortex
fmol/mg p.	femtomoles per milligram of protein
Fro	frontal cortex
GP	globus pallidus
GPCR	G-protein-coupled receptor
GRK	G-protein-coupled receptor kinase
GTP	guanosine 5'-triphosphate
GTPγS	nonhydrolysable GTP analogue
HCl	hydrochloric acid
5-HIAA	5-hydroxyindole-3-acetic acid
Hip	hippocampus
HPLC	high-performance liquid chromatography

5-HT	5-hydroxytryptamine (serotonin)
5-HT_{1A}	serotonin 1A receptor
5-HT_{1B}	serotonin 1B receptor
5-HTP	5-hydroxy-L-tryptophan
5-HTOL	5-hydroxytryptophol
5-HTT	serotonin transporter
HVA	homovanilic acid
IA	interaural
IP	interpeduncular nucleus
IUPHAR	international union of pharmacology
KCl	potassium chloride
K_D	equilibrium dissociation constant
KH_2PO_4	potassium diphosphate, monobasic
KO	knockout (null mutant)
L	radioligand concentration
LD	laterodorsal nucleus, thalamus
L-DOPA	levo-dihydroxyphenylalanine
LC	locus coeruleus
LGP	lateral globus pallidus
LP	lateroposterior nucleus, thalamus
LS	lateral septum
LSD	lisergic acid diethylamide
M	molar solution
mAChR	metabotropic acetylcholine receptor
MAO	monoamine oxidase
Me	medial nucleus, amygdala
$MgCl_2$	magnesium chloride

MHPG	3-methoxy-4-hydroxyphenylglycol
mRNA	messenger ribonucleic acid
3-MT	3-methoxytyramine
_M	micromolar
N	normal solution
nA	nanoampere
NA	noradrenaline
NaCl	sodium chloride
Na_2HPO_4	disodium phosphate
NA_2EDTA	ethylenediaminotetraacetic acid, disodium salt
NaOH	sodium hydroxide
ng/mg p.	nanograms per milligram of protein
nM	nanomolar
nRD	nucleus raphe dorsalis
nRM	nucleus raphe medialis
NSD 1015	3-hydroxybenzyl-hydrazine
OD	optical density
OB	olfactory bulb
[^{3}H]8-OH-DPAT	8-hydroxydipropylaminotetralin, tritium labeled
Par	parietal cortex
PCA	*p*-chloroamphetamine
pM	picomolar
PV	paraventricular nucleus, thalamus
SEM	standard error of the mean
SN	substantia nigra
Spt	septum
SSRI	selective serotonin reuptake inhibitor

TH	tyrosine hydroxylase
Tha	thalamus
Tris	Tris-[hydroxymethyl]-aminomethane
vHip	ventral hippocampus
Vmax	maximal rate (velocity)
VTA	ventral tegmental area
WT	wild-type

Résumé vulgarisé

En notre époque où l'on a appris à manipuler les gènes, il est possible de produire des souris de laboratoire chez lesquelles on a éliminé un gène en particulier, responsable de la production d'une protéine d'intérêt. Ces animaux, que l'on qualifie de knockout, représentent des sujets d'expérience remarquables, lorsque l'on s'interroge sur l'implication de la protéine en question dans le fonctionnement d'une cellule, d'un tissu, d'un organe ou de l'organisme entier, ou encore sur son rôle éventuel dans l'action d'un médicament. C'est ce type de préparation que nous avons utilisé pour examiner les conséquences de la suppression de l'un ou l'autre de deux récepteurs de la sérotonine (5-hydroxytryptamine, 5-HT), dits 5-HT_{1A} et 5-HT_{1B}, sur le métabolisme de ce neurotransmetteur-modulateur dans le cerveau de l'animal, ainsi que les mécanismes moléculaires de son action. La question apparaissait d'autant plus importante que, parmi les 14 sous-types de récepteurs de la 5-HT identifiés à ce jour dans le SNC des mammifères, les sous-types 5-HT_{1A} et 5-HT_{1B} sont impliqués chez l'homme dans des dérèglements émotionnels comme l'anxiété, la dépression, l'aggressivité et l'addiction. Chez les souris knockout 5-HT_{1A}, un comportement de type anxieux a d'ailleurs été décrit, alors que les knockout 5-HT_{1B} montrent une aggressivité exagérée et une tendance accrue à l'auto-administration de cocaine. Au cours de notre étude, nous avons donc accordé une attention spéciale à la contribution potentielle de chacun des

deux récepteurs aux comportements observés, de même qu'à de possibles compensations ou adaptations mutuelles résulant de leur absence constitutive chez l'animal.

Dans un premier temps, nous avons mesuré par chromatographie liquide à haute performance la teneur en monoamines (sérotonine, dopamine, noradrénaline) et leurs métabolites de 16 régions cérébrales et de la moelle épinière chez les souris knockout 5-HT_{1A} et 5-HT_{1B} et la souris sauvage. Chez les knockout 5-HT_{1A}, nous avons ainsi décelé un accroissement de la vitesse de renouvellement de la 5-HT dans plusieurs régions du cerveau, reflétant une augmentation d'activité basale des cellules nerveuses utilisant la 5-HT. Un tel accroissement du métabolisme cérébral de la 5-HT pouvait rendre compte du comportement de type anxieux observé chez le knockout 5-HT_{1A}. Nous avons aussi mesuré une augmentation concomitante de la dopamine et/ou de son turnover, qui pouvait être dûe à un manque d'inhibition des neurones de ce type, ainsi qu'une augmentation de la teneur en noradrénaline dans d'autres régions cérébrales, qui pouvait traduire une diminution d'activité de cette autre espèce de neurones. Chez les knockout 5-HT_{1B}, la teneur en 5-HT est apparue plus faible que chez la souris sauvage dans plusieurs régions du cerveau. De plus, nous avons observé une diminution de la teneur en dopamine, associée à un turnover accru, dans la région dite noyau accumbens. Ces changements du métabolisme de la 5-HT et de la dopamine pourraient expliquer l'aggressivité accrue et la propension pour la cocaine des mutants nuls 5-HT_{1B}.

Dans un deuxième temps, nous avons utilisé des molécules radioactives qui se lient spécifiquement aux récepteurs 5-HT_{1A} et 5-HT_{1B} et la méthode d'autoradiographie pour visualiser et mesurer ces récepteurs dans le cerveau des souris knockout et sauvages. Aucune différence significative de distribution ni de quantité du récepteur conservé n'a été trouvée chez l'un ou l'autre knockout, ce qui excluait toute adaptation mutuelle de cet ordre. Par contre, nous avons détecté des diminutions significatives de la densité de liaison au transporteur membranaire de la 5-HT (5-HTT), lequel est responsable de la recapture de la 5-HT par les neurones qui l'utilisent, dans quelques régions du cerveau des deux knockout. De plus, chez le knockout 5-HT_{1B}, la quantité de 5-HTT est apparue significativement augmentée dans deux régions en particulier, le noyau amygdalo-hippocampique et l'hippocampe ventral. Des mesures immunocytochimiques ont alors révélé un accroissement proportionnel de la densité de l'arborisation axonale des neurones à 5-HT dans ces deux régions, tandis qu'aucune des diminutions n'était associée à ce type de changement. Nous en avons conclu que (1) les récepteurs 5-HT_{1B} ne s'adaptent pas à l'absence des récepteurs 5-HT_{1A}, et vice versa; (2) les 5-HTT font l'objet d'une régulation à la baisse dans plusieurs région du cerveau chez les deux KO; (3) cette régulation à la baisse pourrait contribuer au comportement de type anxieux des souris KO 5-HT_{1A}, en réduisant la disparition (clearance) de la 5-HT dans plusieurs régions du cerveau; (4) l'innervation 5-HT excessive du noyau amygdalo-hippocampique et de l'hippocampe ventral de la souris KO 5-HT_{1B} pourrait jouer un rôle

dans le comportement hyperaggressif de ces mutants et rendre compte de leur meilleure performance lors de certains tests cognitifs; (5) ces innervations 5-HT en excès représentent la première indication d'un contrôle négatif de la croissance des neurones à sérotonine exercé par l'entremise des récepteurs 5-HT_{1B}.

Dans un troisième temps, nous avons voulu déceler et caractériser d'éventuelles capacités adaptatives des récepteurs 5-HT_{1A} et 5-HT_{1B} l'un envers l'autre, en termes de couplage aux protéines G, via lesquelles s'exerce leur action. Pour ce faire, nous avons eu recours à une méthode autoradiographique nouvelle, qui permet de visualiser et mesurer l'incorporation d'une molécule radioactive au sein des protéines G lors de leur activation par un agent pharmacologique qui simule l'action de la 5-HT (agoniste). Tel que prévu, chez la souris sauvage, la 5-carboxyamidotryptamine (5-CT) un agoniste 5-$HT_{1A/1B}$ non sélectif, a stimulé l'incorporation de la molécule radioactive dans plusieurs régions cérébrales munies de l'un et/ou l'autre récepteur, tandis que, chez les knockout respectifs, aucune activation n'a été mesurée dans les régions connues pour exprimer surtout ou exclusivement le récepteur 5-HT_{1A} ou 5-HT_{1B}. Chez les knockout 5-HT_{1A}, l'activation de la protéine G par le 5-CT se comparait à celle observée chez la souris sauvage dans toutes les régions contenant le récepteur 5-HT_{1B}. Chez les knockout 5-HT_{1B}, cependant, elle s'est avérée significativement plus faible que chez la souris sauvage dans la région dite amygdale, lors même que cette région montrait une forte

activation via le récepteur 5-HT_{1A} chez la souris sauvage. Des résultats similaires ont été obtenus dans l'amygdale des knockout 5-HT_{1B} par suite d'activation avec le 8-OH-DPAT, un agoniste sélectif des récepteurs 5-HT_{1A}. De plus, dans ces conditions, l'incorporation de la molécule radioactive est apparue significativement moindre dans la plupart des régions contenant le récepteur 5-HT_{1A}, incluant le principal regroupement de corps cellulaires neuronaux à 5-HT, le noyau raphé dorsalis. Cette diminution de l'efficacité de couplage aux protéines G des récepteurs 5-HT_{1A} en l'absence d'un changement de leur nombre pourrait constituer le premier indice d'une interaction locale, ou cross-talk, entre les sous-types réceptoriels 5-HT_{1A} et 5-HT_{1B}, du moins dans les régions cérébrales où ces deux récepteurs co-existent dans les mêmes neurones.

Qu'il s'agisse de conséquences directes ou de changements compensatoires ou adaptatifs, ces altérations de la structure et du fonctionnement des neurones à sérotonine du cerveau en l'absence constitutive des récepteurs 5-HT_{1A} ou 5-HT_{1B} contribuent, selon toute vraisemblance, aux anomalies de comportement observées chez les knockout respectifs.

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TO MY WIFE

Ana María

Chapter I

GENERAL INTRODUCTION

I.1 THE SEROTONIN (5-HT) NEUROTRANSMITTER

I.1.1 Historical aspects

The discovery of serotonin (5-hydroxytryptamine, 5-HT) can be traced as far back as 1868. Investigators knew that the serum of clotted blood contained a factor capable of producing vasoconstriction. The identity of this factor, however, remained unknown for many years. Beginning in the 1930s, Page and his colleagues were looking for vasoconstrictive substances that might be responsible for cardiovascular hypertension. They eventually isolated the serum factor and called it **serotonin** to indicate its origin from blood *serum* and its effects on vascular muscle *tone* (Rapport, 1948). Meanwhile, Erspamer had independently discovered a substance in gastric mucosa that likewise exerted a contractile effect on vascular and other smooth muscles (Erspamer, 1940). This substance was initially called **enteramine** because it was secreted by enterochromaffin cells of the gastrointestinal tract. Later, enteramine and serotonin were shown to be the same compound (Erspamer and Asero, 1952), now referred to exclusively as serotonin.

The discovery of 5-HT in the nervous system naturally raised the possibility that the compound might serve as neurotransmitter. With the demonstration, in the early sixties, of segregated groups of nerve cell bodies and presumptive axon terminals (varicosities) containing 5-HT in many regions of the rat brain (Dahltröm and Fuxe, 1964, Fuxe, 1965), the evidence

became incontrovertible. Nowadays, this molecule is considered to be an important modulator of chemical neurotransmission throughout the CNS.

I.1.2 Phylogenetic studies

As a neurotransmitter, the ubiquity of 5-HT is not only anatomical but also phylogenetic. This substance is present in virtually all vertebrates as well as in numerous invertebrate species such as sea anemones, planarians, leeches, lobsters, crabs, octopuses and squids (Essman, 1978). The discovery of 5-HT content in neurons of the cnidarian *Renilla koellikeri* (Umbriaco et al., 1990), could make it one of the most ancient of currently known transmitters. It is also found in many plants and fruits, particularly pineapples and bananas. From primates (Azmitia and Gannon, 1986; Törk, 1990) to *Chondrichtyes* (Stuesse and Cruse, 1992), the adult 5-HT system is organized into two subsystems, i.e., a rostral division with cell bodies located in the midbrain and rostral pons and providing projections to the forebrain, and a caudal division located primarily in the medulla oblongata, innervating the brainstem and sending descending projections to the spinal cord. A careful scrutiny of comparative anatomy nevertheless indicates that phylogenetic differences are as important as similarities. In higher mammals, it has been emphasized that the system has evolved to comprise some components with a fast, precise type of neurotransmission, in which 5-HT neurons give rise to few collaterals (Fallon and Loughlin, 1982), have a high proportion of myelinated axons and axon varicosities that are often endowed with synaptic membrane specializations. In lower mammals, the system is

mostly if not entirely diffuse, highly branched, unmyelinated, and with a predominance of nonjunctional innervation in terminal fields (Azmitia, 1986; Descarries et al., 1990; Jacobs and Azmitia, 1992).

I.1.3 Synthesis

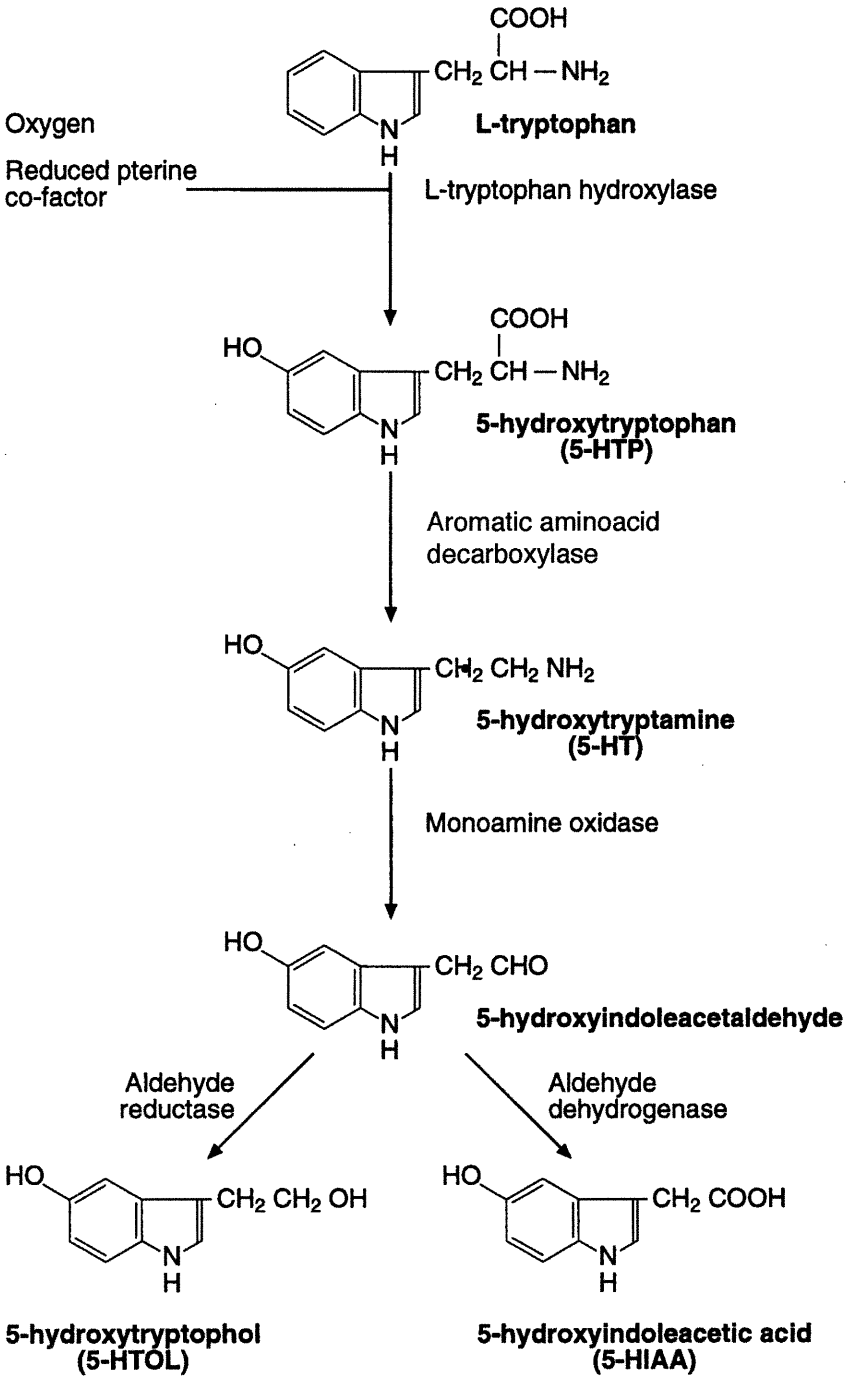
Serotonin is an indolealkylamine whose chemical structure is closely related to that of its precursor, the aminoacid tryptophan. The initial step in the synthesis of 5-HT is the active transport of L-tryptophan from blood into brain (Young and Sourkes, 1977). A rate-limiting step follows, the hydroxylation of L-tryptophan to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (Fig. 1). In the nervous system, this enzyme is exclusively located in the serotoninergic neurons, and may serve as an specific marker for such cells (Feldman et al., 1997). Electrical stimulation of serotoninergic neurons is known to increase tryptophan hydroxylase activity and *in vivo* conversion of tryptophan into 5-HTP (Boadler-Biber et al., 1986). This latter compound undergoes rapid decarboxylation by the enzyme aromatic aminoacid decarboxylase (AADC) to form 5-HT (see Fig. 1). AADC is present not only in serotoninergic but also in catecholaminergic neurons, in which the substrate is DOPA and the reaction products dopamine or noradrenaline.

Regulation of 5-HT synthesis by firing rate also operates in the opposite direction. Several studies have demonstrated that 8-OH-DPAT, a potent agonist at 5-HT_{1A} receptors, inhibits 5-HT synthesis, presumably due to effects on neuronal activity (Fernstrom et al., 1990; Invernizzi et al., 1991).

5-HT neurons are also sensitive to variations in plasmatic tryptophan, and thus dietary changes can regulate 5-HT levels in brain (Fernstrom and Wurtman, 1971). There is abundant evidence in several mammalian species, including human, that increased availability of tryptophan leads to an augmented 5-HT production (Diksic et al., 1991; Westerink and De Vries, 1991). Short-term requirements for increases in 5-HT synthesis are seemingly accomplished by a Ca^{2+}-dependent phosphorylation of tryptophan hydroxylase, changing its kinetic properties (V_{MAX}) without requiring synthesis of more enzyme molecules. In contrast, situations requiring long term increases in 5-HT availability appear to induce a change in tryptophan hydroxylase gene expression.

Figure 1

The biosynthesis and catabolism of 5-HT are depicted in this illustration (reviewed in McGeer et al., 1987). The first and the limiting step of 5-HT synthesis is the hydroxylation of the aminoacid tryptophan by the enzyme L-tryptophan hydroxylase. Follows the convertion of 5-HT to 5-HTP by the enzyme 5-hydroxytryptophan decarboxylase, or aromatic-aminoacid-decarboxylase. 5-HT is catabolized by the action of the enzyme monoamine oxidase, or MAO, to form a very unstable aldehyde, that finally gives rise to the main metabolite 5-HIAA, or 5-hydroxyindole-3-acetic acid. In addition, between 1 to 10% of 5-HT catabolism is the reductive pathway leading to 5-HTOL, or 5-hydroxytryptophol.


COOH
CH2 CH — NH2
N
H
L-tryptophan
Oxygen
Reduced pterine
co-factor
L-tryptophan hydroxylase
HO
5-hydroxytryptophan
(5-HTP)
Aromatic aminoacid
decarboxylase
CH2 CH2 NH2
5-hydroxytryptamine
(5-HT)
Monoamine oxidase
CH2 CHO
5-hydroxyindoleacetaldehyde
Aldehyde
reductase
Aldehyde
dehydrogenase
CH2 CH2 OH
5-hydroxytryptophol
(5-HTOL)
CH2 COOH
5-hydroxyindoleacetic acid
(5-HIAA)

I.1.4 Release

Although some 5-HT is present in the cytoplasm of serotoninergic cell bodies and nerve terminals, most of the neurotransmitter is stored in large dense-core (70-120 nm) or electron lucent (40-60 nm) vesicles (Maley et al., 1990). The mechanism of 5-HT release is generally assumed to be exocytosis, i.e., the discharge from cells of the content of individual vesicles (Jacobs and Azmitia, 1992). Evoked 5-HT release, measured either *in vitro* from brain slices or *in vivo* by means of microdialysis, is sensitive to the Na^+ channel blocker tetrodotoxin and to the presence of Ca^{2+} ions (Auerbach et al., 1989; Carboni et al., 1989; Sharp et al., 1989). The dependence on Ca^{+2} ions and sensitivity to tetrodoxin implies an exocytotic mechanism triggered by action potentials (Zigmond et al., 1999). In addition, some evidence indicates that substituted amphetamines (*p*-chloroamphetamine, or PCA, and fenfluoramine) may induce 5-HT release by a non-exocytotic mechanism (Carboni and Di Chiara, 1989). Consistent with this view, vesicular disruption by reserpine fails to prevent behavioral activation following systemic PCA treatment, despite 90% to 95% reductions in 5-HT concentrations in areas of 5-HT innervation. The mechanism of release might then be a transporter-mediated exchange, by which the membrane 5-HT uptake system would not only transport 5-HT into nerve cells but also carry it from the cytoplasm to the extracellular space (Rudnik and Wall, 1992; Gu and Azmitia, 1993). This would be consistent with earlier observations that

the action of 5-HT releasers can be prevented by pretreatment with 5-HT uptake blockers (Sanders-Bush and Martin, 1982).

I.1.5 Inactivation

Serotonin released into the extracellular space is inactivated primarily by **uptake** of the neurotransmitter through a plasma membrane 5-HT transporter (5-HTT). The uptake process exhibits saturability, a high affinity for 5-HT, Na^+ dependence and requires energy. The driving force for transport is the Na^+ gradient across the membrane. Uptake of 5-HT in the brain has been studied largely *in vitro*, using slice and synaptosomal preparations. The *K*m value for 5-HT is typically about 0.05-0.1 μM for synaptosomes, compared to 0.1-0.5 μM for slices. The 5-HTT has been cloned and sequenced from rat (Blakely et al., 1991) and human (Lesch et al., 1993) brain; it belongs to the same family of 12-transmembrane domain transporters as that described for catecholamines.

The efficiency with which the 5-HTT removes extracellular 5-HT is dependent on several factors, such as the density, subcellular localization and turnover rate of the transporter itself. These factors may vary between 5-HT nuclei, between cells within nuclei and perhaps even among the different projections from given 5-HT neurons. Figure 1 from Ase et al (2001) (Chapter III) illustrates the regional distribution in mouse brain of binding sites for the 5-HTT, labeled with [^{3}H]citalopram. Citalopram displays a high selectivity toward the 5-HTT and only a low affinity (in the μM concentration

range) toward 5-HT_{1A} receptors (for review, see Hyttel, 1994; Sanchez and Hyttel, 1999). At the concentration used in our experiments (2nM), there was no indication that it might label other sites than 5-HTT. Transmitter clearance from the extracellular space will in turn affect the number of 5-HT receptors activated and hence the strength of 5-HT signaling. The 5-HTT is also of particular interest because it is the site of action of a new class of antidepressant drugs collectively called selective serotonin reuptake inhibitors (SSRIs), among which fluoxetine (Prozac™), sertraline and paroxetine (Schloss and Williams, 1998) are the best known.

Another mechanism of 5-HT inactivation is *oxidative deamination*, a reaction mediated by monoamine oxidase (MAO), as shown in Fig. 1. The product of this reaction is 5-hydroxyindole acetic acid (5-HIAA), the primary 5-HT metabolite. 5-HIAA diffuses out of the nerve cells by a probenecid-sensitive acid transport mechanism in the choroid plexus and soon enters the cerebrospinal fluid. Two different MAO isoenzymes have been identified, MAO-A and MAO-B (Bach et al., 1988). Moreover, 5-HT can also be reduced, although in a lesser extent, leading to 5-hydroxytryptophol metabolite, or 5-HTOL. In vitro studies have indicated that the Km of rat brain MAO-A for 5-HT is approximately tenfold lower than that of MAO-B (Fowler and Ross, 1984). Thus arose the idea that 5-HT is preferentially metabolized by MAO-A compared with MAO-B. This was tested by demonstrating that extracellular 5-HIAA in the rat striatum declines after treatment with the selective MAO-A inhibitor clorgyline but not deprenyl (selective inhibitor of MAO-B) (Kato et

al., 1986). The problem with this view is that immunocytochemical studies have identified MAO-B but not MAO-A, in rat serotoninergic neurons of the rat (Willoughby et al., 1988), cat (Kitahama et al., 1986), monkey (Westlund et al., 1985) and human (Konradi et al., 1988). A more recent *in situ* hybridization study has however detected mRNA for MAO-A in the dorsal and medial raphe nuclei of rat (Jahng et al., 1997). Moreover, whereas MAO-B knockout mice do not show alterations in 5-HT metabolism, MAO-A knockout mice display increased levels of 5-HT and decreased levels of 5-HIAA throughout brain (Cases et al., 1995; Grimsby et al., 1997; see also Holschneider et al., 2001). This suggests an important role of MAO-A in 5-HT metabolism at least in mouse.

I.2 ANATOMICAL ORGANIZATION OF SEROTONIN PATHWAYS IN THE CNS

1.2.1 Regional distribution

The distribution of the 5-HT system, as originally described with the fluorescent histochemical method (Dahlsröm and Fuxe, 1964; Fuxe, 1965) consists of nine 5-HT-containing cell body groups (designated B1-B9), all located in the brainstem, and a widespread axonal arborization reaching into all parts of the brain and spinal cord. This method has now been supplanted by more sensitive techniques, such as autoradiography following uptake and storage of [^{3}H]5-HT and more recently by immunocytochemistry using primary antibodies against tryptophan hydroxylase, or 5-HT itself.

The serotoninergic system may be described as two subsystems, one caudal and the other rostral (e.g., Törk, 1990). The **caudal 5-HT subsystem** consists of the B1-B4 cell groups, which are located in the median and paramedian regions of the medulla oblongata and caudal pons. The axons of these cells descend to the spinal cord along several pathways. Those from the raphe magnus (B3) travel in the dorsolateral fasciculus and terminate in the dorsal horn gray matter, particularly in laminae I and II. A second descending projection from the nucleus raphe pallidus (B1) and the nucleus raphe obscurus (B2 and B4) terminates in the ventral horn containing the spinal cord motoneurons. Finally, 5-HT neurons from the rostral ventrolateral medulla (part of B3) innervate preganglionic sympathetic cells in the intermediolateral columns of the thoracic spinal cord. These pathways largely mediate the varied roles of 5-HT in sensory, motor and autonomic functions.

The **rostral 5-HT system** comprises the B5-B9 cell groups, which are associated with the raphe nuclei in rostral pons and mesencephalon, as well as with the caudal linear nucleus, the nucleus pontis oralis, and the supralemniscal region. Early studies showed that the dorsal and median raphe nuclei together account for about 80% of the forebrain serotoninergic terminals, and therefore represent the major source of the forebrain 5-HT innervation (Azmitia, 1978). The ascending pathway originates primarily from the B6-B8 cell groups and supplies projections to virtually the entire brain. 5-HT-containing fibers pass through the midbrain, where they innervate the

superior and inferior colliculus, substantia nigra, ventral tegmental area, and interpeduncular nucleus. A major part of this pathway enters the medial forebrain bundle, from which fibers branch off in several directions. Diencephalic projections terminate in the medial habenula and the various thalamic and hypothalamic nuclei. Other fibers innervate the dorsal and ventral neostriatum (the caudate-putamen and nucleus accumbens), limbic structures such as the amygdaloid complex, the hippocampal formation, the septal nuclei and olfactory turbercle, parts of the olfactory nucleus, and all regions of the neocortex.

Two additional 5-HT projection systems have been identified in the brain (Nieuwenhys, 1985). The first is a major pathway to the cerebellum that terminates in both cerebellar cortex and deep cerebellar nuclei. This projection receives contributions from a number of 5-HT cell groups, most notably from B2, B3 and B5. The second is a widespread system that innervates a number of structures in the pons and medulla oblongata. These structures include the locus coeruleus, the dorsal tegmental nucleus, the inferior olivary nucleus, the nucleus solitarious, the rhombencephalic reticular formation, as well as several cranial nerves nuclei including the nucleus of the trigeminal nerve. It is worth mentioning that connections between some of the raphe nuclei themselves have also been demonstrated (Mosko et al., 1977; Bobillier et al., 1979).

I.2.2 Synaptic versus diffuse transmission by serotonin neurons:

Views of synaptic transmission in the CNS are largely based on the model of the peripheral acetylcholine (ACh) neurotransmission at the neuromuscular junction. At this particular synapse, ACh is released from the vesicle containing axon terminals into an infolded synaptic cleft, where it interacts with receptors collected in regions of the muscle facing the release sites (active zone) and displaying postsynaptic membrane thickenings. The diffusion of ACh is restricted by rapid binding to receptor sites, a process known as **buffer diffusion** (Katz and Miledi, 1973), which also increases the probability of degradation of ACh by acetylcholinesterase (Bartol et al., 1991). In this way, chemical communication involving ACh is mostly restricted to the length of the synaptic cleft, even though there is indirect evidence that some ACh might diffuse further away from the junctions (see Descarries et al., 1997). Other CNS neurotransmitters, particularly the aminoacids glutamate and _-aminobutyric acid (GABA) have also been shown to interact primarily with receptors located on synaptic interfaces (Goda and Stevens, 1994; Borges et al., 1995; Geiger et al., 1997). In contrast, catecholamine dopamine can seemingly *escape* from the vicinity of the synaptic cleft (Garris et al., 1994) and interact with receptors and transporters located at more remote sites (Smiley et al., 1994; Nirenberg et al., 1996, 1997). Thus, the location of release sites as well as the affinities, binding kinetics, and location of receptors, transporters and degradative enzymes are important parameters that determine whether a certain form of

neurotransmission is restricted to the synaptic cleft, promoting hard-wired communication, or can occur in the *extrasynaptic space* (Clements, 1996), allowing for longer range, less specific interactions.

A number of morphological observations have led several authors to suggest the existence of a mode of neuronal communication other than synaptic transmission, which has been called **diffuse** or **volume transmission** (Descarries et al., 1991; Fuxe and Agnati, 1991; Agnati et al., 1995; Zoli et al., 1998). At the origin of this hypothesis was the demonstration of the lack of synaptic membrane differentiation (junctional complexes) on many [^{3}H]5-HT-labeled terminals in rat neocortex (Descarries et al., 1975; Beaudet and Descarries, 1978). This was then interpreted to suggest a non-synaptic release of 5-HT, and its possible action at a distance on a variety of cellular targets in a large sphere of influence, hence the term later coined of "volume" transmission (Descarries et al., 1990; 1991). Moreover, the varicose aspect of the monoaminergic fibers, as initially revealed by fluorescence histochemistry, turned out to represent axon varicosities or boutons en passant in the hundreds of thousands per single 5-HT nerve cell body, emphasizing the diffuseness of the potential effects of 5-HT. Admittedly, there are territories of 5-HT innervation in which all 5-HT varicosities form structurally defined synaptic specializations, such as the pars reticulata of substantia nigra (Moukhles et al., 1997). However, in most CNS regions where this morphological parameter of 5-HT neurons has thus far been examined, a majority of these terminals were found to be non

junctional and to co-exist with their less numerous synaptic counterparts (Beaudet and Descarries, 1981; Descarries et al., 1990; Maley et al., 1990; for a recent review, see Descarries and Mechawar, 2000). Moreover, recent immunoelectron microscopic studies of the 5-HTT (Zhou et al., 1998; see also Tao-Cheng and Zhou, 1999) and of several 5-HT_1 receptor subtypes (Riad et al., 2000) have shown that these targets for 5-HT are not located post-synaptically *strictu sensu*, at sites of postsynaptic membrane specialization, but rather distributed extrasynaptically on the plasma membrane of neuronal somata, dendrites, axons or axon terminals (varicosities), as expected from sites reached by diffusion of the transmitter. More recently, it has been shown by fast-scan cyclic voltametry (Bunin and Wightman, 1999) that 5-HT is able to enter the extracellular space at rates governed by diffusion and at extracellular concentrations closely matching the affinity for the predominant 5-HT receptors in each region. Thus, diffuse transmission is now generally regarded as a predominating complement to classical synaptic transmission in the case of the central 5-HT system.

I.3 SEROTONIN RECEPTORS AND THEIR IMPLICATION IN BEHAVIOR

I.3.1 Early studies

The first suggestion for the existence of multiple 5-HT receptors came from the work of Gadum and Picarelli (1957), who were studying the contractile effect of 5-HT on the isolated guinea pig ileum. They observed that this response was partially antagonized by either morphine or

phenoxybenzamine (Dibenzyline) given alone. Moreover, the effect of 5-HT could be completely blocked by combining appropriate doses of the two drugs. Gadum and Picarelli proposed that their results were due to the existence of two different 5-HT receptors, which they named the M (Morphine) and the D (Dibenzyline) receptors. However, this classification system turned out to be inappropriate for 5-HT receptors in the brain. In 1979, Peroutka and Snyder published a landmark paper suggesting the existence of two receptors in brain tissue, based on binding studies with [^{3}H]5-HT, [^{3}H]LSD and [^{3}H]spiperone as radioligands. They designated as S1 (5-HT$_1$) the receptor with a greater affinity for [^{3}H]5-HT, and S2 (5-HT$_2$) the one with high affinity for [^{3}H]spiperone. In fact, the 5-HT$_2$ sites seemed to correlate quite well with the "D" receptor, and certain neuroleptics displayed high affinity for this site (Leysen et al., 1978).

The 5-HT$_1$ binding site was a new receptor type and proved to be heterogeneous (Pedigo et al., 1981; Pazos et al., 1984). For example, binding of [^{3}H]5-HT to 5-HT$_1$ receptors was displaced by spiperone in a biphasic manner, suggesting that what had been called the 5-HT$_1$ receptor might be a heterogeneous population of receptors. The [^{3}H]5-HT binding site showing a high affinity for spiperone was then termed the 5-HT$_{1A}$ subtype, whereas the component of the [^{3}H]5-HT binding showing low affinity for spiperone was designated as the 5-HT$_{1B}$ subtype. Neither of these binding sites corresponded to the "M" receptor, which in order to retain the 5-HTx system of nomenclature, was called the 5-HT$_3$ receptor (Humphrey et al., 1993).

Subsequently, additional 5-HT receptor subtypes were discovered and classified as follows, according to their sequence homology and intracellular signaling pathways.

I.3.2 Classification

The application of molecular biology techniques has had a major impact on the 5-HT field, allowing the discovery of many additional 5-HT receptors. The current classification system recognized by the International Union of Pharmacology (IUPHAR) includes seven families of 5-HT receptors, 5-HT_{1-7} (see Table 1), comprising a total of fourteen structurally and pharmacologically distinct mammalian 5-HT receptor subtypes (reviewed by Hoyer and Martin, 1997; Barnes and Sharp, 1999). At the molecular level, it has been established that the 5-HT receptor family mostly comprises G-protein-coupled metabotropic receptors with seven putative transmembrane spanning domains, but one member of the family, the 5-HT_3 receptor, is a ligand-gated ion channel.

The receptors belonging to the **5-HT_1 family** (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F}) show a high aminoacid sequence homology, nanomolar affinity for the natural ligand 5-HT, and are negatively coupled to adenylate cyclase. The 5-HT_2 family comprises receptors that stimulate phospholipase C (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}). The heterogeneous group, which stimulates adenylate cyclase, includes 5-HT_4, 5-HT_6 and 5-HT_7 receptors. The 5-HT_5 receptor family includes 5-HT_{5A} and 5-HT_{5B} receptors. The 5-HT_{5A} receptor was recently found to inhibit adenylate cyclase (Francken et al., 1998).

Table 1.

Molecular cloning studies have revealed the existence of 14 different genes, each encoding a distinct 5-HT receptor subtypes (Hoyer et al., 2002). With the exception of the 5-HT_3 receptor, a ligand-gated ion channel, all 5-HT receptors belong to the superfamily of G-protein coupled receptors containing a predicted seven-transmembrane domain structure. The high sequence homology of 5-HT_1 receptor subtypes, coded by intronless genes, is presumably the result of gene duplication during evolution. One single receptor subtype from the 5-HT_1 family may govern multiple efector pathways (Albert and Tiberi, 2001). An even higher level of diversity exists because isoforms have been identified for several of these receptor subtypes (Pauwels, 2000). Up to seven distinct 5-HT_{2C} receptor isoforms were described to be generated by posttranscriptional RNA editing (Burns et al., 1997; Fitzgerald et al., 1999). Many isoforms (denoted with small subscript letters) produced by alterantive splicing, have been identified for the 5-HT_4 and 5-HT_7 receptors (Blondel et al., 1998; Hamblin et al., 1998; Vanhoenacker et al., 2000). Posttranslational events such as, oligomerization and heteromerization (Davies et al., 1999; Xie et al., 1999), multiple G-protein receptor trafficking, as well as cross-talk (Berg and Clarke, 2001) within and possibly between receptor families, contribute considerably the diversity of 5-HT receptor signaling mechanisms.

Current Classification of G-Protein Coupled 5-HT Receptors

Receptor family	Subtypes	Coupling signal	Effector pathways
5-HT$_1$	5-HT$_{1A}$ 5-HT$_{1B}$ 5-HT$_{1D}$ 5-HT$_{1E}$ 5-HT$_{1F}$	Gi/Go	↓cAMP ↑gK ↓gCa ↑PI
5-HT$_2$	5-HT$_{2A}$ 5-HT$_{2B}$ 5-HT$_{2C}$	Gq	↑PLC
5-HT$_3$	5-HT$_{3A}$ 5-HT$_{3B}$ 5-HT$_{3C}$	Channel	Na^+/K^+
5-HT$_4$	5-HT$_{4a}$ 5-HT$_{4b}$ 5-HT$_{4c}$ 5-HT$_{4d}$	Gs	↑cAMP
5-HT$_5$	5-HT$_{5A}$ 5-HT$_{5B}$	Gi	↓cAMP
5-HT$_6$	5-HT$_6$	Gs	↑cAMP
5-HT$_7$	5-HT$_{7a}$ 5-HT$_{7b}$ 5-HT$_{7c}$ 5-HT$_{7d}$	Gs	↑cAMP

I.3.3 SIGNALING BY G-PROTEIN-COUPLED RECEPTORS

I.3.3.1 The many facets of G-protein signaling

A large number of neurotransmitters and hormones exert their effects on cells and organisms by binding to G-protein-coupled receptors (GPCRs). **G-proteins** transduce ligand binding to these receptors into intracellular signals, which underlie physiological responses of tissues and organisms. The nature and amount of G-protein in cells are important in determining the specificity and temporal characteristics of their responses to signals. G-proteins are heterotrimers made up of α, β and γ subunits, and although there are many gene products encoding each subunit (20 α, 6 β and 12 γ), four main classes of G-proteins have been distinguished: Gs, which activates adenylyl cyclase; Gq, which activates phospholipase C; Gi, which inhibits adenylyl cyclase; and G12 and G13, of unknown function. GPCRs have a common body plan of seven transmembrane helices; the intracellular loops that connect these helices form the G-protein-binding domain.

G-proteins are inactive in the GDP-bound, heterotrimeric state and are activated by receptor-catalyzed guanine nucleotide exchange resulting in GTP binding to the α subunit. GTP binding leads to the dissociation of $G\alpha\cdot GTP$ from the $G\beta\gamma$ subunits and to activation of downstream effectors by both the $G\alpha\cdot GTP$ and $G\beta\gamma$ subunits. G-protein deactivation is rate-limiting for turnoff of the cellular response and occurs when the $G\alpha$ subunit hydrolyzes GTP to GDP. It is known that multiple receptors can converge onto a single

G-protein, and, in many cases, a single receptor can activate more than one G-protein, and thus modulate multiple intracellular signals. In other cases, it seems that interaction of a single receptor with a given G-protein is regulated by a high degree of selectivity imparted by specific heterotrimers (reviewed by Bourne, 1997; Gudermann et al., 1997; Hamm; 1998; Wess, 1998). Although the fourteen different 5-HT receptor subtypes might be regarded as providing sufficient diversity to accommodate the wide-ranging physiological roles of 5-HT, it appears that for this and for other neurotransmitters, this is still far too restrictive (Martin et al., 1998).

In characterizing the processes and events mediated by different receptor subtypes, more or less specific pharmacological agonists and antagonists are commonly used. Whereas it is well established that different agonists do not necessarily elicit responses of the same magnitude, it is likely that they select among various signal transduction pathways (Kenakin, 1995). A widely accepted model to describe the activation of GPCRs by agonists is the ternary complex model, which accounts for the cooperative interactions between agonists, receptor and G-protein (De Lean et al., 1980). This model has been recently extended to accommodate the observation that many receptors can activate G-proteins in the absence of agonists (i.e., constitutive Gi dependent activity of the 5-HT_{1A} receptor; Albert et al., 1999, Albert and Tiberi, 2001), and that mutations in different structural domains of receptor can enhance this *constitutive*, or agonist independent activity (Samama et al., 1993; Chidiac et al., 1994). The extended ternary complex

model also accounts for the effects of different classes of ligands (full agonists, partial agonists, silent neutral antagonists) on receptor signaling (Gether and Kobilka, 1998). Pure silent neutral antagonists (with intrinsic activation close to zero) are probably rare; many are actually inverse full agonists or partial agonists. Ligands may demonstrate distinct pharmacological properties depending on which receptor G-protein effector pathway is involved (Pauwels et al., 2000). The concept that GPCRs can couple to different G-protein-effector pathways receives further support from receptor mutagenesis studies. A conserved threonine residue (Thr^{149}) in the second intracellular loop of the 5-HT_{1A} receptor is directly involved in $\beta\gamma$-mediated coupling to Ca^{2+} channels (via $G\alpha o$) and to phospholipase C (via $G\alpha 12$), but plays a minor role in the coupling to $G\alpha i$-mediated inhibition of cyclic AMP accumulation (Lembo et al., 1997). These data suggest that the pharmacological profile of a receptor subtype may be codetermined by the effector pathway that is being considered. **Pathway-dependent constitutive receptor activity** may result from differences in the coupling efficiency between a receptor and its effector pathways, and/or there may be multiple active conformational states of the receptor, each of which has its own level of constitutive activity and couples to an effector pathway. Thus, agonists would preferentially induce or select receptor conformational states that favor activation of one effector pathway over another (Gettys et al., 1994). In the case of 5-HT_{1A} receptors, constitutive receptor activity is apparent toward both $G\alpha i1$ and $G\alpha i3$ proteins (Kellett et al., 1999; Dupuis et al., 2001; see

also Albert and Tiberi, 2001). Thus, partial agonists could be less efficacious than full agonists because they are unable to induce the optimal conformational change in the receptor that regulates contact with the same set of G-proteins.

I.3.3.2 G-protein-coupled receptor kinases

Several mechanisms may regulate the length and strength of GPCR signals. In many cases, a time dependent decrease of the cellular response to the external stimulus occurs despite the continued presence of the signaling agent. This attenuation of signaling is known as **desensitization**. In essence, desensitization of a G-protein-coupled signal can be achieved due to attenuation of signaling at the level of the receptor, the G-protein or the effector system. Proteins considered as the regulators of G-protein signaling may accelerate the hydrolysis of Gα-bound GTP and thus promote deactivation of G-proteins (Berman and Gilman, 1998; Bünemann and Hosey, 1998). Multiple mechanism of receptor desensitization exists, but not all are understood.

Agonist-dependent phosphorylation of GPCRs appears to play an important role in initiating the desensitization of many receptors, and a unique family of protein kinases known as G-protein-coupled receptor kinases, or **GRK**, seem to play a key role in initiating homologous desensitization, i.e.: the situation whereby only activated GPCRs are desensitized. A general model to describe GRK-dependent desensitization was recently developed (see also Bünemann and Hosey, 1999). After

activation of GPCRs by their respective agonists, GRKs specifically phosphorylate the agonist-activated receptors, leaving the non-phosphorylated receptors unaffected. The desensitization is thought to be achieved when 'uncoupling' proteins such as the arrestins (Krupnick and Benovic, 1998) bind to the GRK-phophorylated receptors and cause receptor G-protein-uncoupling by preventing further interactions of the phosphorylated GPCRs with G-proteins.

A second event associated with receptor desensitization is **internalization** of GPCRs from the plasma membrane. Multiple pathways of internalization appear to exist. The best understood are GRK and arrestin dependent. For example, arrestin 2 and 3 bind to clathrin and act as adaptors to facilitate the clathrin-mediated endocytosis of select GPCRs (Ferguson et al., 1996; Goodman et al., 1996). Different consequences of internalization have been reported, depending on the GPCR being studied and the cellular background. In certain cases, such as with the M2 muscarinic acetylcholine receptors (mAChRs) studied in HEK cells, internalization and receptor G-protein-uncoupling have been dissociated and in these cells internalization does not cause desensitization. However, in Chinese hamster ovary (CHO) cells, other studies with M2 mAChRs have shown that internalization is necessary for receptor G-protein uncoupling (Tsuga et al., 1998). For the M4 mAChR, internalization has been suggested to prolong its desensitization (Bogatkewitsch et al., 1996). In marked contrast, internalization of the β2 adrenergic receptor appears to allow for

dephosphorylation, resensitization and recycling to the plasma membrane (Krueger et al., 1997). However, not all of the internalized β2 adrenergic receptors may undergo the recycling process, but rather some may undergo degradation in lysosomes, i.e., a form of down-regulation (Gagnon et al., 1998). The events that allow for the decision to recycle *versus* destroy are poorly understood.

I.3.4 THE 5-HT$_{1A}$ SEROTONIN RECEPTOR SUBTYPE

I.3.4.1 Biomolecular characterization

The **5-HT$_{1A}$ receptor** was the first 5-HT receptor to be fully sequenced. The genomic DNA encoding the human was cloned by Kobilka et al. (1987) and it was later shown to encode for a functional 5-HT$_{1A}$ receptor. The receptor gene is intronless with the typical structure of a protein with seven transmembrane spanning domains, and sites for glycosylation, phosphorylation and presumably palmitoylation. The rat 5-HT$_{1A}$ receptor was also cloned and fully sequenced (Albert et al., 1990), and displays a 89% homology with the human 5-HT$_{1A}$ receptor. Interestingly, Northern blot analysis of various brain regions with a rat cDNA probe have detected the presence of three RNA species of 3.9, 3.6, 3.3 kilobases (Albert et al., 1990).

I.3.4.2 Distribution and subcellular localization

The distribution of the 5-HT$_{1A}$ receptor has been extensively mapped by autoradiography in rat and human brain, using a variety of ligands (Pazos

and Palacios, 1985; Hoyer et al., 1986; Radja et al., 1991; Khawaja, 1995; Kung et al., 1995). Figure 3A,C in Chapter 2 illustrates the regional distribution and density of binding sites for the 5-HT_{1A} receptor in a wild-type 129/Sv strain mouse as mapped with [^{3}H]8-OH-DPAT (for quantitative data, see Ase et al., 2001). The number of 5-HT_{1A} binding sites was found to be high in limbic areas, notably, hippocampus, lateral septum, cortical areas (particularly cingulate and entorhinal cortex), and also in the mesencephalic dorsal and median raphe nuclei.

The distribution of mRNA encoding the 5-HT_{1A} receptor is almost identical to that of the 5-HT_{1A} binding sites (Albert et al., 1990; Chalmers and Watson, 1991; Miquel, et al., 1991; Pompeiano et al., 1992). It is clear that this receptor (5-HT_{1A} heteroreceptor) is located both postsynaptic to 5-HT neurons but also on the 5-HT neurons themselves (5-HT_{1A} autoreceptor) at the level of their soma and dendrites in the raphe nuclei. This is evident from studies of the effect of neuronal lesions on the abundance of 5-HT_{1A} binding sites and mRNA, and studies of the cellular localization of this receptor using light and electron immunocytochemistry (Miquel et al., 1991, 1992; Radja et al., 1991; Kia et al., 1996a,b; Riad et al., 2000). As a heteroreceptor, the 5-HT_{1A} is present on cortical pyramidal neurons, pyramidal and granular neurons of hippocampus (Pompeiano et al., 1992; Burnet et al., 1995), as well as on septal cholinergic neurons (Kia et al., 1996c).

The recent immunoelectron-microscopic study by Riad et al. (2000) has formally demonstrated the predominant plasma membrane localization of the 5-HT_{1A} receptor in the soma/dendrites of both 5-HT neurons in the dorsal raphe nucleus (autoreceptors) and the pyramidal neurons (non 5-HT) in hippocampus (heteroreceptors) (see also Riad et al., 2001).

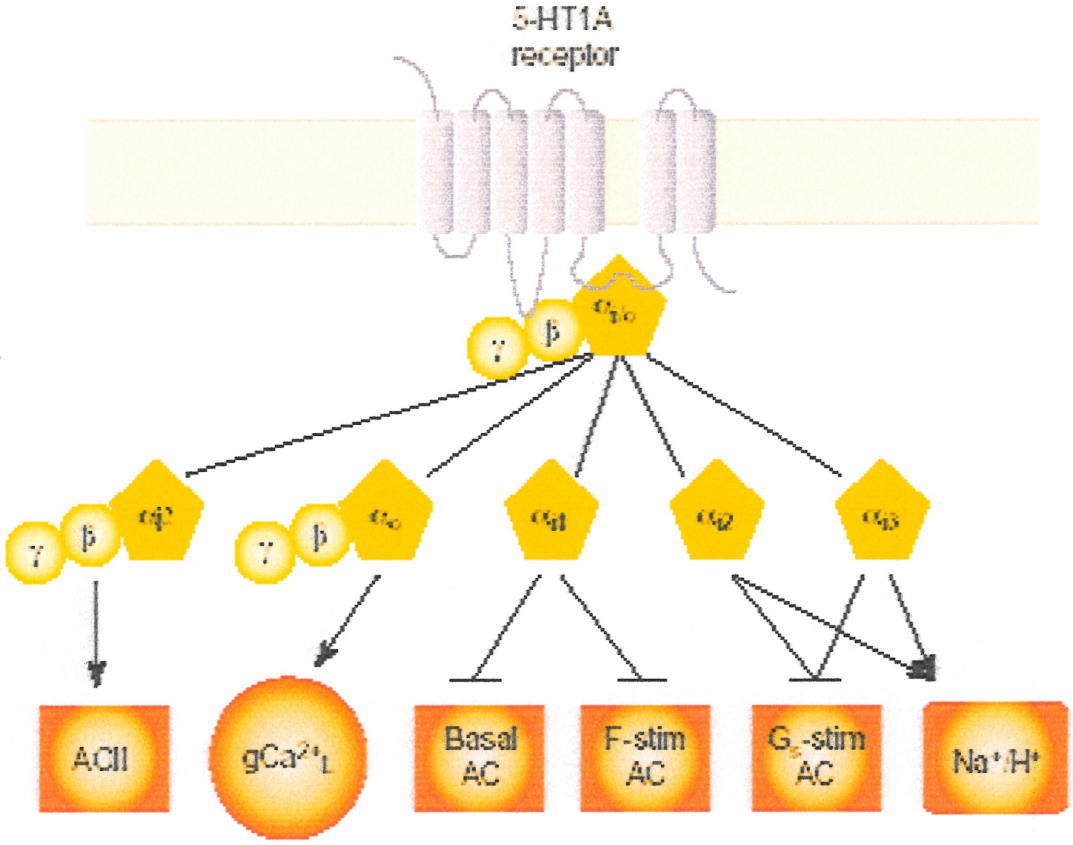
I.3.4.3 G-protein signaling

The 5-HT_{1A} receptor couples negatively via G-proteins (a_i) to adenylate cyclase in both rat and guinea pig hippocampal tissue and cell lines (pituitary GH4Cl cells, COS-7 cells, HeLa cells) stably expressing the cloned 5-HT_{1A} receptor (for review see Saudou and Hen, 1994; Albert et al., 1996; Raymond et al., 1999). Interestingly, despite the high density of 5-HT_{1A} binding sites in the dorsal raphe, 5-HT_{1A} receptors do not appear to result in inhibition of adenylate cyclase in this region (Clarke et al., 1996). Electrophysiological experiments have established that 5-HT_{1A} receptor activation causes neuronal hyperpolarization through the G-protein-coupled opening of K^+ channels, but without the involvement of diffusible intracellular messengers such as cAMP (reviewed by Aghajanian, 1995). Other effects of the 5-HT_{1A} receptor in transfected cell-lines (see Fig. 2) include a decrease in intracellular Ca^{+2}, activation of phospholipase C and increased intracellular Ca^{+2} (for review see Boess and Martin, 1994; Albert et al., 1996). As yet, however, there is no firm evidence for such coupling in brain tissue. Experiments utilising antisense constructs targeted against specific G-proteins subunits (Albert and Morris, 1994) suggest that the

biochemical basis for these diverse effects of the 5-HT_{1A} receptor may depend on the G-protein complement of the particular cells under investigation, but also on the particular isoforms of the effector enzymes that are being expressed (Albert et al., 1996).

Figure 2

The 5-HT_{1A} receptor-signaling pathways are depicted in this illustration (reviewed in Albert and Tiberi, 2001). The 5-HT_{1A} receptor gene expressed in heterologous systems shows a higher degree of G-protein specificity for coupling to effectors. Note that Gαi1 entails inhibition of basal and forskolin-stimulated AC, whereas Gαi2 and Gαi3 inhibit Gs-stimulated AC. Also, Gαi2 and Gαi3 have been shown to mediate 5-HT1A induced Na+–proton exchange. Finally, on cotransfection with Gαi2 and ACII, the 5-HT1A receptor appears to constitutively activate ACII by the release of Gβγ subunits from Gαi2 and a weak coupling of the receptor to Gs. Arrows indicate positive regulation; bars indicate negative regulation. Abbreviations: AC, adenylyl cyclase; gCa2+ L, L-type Ca2+ channel; F-stim, forskolin stimulated; Gs-stim, Gs-stimulated; Na+/H+, sodium–proton exchanger.


5-HT1A
receptor
γ
β
ACII
gCa2+L
Basal
AC
F-stim
AC
Gs-stim
AC
Na+/H+

I.3.4.4 Physiological role(s)

The 5-HT_{1A} receptor is probably the best characterized among all 5-HT receptor subtypes (Hamon et al., 1990). Electrophysiological experiments have established that 5-HT_{1A} receptor activation causes neuronal hyperpolarization, an effect mediated through the G-protein-coupled opening of K^+ channels, and without the involvement of diffusible intracellular messengers such as cAMP (for review see Aghajanian, 1995; Barnes and Sharp, 1999). Of the synthetic 5-HT_{1A} agonists, the compound lysergic acid diethylamide (LSD) and 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) are the most effective in producing suppression of firing activity, not only of dorsal raphe nucleus 5-HT neurons but also of non 5-HT neurons in hippocampus (CA1-3 pyramidal neurons), septum and frontal cortex (Aghajanian et al., 1972; Andrade and Nicoll, 1987; Ashby et al., 1994; Sprose and Aghajanian, 1988; Van den Hoff and Galvan, 1992; Corradetti et al., 1996).

Several compounds previously believed to act as 5-HT_{1A} receptor antagonists at postsynaptic sites have been shown to produce submaximal agonistic effects at presynaptic sites. For example, drugs like WAY 100135, NAN-190 or BMY 7378 block the inhibition induced by 5-HT agonists (Rydelek-Fitzgerald et al., 1990; Sharp et al., 1990; Routledge et al., 1993; Escandon et al., 1994) but they also decrease 5-HT neuron firing activity (Fletcher et al., 1993; Haddjeri and Blier, 1995). Because WAY 100635 has been consistently shown to block the effect of 5-HT_{1A} agonists without

affecting 5-HT neuron firing (Craven et al., 1994; Mundey et al., 1994) it has been designated as a 'silent' antagonist; however, other studies have also demonstrated a dose-dependent increased of 5-HT neuron firing when WAY 100635 (Fornal et al., 1994a,b; Corradetti et al., 1996; Fletcher et al., 1996) or p-MPPI (Bjorvatn et al., 1998) are given alone. Therefore, the 5-HT_{1A} autoreceptors appears to be under some *physiological tone*, at least in some conditions.

I.3.4.5 Behavioral role(s)

Pharmacologically, the 5-HT_{1A} receptor is the best-characterized member of the 5-HT_1 family because of the availability of several selective agonists. These drugs include the tetralin compound 8-OH-DPAT, as well as the azapirone derivates, buspirone, ipsapirone and gepirone. Systemic administration of these compounds to laboratory animals leads to hyperphagia, hypothermia and various behavioral effects that are considered to be predictive of anxiolytic action in human. Another consequence of 5-HT_{1A} agonist administration is the so-called '5-HT syndrome'. Studies in the 1970s described a specific constellation of behavioral effects produced in rats given a 5-HT precursor such as L-tryptophan (with a MAO inhibitor) or 5-hydroxytryptophan. This syndrome typically consisted of (1) resting tremor, (2) muscular hypertonicity or rigidity, (3) reciprocal treading of the forepaws, (4) hindlimb abduction, (5) lateral head weaving and other components such as low body posture, head shaking, hyperactivity and salivation (Jacobs, 1976). There is evidence that a human analogue, with symptoms such as

myoclonus (repetitive muscle spasms), tremor, ataxia, akathisia (extreme restlessness), hyperreflexia, diaphoresis (sweating) and delirium, may occur following administration of an overdose of a 5-HT uptake inhibitor and/or a MAO inhibitor (review by Lejoyeux et al., 1994). It is interesting to note that while 8-OH-DPAT (full agonist) elicits the full 5-HT syndrome, buspirone (partial agonist) and other azapirones given alone elicit only a few components of the syndrome, notably low body posture and hindlimb abduction.

I.3.4.6 Psychiatric disorders associated with the 5-HT$_{1A}$ receptor

Much of the interest in this receptor is related to its possible involvement in the pathogenesis and treatment of anxiety and depression. Indeed, 5-HT$_{1A}$ receptor partial agonists, such as **buspirone** (Buspar™) and its congeners gepirone and ipsapirone, have been demonstrated to have anxiolytic effects. Clinical data indicated that buspirone possessed equal efficacy as diazepam and other benzodiazepines in patients with anxiety (Schuckit 1984; Goa and Ward, 1986). Compared to benzodiazepines, buspirone is free of sedative effect, psychomotor impairment, and does not lead to dependence; but unfortunately, it takes one to four weeks of treatment before clinical anxiolytic improvement is felt. Binding experiments revealed that buspirone and its congeners have no significant affinity for benzodiazepine receptors; rather, they bind to 5-HT$_{1A}$ and D_2 dopamine receptors. In fact, buspirone was initially considered as a potential antipsychotic agent because, like many other neuroleptics, it shows affinity

for D_2 receptors. However, clinical studies have revealed its limited value in psychotic patients. The anxiolytic effects of buspirone could be due to its capacity to inhibit **the spontaneous firing of 5-HT neurons in the raphe nuclei**. In fact, a common effect of benzodiazepines and of the 5-HT_{1A} receptor-related anxiolytics is to inhibit serotoninergic impulse flow.

The classical hypothesis of a **serotoninergic fear/anxiety mechanism** proposes that, in anxiogenic situations, 5-HT neurotransmission increases, whereas a reduction of this transmission is anxiolytic-like in different animal models (for review see Graeff, 1990). The activation of 5-HT_{1A} autoreceptors has been demonstrated to produce anxiolytic effects in several behavioral paradigms in animal studies (Schreiber and De Vry, 1993; Hogg et al., 1994; Jolas et al., 1995; File et al., 1996). The direct administration of 5-HT_{1A} agonists into the dorsal raphe nucleus has been shown to be anxiolytic in the social interaction test (Hogg et al., 1994; Picazo et al., 1995), the plus maze (File and Gonzalez, 1996) and the ultrasonic vocalization fear/anxiety test (Remy et al., 1996; Schreiber and De Vry, 1993). Interestingly, a very recent report has indicated that the administration of 8-OH-DPAT into the dorsal raphe nucleus and the dorsal hippocampus results in anxiolytic and anxiogenic effects, respectively, when assessed by a modified version of the light-dark transitions test (Romaniuk et al., 2001).

Blockade of the 5-HT_{1A} somatodendritic autoreceptors in rodents potentiates the increase in extracellular 5-HT found after administration of

SSRIs. It has therefore proposed that desensitization of the 5-HT_{1A} autoreceptor may underlie the ability of chronic, but not acute SSRI administration, to raise synaptic cleft levels of 5-HT (Piñeyro and Blier, 1999). Co-administration of the 5-HT_{1A} receptor antagonist pindolol is also known to enhance the therapeutic efficacy and thus to shorten the onset of action of SSRIs in patients suffering from depression (Blier and de Montigny, 1994; for review, see also Piñeyro and Blier, 1999).

Studies on the eventual modulations of 5-HT_{1A} receptor function following long-term treatment with 5-HT_{1A} ligands are of considerable clinical interest because of observations that the anxiolytic/antidepressant effects of buspirone, gepirone and ipsapirone become significant only after 2 to 3 weeks of treatment in human (Cott et al., 1988; Robinson et al., 1990). Acute and short-term administrations of 5-HT_{1A} receptor agonists produce a suppression of firing of 5-HT neurons, which results in decreased 5-HT release in projection areas (de Montigny et al., 1984; Sprouse and Aghajanian, 1987; Sharp et al., 1989). However, during sustained treatment, the activity of 5-HT neurons progressively returns to normal as a result of a desensitization of somatodendritic 5-HT_{1A} autoreceptors (Blier and de Montigny, 1990). The gradual recovery of 5-HT neurons activity is consistent with the delayed onset of clinical action of these drugs. Interestingly, the 5-HT_{1A} heteroreceptors in the hippocampus do not desensitize as do their autoreceptor congeners (review in Blier and de Montigny, 1994).

.3.5 THE 5-HT$_{1B}$ SEROTONIN RECEPTOR SUBTYPE

I.3.5.1 Biomolecular characterization

The 5-HT$_{1B}$ receptor gene was also cloned from mouse brain (Maroteaux et al., 1992). This gene is located on mouse chromosome 9 and on human chromosome 6 (Demchyshyn et al., 1992; Jin et al., 1992, Simon-Chazottes et al., 1993). As is the case of the 5-HT$_{1A}$ receptor subtype, there are no introns in its coding sequence. The human 5-HT$_{1B}$ receptor contains a carboxyterminal cysteine that has been implicated as a site for palmitoylation, while the closely related human 5-HT$_{1D}$ receptor lacks this residue (Demchyshyn et al., 1992). Northern blot analysis has shown two RNA transcripts in human brain, which may indicate the use of alternative transcription start sites (Jin et al., 1992).

Because the pharmacology of the **5-HT$_{1B}$ receptor** shows significant differences across species, prefixes are used to denote species specific 5-HT$_{1B}$ receptors: the rat and mouse (rodent) are designated r5-HT$_{1B}$ and the human h5-HT$_{1B}$. The r5-HT$_{1B}$ receptor has been found to have high degree of homology (91.5%) with the human 5-HT$_{1D}$ receptor (Jin et al., 1992). Replacement of the threonine 355 residue in the human 5-HT$_{1B}$ receptor by an asparagine converts the high affinity of the h5-HT$_{1B}$ for pindolol-like-molecules to that of the r5-HT$_{1B}$ receptor (Metcalf et al., 1992; Oksenberg et al., 1992; Parker et al., 1993). These studies indicate that the marked differences in pharmacological profile between the human and rodent 5-HT$_{1B}$ receptors are due to this one aminoacid only. After a recent realignment,

5-HT_{1D} receptors whether expressed in rat, human or other species will merely be called 5-HT_{1D} receptors. It is important to note that these latter receptors are expressed in very low amount in brain. Advice on prefix nomenclature for other species is given by Vanhoutte et al (1996). The nomenclature for the 5-$HT_{1B/1D}$ receptor subtypes used in this thesis conforms to the new guidelines recently adopted by the International Nomenclature Committee, or IUPHAR (see also Hoyer and Martin, 1997).

I.3.5.2 Distribution and subcellular localization

Thus far, receptor autoradiography for the 5-HT_{1B} subtype has been hampered by the lack of specific radioligands for distinguishing between the 5-HT_{1B} and other 5-HT_1-like receptor subtypes. By using non-specific radioligands (i.e., [^{3}H]5-HT, [^{3}H]5-CT, [^{125}I]cyanopindolol and [^{125}I]GTI) in the presence of cold ligands to prevent labeling of unwanted receptors, the density of binding sites for 5-HT_{1B} receptors was demonstrated to be high in the basal ganglia (ventral pallidum, substantia nigra and globus pallidus), deep cerebellar nuclei, subiculum and superior colliculus (Hoyer et al., 1985; Pazos and Palacios, 1985; Boulenguez et al., 1991; Segu et al., 1991; Boschert et al., 1994; Bruinvels et al., 1993; Laporte et al., 1994). Figure 3E,G in Chapter 2 illustrates the distribution and density of these binding sites in a wild-type 129/Sv strain mouse (for quantitative data, see also Ase et al., 2001).

In situ hybridization studies in mouse and rat have revealed the brain distribution of 5-HT_{1B} receptor mRNA (Voigt et al., 1991; Jin et al., 1992;

Maroteaux et al., 1992; Boschert et al., 1994; Bruinvels et al., 1994; Doucet et al., 1995). Hybridization signals were detected in the striatum, cerebellum, hippocampal formation, entorhinal and cingulate cortices, dorsal raphe nucleus, spinal cord and retinal ganglion cells. A characteristic of the regional distribution of the 5-HT_{1B} receptor is the mismatch between the localization of the mRNA and 5-HT_{1B} binding sites. A high 5-HT_{1B} receptor mRNA level is found in the medium spiny neurons of the caudate-putamen, which are known to project to the globus pallidus and substantia nigra. In contrast, no such mRNA is expressed in the globus pallidus and substantia nigra, although these structures exhibit the highest level of 5-HT_{1B} binding sites. It has therefore been concluded that 5-HT_{1B} receptors are localized predominantly on axon terminals (Boschert et al., 1994; Doucet et al., 1995). These findings are in line with post-mortem studies of brain from patients diagnosed to have suffered Huntington's disease, and that exhibit degeneration of striatal neurons, i.e., the density of 5-HT_{1B} receptors was reduced in the substantia nigra (Waeber and Palacios, 1989). Recent observations with specific antibodies raised for the 5-HT_{1B} receptor at the electron microscopic level revealed staining of fine unmyelinated preterminal axons in the substantia nigra (Sari et al., 1997, 1999; Riad et al., 2000). Moreover, the immunoelectron-microscopic observations after gold immunolabeling of the 5-HT_{1B} receptor in rat brain (Riad et al., 2000) have demonstrated its predominant localization on the plasma membrane of fine, unmyelinated, preterminal axons, rather than on the axon terminals

(varicosities) themselves, in both the globus pallidus and substantia nigra. These data have been interpreted to suggest a capacity of this receptor to mediate 5-HT effects on neuronal conduction rather than on 5-HT release.

I.3.5.3 G-protein signaling

Inhibition of adenylate cyclase following activation of 5-HT_{1B} receptors activity was first demonstrated in membrane preparations of calf and rat substantia nigra, which contain a high density of 5-HT_{1B} binding sites (Bouhelal et al., 1988; Schoeffter et al., 1988). Studies on mammalian cells expressing recombinant 5-HT_{1B} receptors confirmed that these receptors are negatively coupled to adenylate cyclase (Hamblin and Metcalf, 1991; Weinshank et al., 1992). The 5-HT_{1B} receptors have been shown to slightly increase intracellular Ca^{+2}. Both the 5-HT_{1B}-triggered inhibition of adenylate cyclase and increased Ca^{+2} are mediated via pertussis toxin-sensitive G-proteins (Maroteaux et al., 1992; Zgombick and Branchek, 1998). There is good evidence in cell lines that the Ca^{+2} increases induced by 5-HT_{1B} receptors result from phospholipase C activation (Albert, 1994). However, whether this occurs in neurons remains an open question (Dickenson and Hill, 1998).

I.3.5.4 Physiological role(s)

The terminal 5-HT autoreceptor regulating exocytotic release of 5-HT was shown more than a decade ago to belong to the 5-HT_{1B} receptor subtype (Engel et al., 1986; Maura et al., 1986). These studies have, however, been

hampered by the lack of specific 5-HT_{1B} agonists and antagonists. The generation of mice lacking specific 5-HT receptor subtypes has helped to define the presumed role of the 5-HT_{1B} receptor (Piñeyro et al., 1995). It has been proposed that its physiological role was to control the amount of 5-HT released into the synaptic cleft as autoreceptor, in order to avoid over stimulation at postsynaptic 5-HT receptor sites. The mechanism is mainly operational when 5-HT transmission is enhanced i.e., when the extracellular 5-HT concentration is elevated (Hjorth and Sharp, 1993). Several studies have however reported increased 5-HT and 5-HIAA concentrations in the extracellular space following administration of the 5-HT_{1B} antagonist GR127935 (Hutson et al., 1994; Rollema et al., 1996; Stenfors et al., 1999); this would seemingly indicate that the terminal 5-HT_{1B} autoreceptor under normal conditions is tonically stimulated.

A very recent study, using the novel selective r5-HT_{1B} receptor antagonist NAS-181, has shown an enhancement of 5-HT synthesis rate and metabolism *in vivo*, in various 5-HT innervated brain regions (Stenfors et al., 2000). It has been proposed that the increased synthesis and hence release of 5-HT induced by the blockade of the terminal 5-HT_{1B} autoreceptors was followed by increased amounts of 5-HT being converted to 5-HIAA after its uptake into the nerve terminals (Ross and Stenfors, 1997). Interestingly, after simultaneous blockade of the somatodendritic 5-HT_{1A} and of the 5-HT_{1B} receptors, 5-HT metabolism was further increased, thus demonstrating an additive or synergistic effect on 5-HT transmission and release. This finding

was in accordance with previous results obtained in guinea pig (Stenfors et al., 1999).

A modulatory role for 5-HT_1-like receptors, other than the 5-HT_{1A} subtype, has also been recently proposed in the control of the somato-dendritic release of 5-HT (for review, see Stanford et al., 2000). It had been previously assumed that somatodendritic 5-HT_{1A} autoreceptors controlled the firing/release of DRN 5-HT cells, whereas 5-$HT_{1B/1D}$ receptors exerted a local control of 5-HT release at nerve terminals only, even as autoreceptors. Similar to the 5-HT_{1A} subtype, mRNAs for both 5-HT_{1B} and 5-HT_{1D} subtypes are known to be present in the DRN, but were considered to encode for receptors destined to the nerve terminals (Boschert et al., 1994; Doucet et al., 1995). However, there is increasing functional evidence to suggest that further 5-HT_1 receptor subtypes, and in particular the 5-HT_{1B} and/or 5-HT_{1D} subtypes, may also modulate 5-HT release within the DRN (Davinson and Stanford, 1995; Piñeyro et al., 1995; for review, see also Stanford et al., 2000). Two compounds have recently become available (SB 216641 and BRL 15572), which appear to be selective as 5-HT_{1B} and 5-HT_{1D} antagonists respectively (Price et al., 1997). Under *in vitro* physiological conditions, Hopwood and Stanford (2001) have used these compounds to confirm that 5-HT release in the DRN might be controlled by 5-HT_{1B} and 5-HT_{1D} as well as 5-HT_{1A}, receptors. Presumably, these receptors, although in small numbers, would couple efficiently to their transduction pathways to generate a functional response. It should however be noted that there are some 5-HT

axon terminals, presumably originating from other raphe nuclei, in the DRN of rat and cat (Descarries et al., 1982; Chazal and Ralston, 1987), which complicates the interpretation of the above results. The latter studies also reported a low incidence of axo-somatic or axo-dendritic synapses made by these 5-HT terminals, suggesting that the amount of 5-HT in the extracellular space might be the determinant factor irrespective of its somato-dendritic or axon terminal origin.

A major role of the 5-HT_{1B} receptors might also be to act as heteroreceptors, as indicated by studies of non-5-HT neurons in which this receptor appears to inhibit release of transmitter. Evidence to this effect has already been reported for acetylcholine (Maura et al., 1989; Consolo et al., 1996), GABA (Johnson et al., 1992), glutamate (Muramatsu et al., 1998) and dopamine (Sarhan et al., 1999). Also noteworthy are the recent findings of Li and Bayliss (1998) indicating that 5-HT caudal raphe neurons receive glutamatergic inputs that are inhibited by presynaptic 5-HT_{1B} receptors. Therefore, inhibition of excitatory synapses onto raphe cells may represent an additional mechanism for the autoregulation of serotoninergic neuronal activity (Li and Bayliss, 1998).

I.3.5.5 Behavioral role(s)

Studies on the *in vivo* effects of 5-HT_{1B} receptor activation have been hampered by the lack of drugs with sufficient selectivity or brain penetration. In mouse, there is some evidence for an involvement of 5-HT_{1B} receptors in the locomotor stimulant effects of the 5-$HT_{1A/1B}$ receptor agonist RU 24969

(Cheetham and Heal, 1993). This has been confirmed by showing that this locomotor response is abolished in mice lacking 5-HT_{1B} receptors (Ramboz et al., 1996). Given the lack of suitable drugs, an important development was the production of 5-HT_{1B} knockout mice (Saudou et al., 1994). The effect of the constitutive inactivation of the 5-HT_{1B} receptor gene on behavior is described below (Section I.4.3).

1.3.5.6 Psychiatric and neurological disorders associated with the 5-HT_{1B} receptor

Pharmacological studies using agonists of low specificity suggest that activation of central 5-HT_{1B} receptors might lead to an increase in anxiety and locomotion as well as to decreases in food intake, sexual activity and aggressive behavior (Kennett et al., 1987; Wilkinson and Dourish, 1991; Chopin et al., 1994). A likely involvement of 5-HT_{1B} receptors in the inhibition of male rat ejaculatory behavior produced by 5-hydroxytryptophan has been demonstrated (Ahlenius and Larson, 1998). It has also been shown that learned helplessness, a behavioral condition induced by exposure to inescapable stress and considered a valid model of stress disorders and depression (Maier et al., 1993), increases 5-HT_{1B} mRNA levels in the rat DRN (Neumaier et al., 1997).

The 5-HT_{1B} receptor has long been assumed to be involved in the pathogenesis of migraine headache and therefore in the therapeutic action of antimigraine agents, of which sumatriptan is the prototype (Moskowitz, 1992; Goadsby, 1998; Hamel, 1999). This compound may act either via

constriction-mediated 5-HT_{1B} receptors on cerebral arteries, or by blocking neurogenic inflammation and nociceptive activity within trigeminal afferent fibers (Moskowitz, 1992; Humphrey and Goadsby, 1994). It is currently believed that the trigger for headache, which remains unknown, activates perivascular trigeminal axons to release vasoactive neuropeptides that promote neurogenic inflammation (vasodilation, plasma extravasation and mast cell degranulation). Orthodromic and antidromic conduction along trigeminal-vascular fibers can spread inflammatory responses to adjacent tissues, and thus transmit nociceptive information towards the trigeminal nucleus caudalis as well as to higher brain centers for pain perception (Moskowitz, 1992). Experimental data support the hypothesis that sumatriptan activates inhibitor receptors, presumably of the 5-HT_{1B} and/or 5-HT_{1D} subtypes, on perivascular fibers, therefore blocking neurogenic inflammation. Furthermore, sumatriptan has been shown to inhibit *c-fos* expression within the trigeminal nucleus caudalis.

The 5-HT_{1B} receptor could play a role in depression (Bridley and Moret, 1993; Halazy et al., 1997; Pauwels, 1997). Activation of 5-HT_{1B} autoreceptors at the 5-HT terminals suppresses synthesis (Hjorth et al., 1995) and release (Hoyer and Middlemiss, 1989) of 5-HT in rat brain resulting in decreased 5-HT transmission (Göthert and Schlicker, 1997). Both auto- and hetero-5-HT_{1B} receptors have been shown to be desensitized after long-term treatment with antidepressants (Blier et al., 1987; 1990; Briley and Moret, 1993). Therefore, blockage of terminal 5-HT_{1B} receptors by selective

antagonists has been proposed as a new approach toward the design of potentially more efficient and/or fast-acting antidepressant drugs, since acute 5-HT_{1B} receptor blockage should, in theory, immediately elevate terminal 5-HT release (reviewed by Blier and de Montigny, 1998).

I.4 NULL MUTANT (KNOCKOUT) MICE

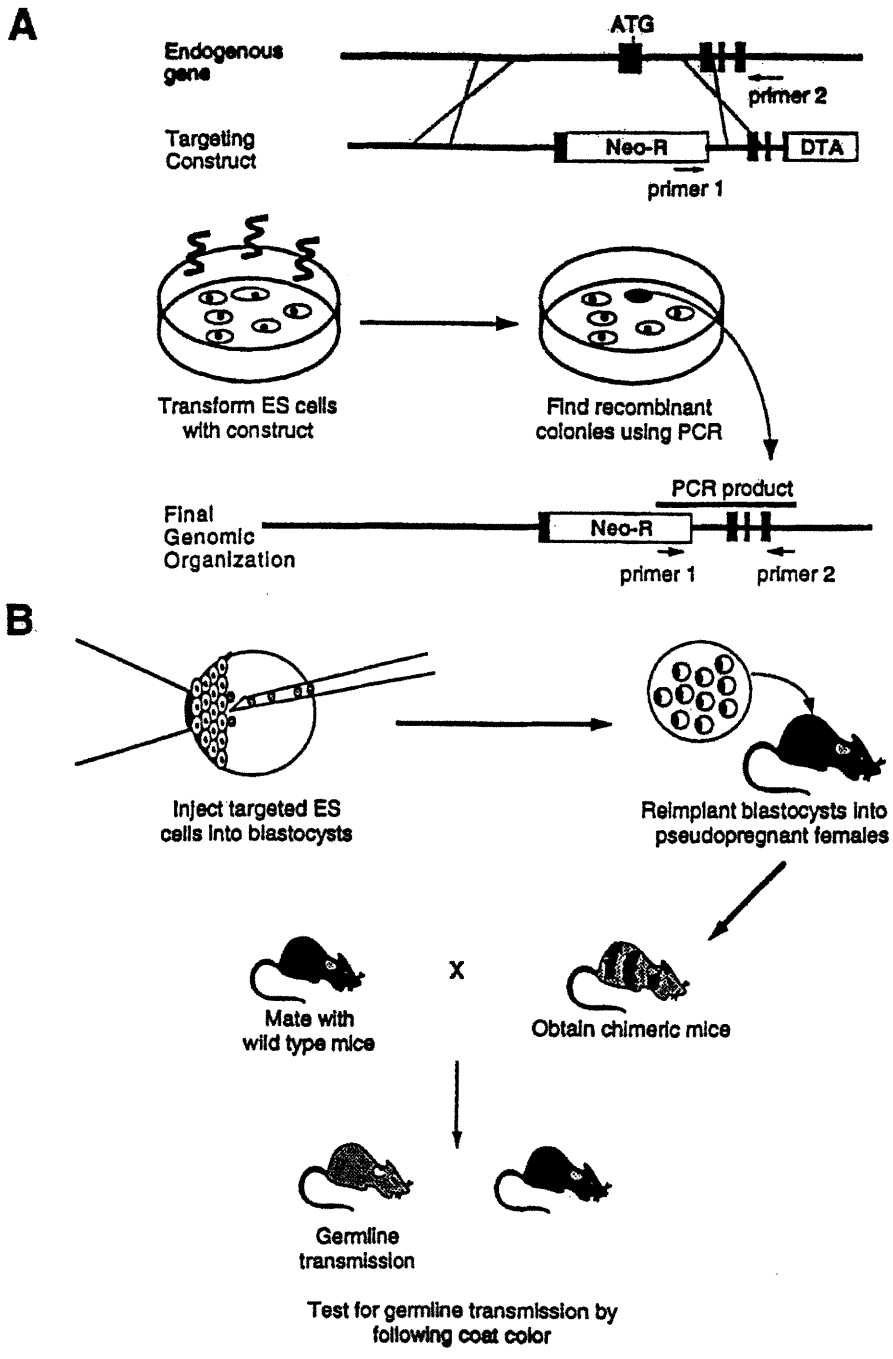
I.4.1 Gene targeting techniques

In principle, there are two ways to generate mutant mice (reviewed by Picciotto and Wickman, 1998; Brusa, 1999). A first approach is to microinject a foreign gene (transgene) into the fertilized egg, to give rise to a **gain of function** mutation derived from overexpression of the transgene. A second method is gene targeting by homologous recombination in **embryonic stem** (ES) cells, whereby it is possible to mutate one defined locus in the mouse genome. As the name suggests, **homologous recombination** is the *in vivo* exchange of a genomic sequence between homologous fragments of DNA. This latter approach is the classical way of generating **knockout** (KO) mice, in which specific genes are ablated. Alternatively, it may be used to knock in a mutated gene in a specific locus. Based on the finding that homologous recombinant could take place in somatic cells (Folger et al., 1982) combined with the establishment of cultured undifferentiated ES cells (Evans and Kaufman, 1981), it has been possible to generate mutations *in vitro* under controlled conditions and then to transfer these mutations to a whole mouse.

In a typical gene targeting experiment (see Fig. 3), ES cells are transfected by electroporation with a linearized, manipulated genomic DNA fragment (referred as a targeting vector), and selected for the rare homologous recombination events among the high background of non-homologous integration. Depending of the targeting vector, several variants of the technique are possible, thus allowing for deletions, point mutations or replacements. A positive-negative selection strategy is commonly used to increase homologous recombination frequency by several folds (Capecchi, 1989). A neomycin-resistant gene (*neo*) is introduced within the homologous region as a positive selection marker that confers resistance to geneticin (G418), and the tymidine kinase gene from herpes simplex virus (HSV-TK) is introduced outside the homologous region as a negative marker for non-homologous integration, conferring sensitivity to gancyclovir (Fig. 3).

Figure 3

Classical gene knockout. (**A**) Wild-type allele, targeting vector and targeted allele following *homologous recombination* (HR). A manipulated genomic DNA fragments (targeting vector) is introduced into *embryonic stem* (ES) cells by electroporation, and subsequent positive–negative selection strategy is commonly used to increase HR versus spontaneous integration events by several folds. Neomycin-resistant gene, or Neo-R is introduced into the homologous region as a positive selection marker conferring resistance to geneticin or G418, and diphtheria toxin (DTA) or thymidine kinase genes can be included outside of the homologous region as a negative marker for non-homologous integration, conferring sensitivity to gancyclovir. HR can be detected by polimerase chain reaction (PCR) screening, using primers. (**B**) Targeted ES cell clones are injected into *host blastocysts* (3.5-day embryos), which are reimplanted in the uterus of pseudopregnant foster mothers. ES cell lines are usually derived from *129 mouse strain*, and the host blastocysts are from C57B1/6 mice. This choice allows the identification of chimeric mice by the contribution of the dominant Agouti coat color derived from the 129 phenotype. If ES cells have contributed to the germ line, the mutation is transmitted to the offsprings and homozygous knockout mice are generated by subsequent matings. (Reproduced with permission from Picciotto and Wickman, 1998).


A
ATG
Endogenous gene
primer 2
Targeting Construct
Neo-R
DTA
primer 1
Transform ES cells with construct
Find recombinant colonies using PCR
PCR product
Final Genomic Organization
Neo-R
primer 1
primer 2
B
Inject targeted ES cells into blastocysts
Reimplant blastocysts into pseudopregnant females
X
Mate with wild type mice
Obtain chimeric mice
Germline transmission
Test for germline transmission by following coat color

After electroporation and antibiotic selection, a suitable number of colonies (generally between 50 and 500) are picked for DNA isolation and are screened by southern blotting or PCR to detect ES cell colonies in which the DNA is correctly recombined. Targeted ES cell clones are injected into 3.5-day-old blastocysts that are then reimplanted in the uterus of pseudopregnant foster mothers. Mostly used ES cell are derived from the 129 mouse strain, with host blastocysts are from C57B1/6 mice. This choice allows the identification of chimeric mice (mice developed in part from the targeted ES cells and in part from cells of the donor blastocysts) by the contribution of the dominant Agouti coat color derived from the 129 phenotype. If ES cells contribute to the germ line, the mutation is transmitted to the offspring (50% probability). These mice are typically identified by PCR amplification of a portion of the targeted allele from tail biopsy DNA. Mice containing one wild type and one targeted allele (heterozygotes) can then be intercrossed to generate mice homozygous for the targeted allele (knockouts). Generally, the targeted mutation is inherited in a classical Mendelian fashion.

The rationale for generating a mouse bearing an inactivation-mutation of a gene is dependent on the presumed function of the gene, itself often defined from previous work revealing the structure of the protein it encodes, its expression pattern, and findings from pharmacological studies. Most research using the knockout strategy is intended at inferring the function of a gene from the dysfunction that occurs in its absence. Mice lacking key

components of the 5-HT system have been used to create inactivation-mutations, and thus, to dissect the role of some of the fourteen 5-HT receptor subtypes as well as multiple enzymes and proteins that influence 5-HT metabolism and release (reviewed by Gingrich and Hen, 2001). Studies pertaining to the mice lacking 5-HT_{1A} or 5-HT_{1B} serotonin receptors are summarized in the following sections.

I.4.2 The 5-HT_{1A} knockout mouse

At present three different lines of mice lacking the 5-HT_{1A} receptor have been produced by independent research groups (Parks et al., 1998; Ramboz et al., 1998; Heisler et al., 1998). While all lines were created through homologous recombination at the 5-HT_{1A} gene in ES cells derived from strain 129, differing breeding strategies have been pursued by the three laboratories. Thus, the 5-HT_{1A} mutation has been bread to 129/Sv (Ramboz et al., 1998), C57BL/6 (Heisler et al., 1998) and Swiss-Webster mice (Parks et al., 1998).

In accord with the anxiolytic effects of 5-HT_{1A} agonists, all three 5-HT_{1A} KO mice display increased anxiety-related behaviors. These mice appear insensitive to the anxiolitic/sedative effect of benzodiazepines, presumably because of decreases in $GABA_A$ receptor α_2-subunit expression and benzodiazepine binding sites (methyl-^{3}H-flunitrazepam) (Sibille et al., 2000). They fear open space and avoid the center of the open field apparatus. In the open field, they display a significant effect on time spent, distance traveled and number of entries into the center area of the enclosure (Heisler et al.,

1998; Ramboz et al., 1998; Parks et al., 1998). In the elevated plus mice, they face a conflict between an aversion to the open arms of the maze and the motivation to explore this compartment. As expected, time spent, number of entries and head-dips in the open arms show genotype differences (Ramboz et al., 1998).

Marked genotype differences are also found in the response to the presentation of a novel object, with knockout exhibiting greater latencies than wild-type mice to approach the object and time spent in the nest area (Heisler et al., 1998). A significant genotype effect has also been observed in horizontal and vertical activity in the presence of the object. In the tail-suspension test, an assay used to evaluate the potential antidepressant efficacy of drugs, the null mutants exhibited an 85% reduction in immobility (Heisler et al., 1998). Antidepressant drugs, including SSRIs, will reduce the immobility displayed by mice following unsuccessful attempts to escape when suspended by the tail (Steru et al., 1985; van der Heyden et al., 1987). Mice, when placed into a water-filled tank, show stress-induced increases in mobility that can be quantified by measuring swimming and climbing activity. With habituation, they become progressively less mobile during the 6 min test, assuming an almost immobile floating position. Specifically, whereas the wild-type mice become progressively more immobile, the mutants maintain the same level of activity throughout the test (Parks et al., 1998; Ramboz et al., 1998). Performance in the rotarod apparatus (fall latencies), a test for motor and spatial coordination (Hamm et al., 1994), as

well as whole locomotor activity in the open field (total distance travel, rearing and nose pokes), show no difference between mutant and wild-type animals, ruling out differences in motor behavior (Parks et al., 1998). In addition, intermediate phenotypes are found in heterozygotes for the 5-HT_{1A} mutation.

Deficits in hippocampal-dependent learning and memory tests, such as the hidden platform (spatial) version of the Morris water maze and the delay version of the Y maze, have also been found in KO mice from the Swiss-Webster strain (Sarnyai et al., 2000). The performance of these KO mice was however not impaired in non hippocampal memory tasks. Furthermore, paired-pulse facilitation in the dentate gyrus of the hippocampus was impaired in this mutant. Finally, these mutants, as compared with wild-type animals, displayed higher limbic excitability manifested as a lower seizure threshold and higher lethality in response to kainic acid administration (Sarnyai et al., 2000).

It has been shown in heterozygotes for the 5-HT_{1A} mutation that the the total number of 5-HT_{1A} receptors (binding sites analysis with two different radioligands: [^{125}I]-*p*-MPPI and [^{3}H]8-OH-DPAT) is close to 50% of wild-type, suggesting a lack of transcriptional or post-transcriptional control to adjust receptor levels in these heterozygotes (Ramboz et al., 1998). The 5-HT_{1A} knockout mice, therefore, provide a robust model relevant to the 5-HT regulation of affect. Yet, the respective contributions of the 5-HT_{1A} autoreceptors and heteroreceptors to the mutant phenotype remain to be

determined. This issue will possibly be addressed in the future, following the development of animals with region-specific 5-HT_{1A} gene inactivation.

I.4.3 The 5-HT_{1B} knockout mouse

The initial phenotypic abnormality observed in mice lacking the 5-HT_{1B} receptor was an enhancement of aggressive behavior in several paradigms, such as the resident-intruder and maternal aggression tests (Saudou et al., 1994; see also Ramboz et al., 1996). Various aspects of aggressive behavior have been observed in mice and other species. These include establishment of dominance (to secure access to food and female mating partners), predatory violence (attacking other species to provide nourishment), defensive aggression (responding and repelling attacks by predators or other members of the same species), to name but a few. In the resident intruder test, a male intruder mouse is placed in the home cage of an animal (resident mouse) that has been housed individually. Mutant resident mice display shorter attack latencies and more frequent attacks on intruder mice than do wild-type residents. Heterozygous animals show an aggressive phenotype of intermediate intensity. Interestingly, a class of mixed 5-$HT_{1A/1B}$ receptor agonists, termed *serenics*, reduces aggressive behavior in animal models, without promoting sedation (Olivier et al., 1990). This has led to the hypothesis that 5-HT_{1B} receptor may mediate the action of these compounds.

Psychostimulant addiction has long attracted more attention on dopamine (DA) than on other neurotransmitter systems. The prevailing theory is that elevated extracellular DA is the primary mediator of the

powerful reinforcing effects of cocaine and other addictive drugs. However, recent studies have re-evaluated the dopamine hypothesis of cocaine abuse and proposed that the 5-HT system may play a role in the rewarding properties of this drug (Parsons et al., 1998; Rocha et al., 1998a; Gainetdinov et al., 1999; for review, see also Caine, 1998; White, 1998). The 5-HT_{1B} KO mice display an increased locomotor response to cocaine as well as an increased propensity to self-administer cocaine as measured by a fixed and progressive ratio schedule (Rocha et al., 1997; 1998b). These results are in sharp contrast with the effects of the 5-HT_{1B} antagonist GR127935 on cocaine responses: an acute administration decreases the locomotor effect of cocaine and has no effect on cocaine self-administration (Harrison et al., 1999; Scearce-Levie et al., 1999). Chronic administration of GR127935 has no effect on the locomotor response to cocaine either (Hen's group unpublished results). However, this mutant displays less cocaine-induced place preference that wild-type mice.

The 5-HT_{1B} KO mice differ from wild-type mice in locomotor/exploratory activity only when the environment is relatively "rich", given its internal structure (Y-maze) or the addition of inner variety (objects in the open field) (Malleret et al., 1999). Test of spontaneous alternation in a Y-maze reveal that the null mutant has increased locomotor behavior and increased entries into the maze arms. In a novel object exploration task, this mutant spends significantly more time in the vicinity of a novel object than in the empty area. In the Morris water maze, a paradigm testing spatial

(place) memory, 5-HT_{1B} KO mice exhibit a more rapid reduction of escape latencies in a task requiring them to locate a submerged platform. This has not been observed, however, in the visual (cue) version of the task. In the transfer test, mutant mice also relearn the new position of the platform faster than wild-type, and this has been interpreted as better cognitive flexibility. This flexibility requires the animal not to perseverate in choosing a wrong solution, and thus reorganize its behavior to solve the new spatial problem. The performance in contextual fear conditioning does not reveal any difference between genotypes. It was concluded from these results that 5-HT_{1B} KO mice display better performance than wild-type on the more cognitively demanding tasks (e.g., water maze), but not on less demanding tasks (e.g., contextual fear conditioning) (Malleret et al., 1999). Interestingly, rat receiving a stimulation of hippocampal (CA1) 5-HT_{1B} receptors were found to be impaired in a spatial learning task (Buhot et al., 1995), and exhibited neophobic reactions in an object exploration task (Buhot and Nali, 1995).

I.4.4 The next generation of mutant mice: inducible and tissue-specific knockouts

As with transgenic studies, functional compensation may occur in a knockout mouse to mask or distort the phenotype resulting from the chronic absence of an endogenous gene. This is of particular concern when targeting a member of a large family of related genes, and has been observed, for example, in mice lacking the transcription factor CREB (Blendy, et al., 1996). Alterations in development can take place not only at the molecular level,

through changes in gene expression, but also at the neuroanatomical level. For example, lack of the dopamine D_1 receptor leads to altered morphology of the striatum, in which the striosomes seen in wild-type mice are absent (Xu et al., 1994).

These scenarios make it difficult to determine the role of a protein of interest in an adult organism. To overcome developmental phenotypes, spatial or temporal control of gene ablation can be achieved through the application of either the *E. coli* bacteriophage **P1 enzyme Cre recombinase** (Tsien et al., 1996; Sauer, 1998) or the **tetracycline**-dependent control of transgenic expression in the CNS (Gossen and Bujard, 1992; Stark et al., 1998). Thus, the strengths of the classical knockouts approach must be tempered by caution in the interpretation of the observed phenotypes. Some of this potential confounds will certainly be obviated as new developments in technology are refined that allow for the temporal and spatial control of the gene knockout (Gingrich and Hen, 2000). This technology will allow a more precise definition of the role of the gene in development at different stages of maturation, including the loss of gene in adult animals (van der Neut, 1997; Stark et al., 1998). Hen's group is currently applying this technique to generate mice lacking either auto- or hetero- 5-HT_{1A} receptors.

I.5 NEUROCHEMICAL APPROACHES

I.5.1 Measurement of monoamine turnover

Measures of neurotransmitter concentration alone are often of little utility in determining whether a particular pharmacological or environmental manipulation has influenced the activity of that transmitter system. Even though stimulation of a neuronal pathway can increase the neurotransmitter synthesis within that pathway, endogenous tissue concentrations may show little change if the rate of catabolism increases to a similar degree. A better approach is to obtain some index of the rate of usage of the neurotransmitter, which is usually called **turnover**, by measuring neurotransmitters and their metabolites simultaneously in the same tissue with high performance liquid chromatography (HPLC) procedures (see Ase et al., 2000a).

Depending on what is being measured, turnover may refer to the rate of transmitter synthesis, catabolism, or sometimes release. A simple approach is to determine, in addition to that of the monoamine themselves, the regional concentrations of HVA, DOPAC and 3-MT in the case of DA, MHPG in the case of NA, or 5-HIAA in the case of 5-HT (for review, see Sharman, 1981). It is then possible to calculate [metabolite]/[transmitter] ratios (Hallman and Jonsson, 1984). Alternatively, other non-isotopic methods can be used to estimate monoamine turnover. Example of this general approach include (1) inhibiting tyrosine hydroxylase (TH) with α-methyl-*p*-tyrosine (AMPT) and then determining the rate of DA and NA

degradation by examining the subsequent decline in transmitter concentration over time (Javoy and Glowinski, 1971; Widerlöv and Lewander, 1978), (2) inhibiting aromatic aminoacid decarboxylase (AADC) with NSD 1015 or another suitable compound, and then determining the rate of tyrosine or tryptophan hydroxylation by measuring DOPA or 5-HTP accumulation, respectively (Carlsson et al., 1972; Duda and Moore, 1985; Broadhurst and Briley, 1988), and (3) blocking efflux of DOPA, HVA, MHPG and 5-HIAA with probenecid and measuring their accumulation within the brain (Meek and Neff, 1973; Elchisak et al., 1977; Curzon, 1981). Finally, isotopic methods can be used in which either [^{3}H]tyrosine or [^{3}H]tryptophan is administered and the rate of formation of [^{3}H]DA and [^{3}H]NA, or [^{3}H]5-HT are respectively determined (Costa et al., 1972; Curzon, 1981).

Most investigators currently used either assessment of metabolite levels or ratios, monoamine disappearance rate following TH or Tryptophan hydroxylase inhibition, or DOPA and 5-HTP-accumulation rate following AADC inhibition. Although measurement of monoamine metabolites is easily accomplished and does not involve any pharmacological interference with the system dynamics, one should remember that **turnover studies are usually aimed at providing a biochemical index of functional activity.** Yet, as a consequence of the intracellular location of monoamine oxidase (MAO), significant amounts of metabolites may be generated from monoamines that are never released (Commissiong, 1985; Khun et al., 1986; Auerbach et al., 1989). The two other approaches can be criticized because their validity rests

in part on the assumption that monoamine synthesis or degradation will not be affected by disruption of the normal biochemical pathway. Nevertheless, the DOPA accumulation method has become particularly attractive because it can be combined with microdialysis to assess *in vivo* rates of tyrosine hydroxylation in freely moving animals (Westerink and De Vries, 1991).

A major limitation of the biochemical determination of regional monoamine levels in brain is the incapacity to discriminate between extracellular and intracellular levels of the transmitter or its metabolites. Also, since the measurements are post-mortem, direct correlations cannot be made between behavior and metabolism. However, 5-HT turnover ratios seem to correlate well with other biochemical parameters and electrophysiological assessments of 5-HT function. This has been shown, for example, after 5-HT_{1A} receptor activation, which reduces [5 HIAA]/[5-HT] ratios and also the firing of 5-HT neurons, 5-HT synthesis and 5-HT release in projection areas of the raphe nuclei (reviewed by Barnes and Sharp, 1999; Piñeyro and Blier, 1999).

I.5.2 Receptor autoradiography

Receptor mapping has been of considerable help to understand some of the mechanisms of action of various drugs. Aside from solving pharmacological issues, this technique provides a unique view of the biochemical organization of the brain. The maps yield information on potential sites of action for the different endogenous transmitters. **Autoradiography**, compared to radioligand binding techniques with tissue

homogenates, has the advantage of preserving tissue anatomy and can consequently produce a more precise, discrete and quantitative cartography of receptors, uptake sites or transporters, as well as mRNAs for peptides and neurotransmitter enzymes. This approach has been used in the past two decades for the study of CNS receptors, and several reviews have already dealt with its different technical and theoretical aspects (Palacios and Wamsley, 1982; Wamsley, 1984; Kuhar et al., 1986; Perretti-Rennucci et al., 1991; Reader and Strazielle, 1999).

The two main applications of autoradiography are to **localize** and **quantify radioligand binding sites**; the first objective relies on the identification of the anatomical structures where the ligand is bound. These labeled regions can then be studied with the help of anatomical atlases and of cresyl violet-stained sections to interpret the information of autoradiographic films or emulsion-coated histological sections, respectively. The second objective is to quantify receptor densities, and for this purpose the optical densities, or grain counts can be converted to molar amounts of ligand bound by reference to standard curves generated by co-exposing the tissue sections with known amounts of radioactivity. After subtraction of background, this procedure allows to establish the relationship between optical densities and the actual amounts of bound ligand (Unnerstall et al., 1982; Niehoff, 1986; Reader and Strazielle, 1999). The general equation to calculate molar quantities of bound ligand is:

$$\text{mmol / mg p} = OD_A \times [(\text{DPM / mg p}) / (\text{OD) B}] \times [\text{mmol / (Ci) / } (2.22 \times 10^{12} \text{ DPM})]$$

In this equation, OD_A is the optical density over the region studied, factor B is the slope of the standard curve, and factor C is the specific activity of the ligand (Unnerstall et al., 1982). Alternatively, using commercial calibration scales, the values of each known standard, usually in nCi/mg, can be converted into the appropriated units, such as femtomoles per milligram (fmol/mg), and thereafter the OD values extrapolated from this standard calibration curve. The equation that will allow OD values to be transformed into molar bound values can be expressed as $[(nCi/mg \times 10^{-12})]$; where the values of each standard concentration in nCi/mg is corrected for the decay between the time of manufacture and exposure day, and C is the specific activity of the radioactive ligand, also been corrected for decay. Protein concentration in brain sections has been determined to be 0.3 mg per mg of tissue section; thus, molar concentrations per mg of tissue dry weight can be converted to molar concentrations per mg of protein (Unnerstall et al., 1982).

In order to estimate the number of binding sites, the experiments should be carried out at radioligand concentrations that saturate all the specific binding sites. This can be accomplished by complete saturation binding isotherms thereafter analyzed with conventional mathematical procedures, or using a single radioligand concentration, but ensuring that all available sites have been occupied by the radioligand. This second procedure implies the use of a radioligand concentration 10-20 fold its K_D value determined for the tissue-binding site. Thereafter, densities of specific

binding can be converted to maximum binding capacities, or B_{MAX} values, by the equation $\mathbf{B_{MAX} = B \times (K_D + L) / L}$, where B is the specific bound, K_D the dissociation constant determined by the saturation binding curves and L the radioligand concentration (Boyson et al., 1986; Reader et al., 1988; Huang et al., 1997).

In membrane preparations, there often seems to be a marked heterogeneity in the affinity of binding sites labeled with receptor agonists. Presumably as a consequence of their ability to activate the receptor, agonists bind with a higher affinity to G-protein coupled and a lower affinity to uncoupled receptors. Antagonists do not distinguish between G-protein coupled and uncoupled receptor sites. The effects of antagonists apparently depend solely on their affinity for the receptor, which can be measured in both functional and binding assays. In contrast, the response to agonists in functional assays will depend not only on their affinity for the receptor but also on their efficacy. In radioligand binding autoradiography, the situation is further complicated by the fact that agonist binding is tightly dependent on the physico-chemical conditions of the binding assay. Alterations in density of binding sites must therefore be interpreted with caution, especially after agonist labeling, since they may theoretically reflect changes in affinity (Kd) as well as or rather than number (Bmax) of receptors. This drawback can be overcome by concomitant assays in membrane preparations.

The majority of imaging systems used for quantitative binding autoradiography, are based on densitometric measurements of radioactive-

sensitive films that have been exposed to a radiation source originating from tissue sections that were incubated with a radioactive ligand. As radioactivity of the label and/or the exposure time increases, the response of the emulsion saturates (Rogers, 1979; Niehoff, 1986); therefore, the relationship between the OD, or silver grain counts, and the tissue radioactivity very often may not be linear. To obtain reproducible and comparable measurements, the scanning conditions always have to be identical. A constant enlargement of the image must be kept, which will depend on the height between the camera and the film, as well as on the focal point of the video camera for a same diaphragm aperture (Reader and Strazielle, 1999). The shading distortion has to be corrected, and the densities of the non-specific labeling have to be digitally subtracted from the corresponding total binding values to obtain quantitative readings of specific binding (Ramm, 1990; Ramm et al., 1984). For the studies concerning mouse brain, the structures can be identified according to the anatomical landmarks and the nomenclature of a mouse stereotaxic atlas (Franklin and Paximos, 1997).

I.5.3 GTPγS autoradiography

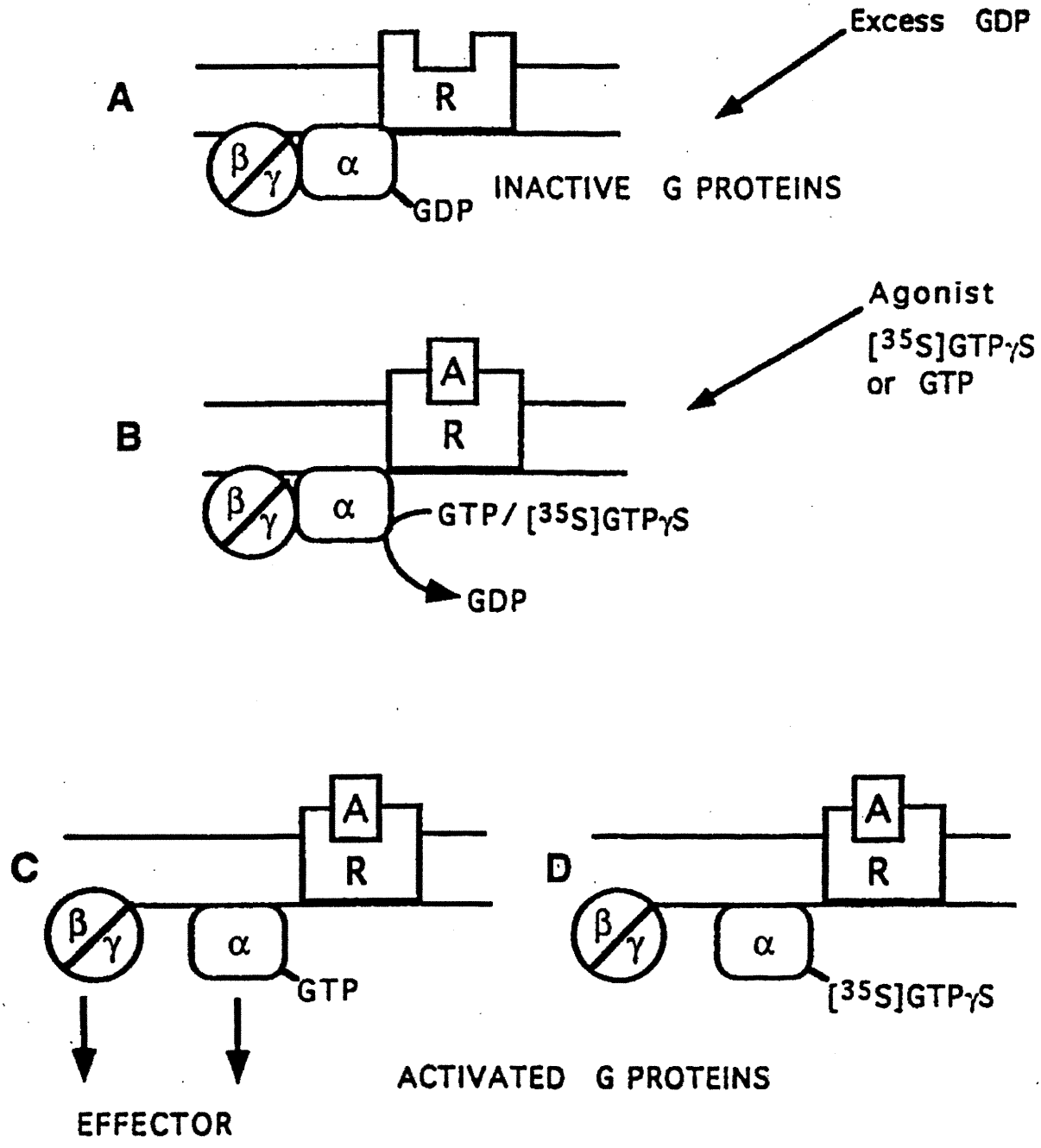
As already mentioned, ligand-binding autoradiography is an important technique to identify and quantify neurotransmitter receptors and transporters across the brain, but provides little information regarding the functional relevance of these sites. In fact, it does not establish which of the labeled receptor sites are actually coupled to intercellular signaling mechanism. This section describes the recent development, by Sim and

collaborators (1995), of an *in vitro* autoradiographic technique for visualizing and measuring receptor-stimulated [^{35}S]GTPγS binding in brain sections (see also Sóvágó et al., 2001).

A large number of neurotransmitters and hormones produce their biological effects via G-protein coupled receptors, or GPCRs (Birnbaumer et al., 1990) and receptor-G-protein activation has been extensively reviewed (Gilman, 1987). Each G-protein is a heterotrimer composed of three subunits, α-, β- and γ- (see Fig. 4).

Figure 4

Schematic diagram of the mechanism of agonist-stimulated [^{35}S]GTPγS incorporation. (**A**) Addition of excess GDP shifts the G-proteins into the inactive state. (**B**) Agonist binds to the receptor, which increases the affinity of the G-proteins for GTP or [^{35}S]GTPγS. (**C**) Activation of the G-proteins by GTP activates effectors, or; (**D**) Activation of the G-proteins by [^{35}S]GTPγS provides labeled G-proteins. (Reproduced with permission from Sim et al., 2000).


A
R
Excess GDP
β
γ
α
GDP
INACTIVE G PROTEINS
B
A
R
Agonist
[35S]GTPγS
or GTP
β
γ
α
GTP/ [35S]GTPγS
GDP
C
A
R
β
γ
α
GTP
EFFECTOR
D
A
R
β
γ
α
[35S]GTPγS
ACTIVATED G PROTEINS

Initially, G-proteins are inactive in the GDP-bound heterotrimeric state, as illustrated in Figure 4A (Gilman, 1987; Bourne et al., 1990). The binding of agonist activates its receptor; this activation changes the conformation of the G-protein, increasing the affinity of the α-subunit for GTP (guanosine 5'-triphosphate) and decreasing its affinity of GDP (Fig. 4B). GTP binding activates the α-subunit, and decreases its affinity for βγ, causing βγ to dissociate from α (Fig. 4C). The receptor and α then dissociate, enabling α and βγ to interact downstream with effectors such as enzymes and ion channels. The α-subunit is spontaneously inactivates by an intrinsic GTPase that hydrolyzes GTP to GDP, increasing the affinity of α for βγ and thus completing the cycle. The reaction is catalytic, since each receptor can activate multiple G-proteins, resulting in amplification of the receptor signal into an intracellular response.

Using autoradiographic techniques, GTP incorporation, stimulated by multiple GPCRs, can be localized in adjacent sections using a single radioligand, [^{35}S]GTPγS (Sim et al., 1995). Fortunately, with the family of GPCRs, signal transduction is mediated at the point at which receptors activate the α-subunits of G-proteins to bind GTP. The stimulation of [^{35}S]GTPγS binding to G-proteins by receptor agonists is based upon the G-protein activation cycle discussed above. Initially, an excess of GDP is added to the assay to inactivate G-protein α-subunits and reduce basal [^{35}S]GTPγS binding. [^{35}S]GTPγS and agonist are then added to activate the G-protein coupled to the receptor of interest. Receptor activation decreases

the α-subunit affinity for GDP but increases its affinity for GTP, so the G-protein incorporates GTP, or [^{35}S]GTPγS (Fig. 4D). This latter analog is favored because it has a high affinity for G-protein and is resistant to GTPase activity (Wieland and Jacobs, 1994). *In vivo*, the α-subunit GTPase hydrolyzes GTP to GDP; however, [^{35}S]GTPγS is resistant to hydrolysis and remains bound. The increase in bound [^{35}S] induced by agonist drugs can then be measured by autoradiography, or assessed by liquid scintillation spectrometry.

Autoradiography of [^{35}S]GTPγS has several advantages over traditional radioligand receptor autoradiography (Sim et al., 1995). First, since [^{35}S]GTPγS is the radiolabeled ligand used in this technique, and unlabeled agonists are used to activate G-proteins via specific receptors, [^{35}S]GTPγS autoradiography is not limited by the availability of radiolabeled ligands. Second, [^{35}S]GTPγS requires a much shorter film exposure time than ^{3}H-labeled compounds. Finally, multiple receptor analysis is easily performed by incubating sequential sections with a variety of agonists. Thus, simultaneous information regarding several receptor systems can be collected from the brain of a single experimental animal. Perhaps the most important aspect of this methodology is that [^{35}S]GTPγS autoradiography demonstrates functional activity at the level of signal transduction, rather than binding at the level of receptor. The most obvious limitation of this technique is the difficulty to detect certain classes of receptors because of low receptor density and/or low efficiency of coupling.

Thus far, the assay has not been applicable with success to Gs-coupled system in brain sections. This is because the relative abundance of G-proteins in the brain is Go > Gi > Gs (Sternweis and Robishaw, 1984). Thus, the levels of Gs may be too low to produce detectable [^{35}S]GTPγS binding. Another limitation is the specificity of the technique for individual G-proteins. Although the [^{35}S]GTPγS binding assay is highly specific for a given receptor by the use of selective agonists, there are currently no methods to determine which type of G-protein(s) the receptor activates in the tissue section. Thus, the [^{35}S]GTPγS binding signal results from the activation of the pool of G-proteins coupled to the receptor of interest, which may be a heterogeneous G-protein population.

Thus far, this technique has allowed for the study of [^{35}S]GTPγS-binding to μ-opiod, canabinoid, γ-aminobutyric acid (GABA), muscarinic (Sim et al., 1996), 5-HT$_{1A}$ (Sim et al., 1997; Dupuis et al., 1998) and D_2 (He et al., 2000) activated receptors in rat brain slide-mounted sections. Although these studies were restricted to receptors that are specifically coupled to Gi/o, a more recent study has succeeded in measuring the coupling of 5-HT$_{2A/2C}$ receptors to Gp proteins in rat brain sections (Adlersberg et al., 2000).

I.5.4 Autoradiographic and immunocytochemical measurement of 5-HT innervation density

When detecting changes in transmitter metabolism or in receptor or transporter density at the regional level, questions arise about the cellular localization of these changes, knowing that the neurons in cause or affected generally represent but a fraction of the diversified population in the region examined. More specifically, in the face of lowerings or increases in monoamine content and/or in receptor and transporter density, one may immediately ask whether such changes reflect some structural alteration of the neuronal system in question, such as a decrease or increase in innervation density, for example. The autoradiographic method, as well as a newly developed technique for the analysis of light microscopic immunocytochemical results provides a mean to assess this parameter in the case of the 5-HT system.

Light microscope autoradiography after uptake and storage of [^{3}H]serotonin may be used to directly count 5-HT axon terminals and varicosities in brain regions containing this neurotransmitter in normal or lesioned animals. This method has been applied successfully to quantify the density of 5-HT innervation in several brain regions of intact rat or rat treated with cytotoxin (Mrini et al., 1995). An alternative approach would be to use quantitative ligand binding autoradiography of [^{3}H]citalopram or [^{3}H]cyanoimipramine, which, at least in wild-type animals, were proven to be highly specific and reliable markers of 5-HT innervation density (Descarries

et al., 1995). Recently, however, it has become possible to measure 5-HT axon density more directly and readily, by using a 'semi-computerized' method to estimate the length of labeled axons in brain sections processed for **light microscopic immunocytochemistry** (Mechawar et al., 2000; Ase et al., 2001; Aznavour et al., 2001). This method, developed in our laboratory to evaluate the density of innervation by acetylcholine (ChAT-immunostained) neurons, allows estimating the actual length of immunostained axons as well as their number of varicosities per mm^3 of tissue. It provided a simple mean to measure the same parameters in brain sections immunostained for 5-HT, as described in Chapter IV.

I.6 RESEARCH PROJECT

1.6.1 General goal

Central 5-HT transmission has been associated with anxiety, depression, as well as a group of impulse-related disorders, including aggression and substance abuse (Lucki, 1998). It is also well established that 5-HT_{1A} (somatodendritic) and 5-HT_{1B} (terminal) receptors behave as autoreceptors and heteroreceptors, and control the firing and release of 5-HT and non 5-HT neurons, respectively. Important clues into the role of these receptors in behavior have come from the study of the corresponding null mutants. As already mentioned, 5-HT_{1A} KO mice from three different strains were shown to display an anxious-like phenotype in several behavioral tests, such as the elevated plus and zero mazes, the light-dark choice, the open

field and the forced-swimming test (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). In 5-HT$_{1B}$ KO mice, an enhancement of aggressive behavior was observed in the resident-intruder and isolation-induced or maternal aggression tests, as well as a high vulnerability to cocaine (Saudou et al., 1994; Rocha et al., 1998b). The general goal was to investigate in KO mice the consequences of a chronic ablation of 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor genes on 5-HT transmission in CNS. The two null mutants provided a unique *in vivo* model to characterize adaptive regulations of the 5-HT system as well as modifications of other chemically-defined systems, known to interact with 5-HT neurons.

1.6.2 Specific objectives

In a first step, tissue levels of monoamines and their main metabolites were measured in discrete brain regions and spinal cord of WT, 5-HT$_{1A}$ KO and 5-HT$_{1B}$ KO mice, by means of high-performance liquid chromatography. This method allowed us to search for possible abnormalities in transmitter content and metabolism. An indirect measure of 5-HT or DA turnover could be obtained by calculating the ratios [5-HT-metabolites]/[5-HT] and [DA-metabolites]/[DA], respectively. The resulting values could then be viewed as indices of neuronal activity and/or transmitter release (Shannon et al., 1986; Stenfors et al., 2000).

In a second step, Receptor-binding autoradiography was used to localize and quantify densities of 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors throughout the brains of mutant and WT mice, using [^{3}H]8-OH-DPAT and [^{125}I]cyanopindolol

as radioligands. The density of 5-HT transporters was also measured with [^{3}H]citalopram as specific probe of uptake sites. As alterations in 5-HT transporter were observed, it was of interest to estimate the density of 5-HT axons and axonal varicosities by light microscopic immunocytochemistry, to determine whether these changes reflected structural alterations of the 5-HT innervation.

Finally, [^{32}S]GTPγS autoradiography was applied to examine the functional state of the remaining 5-HT$_1$ receptor subtype in both KO mice. As no changes in density of 5-HT$_{1B}$ receptors had been found in 5-HT$_{1A}$ KO mice, nor of 5-HT$_{1A}$ receptors in the 5-HT$_{1B}$ KO, this approach allowed to search for possible mutual adaptations in signaling capacity of these two GPCRs, in addition to biochemical and anatomical changes directly imputable and vice-versa autoradiographic methods measure different parameters in the receptor cascade, these experiments allowed us to search for mutual adaptations of these two G-protein-coupled autoreceptors toward one another. In addition, anatomical and biochemical changes directly or indirectly imputable to their constitutive absence.

Chapter II

Altered serotonin and dopamine metabolism in the CNS of serotonin 5-HT_{1A} or 5-HT_{1B} receptor knockout mice

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As first author, I carried out most of the experiments, analyzed the data, and contributed in a significant way to all the steps toward the writing of the article.

Résumé: Nous avons mesuré par chromatographie liquide à hautre pression la sérotonine (5-HT), la dopamine (DA), la noradrénaline, ainsi que les métabolites de la 5-HT et de la DA, dans 16 régions du cerveau et la moelle épinière de souris knockout (KO) 5-HT_{1A} ou 5-HT_{1B} et sauvages (WT) de la souche 129/Sv. Chez les KO 5-HT_{1A}, la teneur en 5-HT s'est avérée inchangée dans toutes les régions, en présence de métabolites 5-HT plus élevés que chez le WT, dans les noyaux raphé dorsalis et medianus, le bulbe olfactif, la substance noire et le locus coeruleus. Ce résultat signifiait une augmentation du renouvellement (turnover) de la 5-HT, donc une augmentation de l'activité de base des neurons 5-HT du raphé avec augmentation probable de leur libération somatodendritique et terminale de 5-HT. Il pouvait expliquer le comportement de type anxieux décrit chez la souris KO 5-HT_{1A}. Une augmentation concomitante de la teneur et DA et/ou de son turnover dans les mêmes régions a été interprétée comme résultant d'une désinhibition des neurones DA, alors que des augmentations de la teneur en noradrénaline dans plusieurs territoires de projection du locus coeruleus signifiaient peut-être une diminution d'activité de ses neurones. Chez le KO 5-HT_{1B}, la concentration de 5-HT est apparue significativement plus faible que chez le WT, dans le noyau accumbens, le locus coeruleus et la moelle épinière; elle l'était probablement aussi dans quelques autres territoires d'innervation 5-HT. Une diminution de la DA, associée à une augmentation de son turnover, a été mesurée dans le noyau accumbens. De tels changements du métabolisme de la 5-HT et de la DA peuvent rendre compte de l'aggressivité excessive rapportée chez la souris KO 5-HT_{1B} et de sa propension à s'auto-administrer la cocaine. Ainsi, des altérations fortement contrastées du métabolime cérébral des monoamines pourraient sous-tendre les phénotypes comportementaux quasi opposés de ces deux mutants nuls.

Altered Serotonin and Dopamine Metabolism in the CNS of 5-HT_{1A} or 5-HT_{1B} Receptor Knockout Mice

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Abbreviations used: DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindole-3-acetic acid; 5-HT (5-hydroxytryptamine), serotonin; 5-HTOL, 5-hydroxytryptophol; HVA, homovanilic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol; 3-MT, 3-methoxytyramine; NA, noradrenaline; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin.

Abstract: Measurements of serotonin (5-HT), dopamine (DA) and noradrenaline, and of 5-HT and DA metabolites, were obtained by HPLC from 16 brain regions and the spinal cord of 5-HT_{1A} or 5-HT_{1B} knockout and wild-type mice of the 129/Sv strain. In 5-HT_{1A} knockouts, 5-HT concentrations were unchanged throughout, but 5-HT metabolites were higher than wild-type in dorsal/medial raphe nuclei, olfactory bulb, substantia nigra and locus coeruleus. This was taken as an indication of increased 5-HT turnover, reflecting an augmented basal activity of midbrain raphe neurons and consequent increase in their somatodendritic and axon terminal release of 5-HT. It provided a likely explanation for the increased anxiety-like behavior observed in 5-HT_{1A} knockout mice. Concomitant increases in DA content and/or DA turnover were interpreted as the result of a disinhibition of DA, whereas increases in noradrenaline concentration in some territories of projection of the locus coeruleus could reflect a diminished activity of its neurons. In 5-HT_{1B} knockouts, 5-HT concentrations were lower than wild-type in nucleus accumbens, locus coeruleus, spinal cord and probably in several other territories of 5-HT innervation. A decrease in DA, associated with increased DA turnover, was measured in nucleus accumbens. These changes in 5-HT and DA metabolism were consistent with the increased aggressiveness and the supersensitivity to cocaine reported in 5-HT_{1B} knockout mice. Thus, markedly different alterations in CNS monoamine metabolism may contribute to the opposite behavioral phenotypes of these two knockouts.

Key words: 5-HT_{1A} knockout — 5-HT_{1B} knockout — Brain and spinal cord — Monoamines — Metabolites — Turnover

Running title: Monoamines in 5-HT_{1A} and 5-HT_{1B} knockout mice

There are seven families and at least fourteen subtypes of serotonin (5-hydroxytryptamine; 5-HT) receptors thus far identified in mammalian CNS (for reviews, see Saudou and Hen, 1994; Barnes and Sharpe, 1999). Among these, the 5-HT_{1A} and 5-HT_{1B} subtypes of the 5-HT_1 family have received the most attention, as putative targets for drugs used in anxiety, depression and migraine (e.g., Peroutka 1993; Blier and de Montigny, 1999; Hamel, 1999). Both these receptors function as auto- as well as heteroreceptors. The 5-HT_{1A} receptors are somatodendritic and predominate on the membrane of 5-HT (e.g., nucleus raphe dorsalis) and non-5-HT (e.g., hippocampus) neurons (Riad et al., 2000; see also Kia et al., 1996a,b). Their activation inhibits neuronal firing (Sprouse and Aghajanian, 1987; Dong et al., 1997) through a hyperpolarization mediated by a G-protein-coupled opening of K^+ channels and reduction of a high threshold Ca^{2+} current (for reviews, see Aghajanian, 1995; Piñeyro and Blier, 1999). As a consequence, there is a reduction in the release of 5-HT (Sharp et al., 1989) or of other transmitters, such as acetylcholine (Bianchi et al., 1990; Kia et al., 1996c), noradrenaline (NA) (Done and Sharp, 1994; Hajós-Korcsok and Sharp, 1999) and glutamate, presumably. The 5-HT_{1B} receptors also are capable of modulating the release of 5-HT (Piñeyro et al., 1995; Engel et al., 1996) and of other transmitter/modulators, including acetylcholine (Maura et al., 1989; Consolo et al., 1996), dopamine (Sarhan et al., 1999), GABA (Johnson et al., 1992) and glutamate (Li and Bayliss, 1998). This presumably involves a different mechanism, however, since recent immunoelectron microscopic studies of

their subcellular distribution have shown that they are localized mainly to the plasma membrane of fine, preterminal axons in substantia nigra and globus pallidus, suggesting a mediation of 5-HT effects on axonal conduction rather than neuronal firing (Riad et al., 2000).

In recent years, knockout mice produced by homologous recombination have been made available to study the role and function of 5-HT_{1A} and 5-HT_{1B} receptors (Saudou et al., 1994; Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). In the wild-type mouse brain, as in the rat, the 5-HT_{1A} receptors are predominantly found in the raphe nuclei and limbic regions (frontal and entorhinal cortex, dentate gyrus, CA1 and CA3 fileds of the Ammon's horn, lateral septum, amygdala), when visualized by ligand binding autoradiography (Pazos and Palacios, 1985; Laporte et al., 1994). In contrast, 5-HT_{1B} receptors binding sites are concentrated in extrapyramidal areas (caudate-putamen, substantia nigra, globus pallidus) and to a lesser extent in the dorsal subiculum, lateral geniculate nucleus, superior colliculus, central grey and deep cerebellar nuclei (Pazos and Palacios, 1985; Boschert et al., 1994; Langlois et al., 1995). In the 5-HT_{1A} receptor knockouts (1A −/−), the phenotype is characterized by increased anxious-like behavior (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). The 5-HT_{1B} knockouts (1B −/−) have been reported to be more aggressive than wild-type (+/+) mice of the same background strain (Saudou et al., 1994), more active in the open field (Ramboz et al., 1996) or in a novel environment

(Malleret et al., 1999), and more inclined to self-administer cocaine (Rocha et al., 1998).

The present study was aimed at a better understanding of the functional roles of 5-HT_{1A} and 5-HT_{1B} receptors through examination of the consequences of their constitutive absence on regional CNS content in monoamines and their major metabolites. In 16 microdissected brain regions and the spinal cord of 1A –/–, 1B –/– and +/+ mice, the compounds 5-HT, DA and NA, as well as some of the major metabolites of 5-HT and DA, were measured using HPLC with electrochemical detection. In addition, the ratios between the regional concentrations of the respective metabolites and 5-HT and DA were determined as *turnover indices*, allowing for some assessment of the functional state of these neurons.

MATERIAL AND METHODS

Animals

The 5-HT_{1A} knockout and wild-type mice in the present study had exactly the same genetic background: 129/SvEvTac. These 5-HT_{1A} knockouts were produced from 129/SvEvTac ES cells and the resulting chimeras were bred with 129/Sv/EvTac females (Ramboz et al., 1998). The 5-HT_{1B} knockouts, in contrast, were initially produced on distinct genetic background: 129/SvPas X 129/Sv-ter (Saudou et al., 1994). However, these mice were backcrossed for six generations with 129/SvEvTac mice (Phillips et al., 1999). As a result, the present 5-HT_{1B} knockouts had a genetic background very similar

(although not exactly identical) to the wild-type and 5-HT_{1A} knockouts. The mice were housed four per cage in a colony room and maintained on a 12-h light/dark cycle with food and water available *ad libitum*. All animal procedures were in strict accordance with the Canadian Council on Animal Care, *Guide to the Care and Use of Experimental Animals* (2^{nd} edition) and the protocols approved by the *Comité de Déontologie pour l'Experimentation sur des Animaux* at the Université de Montréal.

Chemicals

5-HT HCl, NA HCl, 5-hydroxytryptophol (5-HTOL), 5-hydroxyindole-3-acetic acid (5-HIAA), DA HCl, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA), 3-methoxytyramine (3-MT) and 3-methoxy-4-hydroxyphenylglycol (MHPG) were purchased from Research Biochemicals Inc (Natick, MA, U.S.A.). The compounds Tris-(hydroxymethyl)-aminomethane 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) and isoproterenol were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and sodium octyl sulphate from Eastman Kodak (Rochester, NY, U.S.A.). [^{3}H]8-OH-DPAT (137 Ci/mmol) was purchased from New England Nuclear / Du Pont (Boston, MA, U.S.A.), and [^{125}I]cyanopindolol (2,200 Ci/mmol) from Amersham (Arlington Heights, IL, U.S.A.).

Receptor autoradiography

Autoradiographic observations were made in three groups of 3-month-old 129/Sv mice (three males and three females in each group): 1A –/–, 1B –/–

and +/+. After the animals were killed by decapitation, the brain was quickly removed, frozen in 2-methylbutane cooled to –40°C with liquid nitrogen, and stored at –80°C. Transverse sections (20 _m thick) were serially cut with a cryostat, thaw-mounted onto gelatin-coated slides, and kept at –80°C until used. For the labeling of 5-HT_{1A} receptors, the sections were preincubated at 25°C for 15 min in 50 m*M* Tris-HCl buffer (pH 7.4) and then incubated for 60 min in the same buffer with 2 n*M* of the agonist compound [^{3}H]8-OH-DPAT (Radja et al., 1991,1993; Reader et al., 1995). The 5-HT_{1B} receptors were labeled as follows with the antagonist [^{125}I]cyanopindolol (Boschert et al., 1994). After a preincubation at 25°C for 30 min in a 170 m*M* Tris-Cl buffer (pH 7.6) containing 150 m*M* NaCl, the slides were incubated for 120 min in the same buffer containing in addition 100 p*M* of [^{125}I]cyanopindolol together with 100 n*M* of 8-OH-DPAT (5-HT_{1A} blocker) and 30 μ*M* isoproterenol (β-adrenergic blocker).

Incubations were always carried out in triplicate, two slides being used for total binding and a third slide incubated with 10 μM serotonin creatinine sulfate to determine nonspecific labeling (Marcinkiewicz et al., 1984; Vergé et al., 1986; Radja et al., 1991, 1993; Boschert et al., 1994). Following incubations, the slides were washed in cold buffer, rinsed in distilled water, air-dried, and exposed to Hyperfilm™ (Amersham; Arlington Heights, IL) together with either [^{3}H]- or [^{125}I]-microscales™ (Amersham). Exposures lasted 2 weeks ([^{3}H]) or 24 hours ([^{125}I]), and development was in Kodak D-19. Optical density measurements were carried out with an MCID™ image

analysis system (Imaging Research, St-Catherines, Ontario, Canada). Specific binding was obtained by digital subtraction of nonspecific labeling from total binding. Standard curves generated from the [^{3}H] or the [^{125}I] microscales were used to convert optical densities into femtomoles per milligram of protein (Unnerstall et al., 1982; Reader and Strazielle, 1999).

Biochemical measurement of monoamines and their metabolites

HPLC measurements were carried out in three groups of 3-month-old mice (four males and four females in each group): 1A –/–, 1B –/– and +/+. After decapitation, the brain and spinal cord were removed and frozen as described above. On a cold plate, each brain was then cut into 0.5–1.0 mm-thick coronal slices, from which 16 regions were microdissected by hand under a binocular microscope: olfactory bulb, cingulate, frontal and entorhinal/piriform cortex, nucleus accumbens, septum, caudate-putamen, globus pallidus, thalamus, hypothalamus, amygdala, hippocampus, substantia nigra, dorsal/medial raphe nuclei, locus coeruleus, and cerebellum (Fig. 1). The spinal cord was processed as a whole.

Each sample was rapidly weighed, homogenized in a mobile phase of 0.1M monochloroacetic acid (adjusted to pH 3.3) with 2.0 mM Na_2EDTA, and centrifuged at 39,000 x g for 45 min at 4°C (Reader and Grondin, 1987). The pellets were resuspended in 1M NaOH overnight for protein assay (Lowry et al., 1951), and the supernatants filtered (GS, Millipore, Bedford, MA, U.S.A.; 0.45-µm pores) and stored at –80°C until the actual analysis was carried out,

usually within 48 h. The measurements were made by HPLC with electrochemical detection, as previously described (Reader and Grondin, 1987; Ase et al., 1999). Briefly, the filtered supernatants were injected into a 3 μm particle-size HPLC analytical column (100 x 4.6 mm; Adsorbosphere CatecholamineTM; Althech; Deefield, IL, U.S.A.), and an electrochemical detector (EE&G M-400, Princeton Applied Research, NJ, U.S.A.) was used with a working electrode at an oxidation potential of +680 mV. The peaks generated from the compounds were recorded, and their heights integrated with a Hewlett Packard 3392A integrator. The NA metabolite MHPG was not quantified since its peak overlapped with the solvent front of tissue samples. For every chromatographic run, external standards containing 0.5 ng of each of the authentic compounds were injected to quantify peak heights and determine retention times (Fig. 2A). Representative chromatograms from dorsal/medial raphe nuclei, septum and globus pallidus of 129/Sv mice are shown in Fig. 2B–D. Turnover indices of 5-HT and of DA were obtained as the ratios of concentration of the respective metabolites over 5-HT and DA.

Statistics

The biochemical results were tabulated as means ± S.E.M. in nanograms per milligram of protein. The statistical comparisons between mutants and wild-type mice were made by analysis of variance followed by Fisher's F-distribution tests (Barlow, 1983; Frank and Althoen, 1994). Because of the

great number of measurements carried out in this study, only differences with $p < 0.01$ were considered significant.

RESULTS

Absence of 5-HT_{1A} or 5-HT_{1B} receptor binding in the respective knockout mice

Detailed results of these and other radioligand binding experiments will be reported separately. In the present context, the purpose was merely to verify the absence of the deleted receptors in the knockouts. As expected, throughout the brain, there was a total lack of specific binding of [^{3}H]8-OH-DPAT to 5-HT_{1A} receptors in 1A −/− mice (Fig. 3B and D), and of [^{125}I]cyanopindolol to 5-HT_{1B} receptors in 1B −/− mice (Fig. 3F and H). In contrast, in wild-type mice, intense [^{3}H]8-OH-DPAT labeling was apparent in the midbrain raphe nuclei and hippocampus (Figs. 3A and C), and [^{125}I]cyanopindolol labeling in the globus pallidus and substantia nigra (Fig. 3E and G).

Monoamines and their metabolites in the CNS of wild type mice

In wild-type as well as knockout mice, there were no gender differences in the measurements of monoamines and their metabolites. As shown in Table 1, endogenous 5-HT and its metabolites were present throughout the brain and spinal cord of +/+ mice. As in other species of mice (e.g., Ase et al., 2000), 5-HT concentrations varied up to ninefold between the 17 regions

examined. The lowest 5-HT levels were measured in cerebellum, and the highest in caudate-putamen and nucleus accumbens. The 5-HT metabolites, 5-HIAA and 5-HTOL, showed comparable interregional variations (six- to 10-fold differences); their lowest levels were in olfactory bulb and cerebellum, and the highest in thalamus and hypothalamus (5-HIAA) and then the dorsal/medial raphe nuclei (5-HTOL). NA was detected in all regions (Table 1), with variations up to 10-fold between lowest (olfactory bulb) and highest (locus coeruleus and hypothalamus) levels.

DA and its metabolites (Table 2) were also present in all regions, although barely detectable in the cerebellum. DA concentration was several hundredfold higher in caudate-putamen than spinal cord, hippocampus or frontal cortex. Parallel differences were found in the regional levels of DA metabolites. Indeed, a linear regression analysis indicated a very good correlation between the regional DA levels and those of each of its three metabolites ($r = 0.986$ for DA versus DOPAC; 0.976 for DA versus HVA; 0.982 for DA versus 3-MT; $p < 0.001$).

As indicated in Table 3, the ratios [5-HT metabolites] / [5-HT] ranged from 3.2 in cerebellum to 1.0 in caudate-putamen. DA turnover appeared more variable, with [DA metabolites] / [DA] ratios from 6.1 in frontal cortex to 0.9 in caudate-putamen. In the majority of regions examined, except nucleus accumbens and globus pallidus, the DA turnover index was higher than that of 5-HT.

Monoamines and their metabolites in the CNS of 1A –/– mice

There were few statistically significant differences in regional 5-HT concentration between 1A –/– and +/+ mice in the CNS regions examined (Table 1). In contrast, 5-HIAA content was elevated significantly in five brain regions of 5-HT1A knockouts, namely, in olfactory bulb (+ 193%), substantia nigra (+ 133%), dorsal/medial raphe nuclei (+ 110%) and locus coeruleus (+ 86%) and thalamus (+ 47%). Levels of 5-HTOL were elevated similarly in substantia nigra (+ 124%), dorsal/medial raphe nuclei (+ 105%), and probably in locus coeruleus (+ 61%), but not in olfactory bulb and thalamus. As evidenced by high [5-HT metabolites] / [5-HT] ratios (Table 3), 5-HT turnover was considerably increased in these four regions (olfactory bulb, + 87%; substantia nigra, + 138%; dorsal/medial nuclei, + 79%; locus coeruleus, + 103%).

The NA content was increased (+ 74%) in olfactory bulb, thalamus (+ 57%), substantia nigra (+ 63%) and cerebellum (+ 52%) of 1A –/– mice. There were also differences in DA, DOPAC and HVA levels, as indicated in Table 2. DA was increased in caudate-putamen (+ 55%), DOPAC in olfactory bulb (+ 120%) and HVA in substantia nigra (+ 59%). In spite of the DA and 3-MT increases in caudate-putamen, DA turnover ([DA metabolites] / [DA]) was apparently unchanged in this region. However, it was markedly increased in olfactory bulb (+ 81%) and substantia nigra (+ 88%), as well as in the dorsal/medial raphe nuclei (+ 89%) (Table 3).

Monoamines and their metabolites in the CNS of 1B –/– mice

As indicated in Table 1, 5-HT concentration was significantly lower than that in the wild-type in three CNS regions of the 5-HT1B knockouts, namely the nucleus accumbens (– 46%), locus coeruleus (– 43%) and spinal cord (– 38%). Other regions (septum, caudate-putamen, globus pallidus, thalamus, hypothalamus and entorhinal/piriform cortex) showed 5-HT levels >30% lower than those in wild-type, but which could not be demonstrated to be significantly different because of greater individual variability in these measurements. 5-HT turnover was generally increased in these regions, but only in the septum (+ 53%) and spinal cord (+ 62%) were these differences statistically significant. NA concentration was unchanged throughout. DA was decreased in the nucleus accumbens (– 34%), entorhinal/piriform cortex (– 38%) and dorsal/medial raphe nuclei (– 38%) (Table 2). DOPAC, HVA and 3-MT were unchanged in all regions (Table 2) and DA turnover was increased (+ 32%) in the nucleus accumbens (Table 3).

DISCUSSION

This study revealed major changes in monoamine metabolism in discrete CNS regions of both 1A –/– and 1B –/– mutant mice of the 129/Sv strain.

Mouse lacking the 5-HT$_{1A}$ receptor

In 1A –/– knockouts, 5-HT concentration was comparable to wild-type mice throughout the brain and spinal cord, but the higher [5-HT metabolites]

/ [5-HT] ratios in dorsal/medial raphe nuclei, olfactory bulb, substantia nigra and locus coeruleus were indicative of increased 5-HT turnover and thus, presumably, of increased 5-HT release (Shannon et al. 1986; Rollema et al. 1996; Ross and Stenfors, 1997; Stenfors et al., 1999). As these increases involved the nuclei of origin of 5-HT neurons (dorsal/medial raphe nuclei) as well as three of their territories of innervation, it could be assumed that they reflected increases in both the somatodendritic (Hery et al., 1982) and axon terminal release of 5-HT. It is well established that somatodendritic 5-HT_{1A} autoreceptors mediate an inhibition of the firing and release by 5-HT neurons (Hjorth and Magnusson, 1988; Hamon et al., 1990). This inhibitory effect has been demonstrated to be present under basal conditions, as shown by increases in the spontaneous firing of 5-HT neurons in vivo or in vitro after systemic administration or superfusion of the highly potent and selective 5-HT_{1A} antagonist, WAY 100635 (Fornal et al., 1996; Corradeti et al., 1996) or p-MPPI (Bjorvatn et al., 1998). In the constitutive absence of this receptor, the increased 5-HT turnover in the nuclei of origin and territories of projection of 5-HT_{1A} knockouts was therefore likely to result from an enhanced tonic activity (lack of autoinhibition) of 5-HT neurons.

The increased 5-HT turnover in olfactory bulb could be explained on this basis, because this 5-HT innervation has been shown to originate from both the dorsal and median raphe nuclei in the mouse, as in the rat (Araneda et al., 1980ab; McLean and Shipley, 1987). Studies in neonate rats have indicated that this 5-HT input is important for the acquisition or expression

of olfactory-based learned behavior, and facilitates conditioned olfactory learning induced by NA (McLean et al., 1993; Price et al., 1998). Whether these behaviors are affected in the 1A –/– mice remains to be determined. If they are not, it would be of interest to identify the adaptative mechanism responsible for such a takeover of function.

In substantia nigra, pars reticulata, a double-labeling immunoelectron microscopic study has shown many 5-HT terminals to be in synaptic contact with tyrosine hydroxylase-positive as well as –negative, somata and dendrites (Corvaja et al., 1993). Facilitation of basal DA release by 5-HT itself, or by 5-HT agonists, has been demonstrated repeatedly in various subcortical areas, both *in vitro* and *in vivo* (Jiang et al., 1990; Benloucif and Galloway, 1991; Galloway et al., 1993; Parsons and Justice, 1993; Iyer and Bradberry, 1996). In the pars reticulata of substantia nigra, 5-HT has been shown to inhibit GABA release through 5-HT_{1B} receptors (Johnson et al., 1992; Stanford and Lacey, 1996) located on the preterminal axons of striatonigral neurons (Boschert et al., 1994; Doucet et al., 1995; Riad et al., 2000). The enhanced tonic activity of midbrain 5-HT neurons in 1A –/– mice should therefore entail both a direct facilitation and concomitant disinhibition of the nigral DA neurons. The ensuing disinhibition of the nigral DA neurons could in turn account for the elevation of DA or its metabolites in the caudate-putamen as well as other DA-innervated brain regions. The higher DA turnover in olfactory bulb and dorsal/medial raphe nuclei might result from similar mechanisms operating within these regions, in which DA

neurons are present, albeit in smaller number than in the substantia nigra (Descarries et al., 1982; Björklund and Lindvall, 1984).

The increased 5-HT turnover in the locus coeruleus drew attention to the anxious-like behavior in 5-HT_{1A} knockout mice. In rodents as well as in primates, anxious-like behaviors and increased responses to stressful stimuli have generally been associated with increased activity in this noradrenergic nucleus (review in Charney et al., 1995). The NA concentration was unchanged in locus coeruleus, but it showed an increase in olfactory bulb, thalamus, substantia nigra and cerebellum, all regions innervated by the locus coeruleus. Even if the interpretation of these data was somewhat hampered by the lack of measurement of NA metabolites, it was plausible that such changes reflected a diminished activity of NA neurons in the locus coeruleus, secondary to the constitutive increase in serotoninergic tone (Haddjeri et al., 1997; Kaehler et al., 1999).

In rodents, it is generally assumed that activation of 5-HT_{1A} autoreceptors produces its anxiolytic-like effects via the suppression of 5-HT neuron activity and consequent decrease in 5-HT release (Sprouse and Aghajanian, 1987; Andrews et al., 1994; De Vry, 1995). The converse enhancement of anxiety observed in 1A –/– mice might be secondary to the increased activity of 5-HT neurons in the dorsal/medial raphe nuclei. The associated increases in DA and NA contents, and particularly in DA turnover, might also contribute to the abnormal behavioral phenotype in these mice.

Mouse lacking the 5-HT$_{1B}$ receptor

In 5-HT$_{1B}$ knockouts, the decreased 5-HT concentration in spinal cord, nucleus accumbens, locus coeruleus, and presumably other territories of 5-HT innervation also was associated with normal levels of 5-HT metabolites. This suggested a dysregulation in the biosynthesis of 5-HT at the level of the nerve terminals in the absence of 5-HT$_{1B}$ autoreceptors. Other regions of 5-HT projection did not show such changes, in keeping with the results of a microdialysis study (Trillat et al., 1997), which did not demonstrate significant differences in the basal or potassium-evoked release of 5-HT in the frontal cortex and ventral hippocampus of 1B –/– mice. The lack of altered 5-HT turnover in the raphe nuclei was also consistent with recent electrophysiological findings of Evrard et al. (1999), who found no changes in the spontaneous firing of the raphe 5-HT neurons, or in the potency of the agonist 8-OH-DPAT to inhibit these cells, in 1B –/– mice.

The few changes in DA metabolism observed in 1B –/– mice were not as readily explained as in 1A –/– mice. The decreased DA concentration in nucleus accumbens was associated with an increased DA turnover, perhaps as a consequence of the augmented 5-HT turnover in this region. Indeed, concordant microdialysis studies have demonstrated a 5-HT-induced increase in extracellular DA in rat nucleus accumbens upon local application of 5-HT or *in vivo* stimulation of the dorsal raphe nucleus (Parsons and Justice, 1993; De Deurwaerdere and Spampinato, 1999). Furthermore,

recently, both basal extracellular and cocaine-evoked DA levels were shown to be increased in the nucleus accumbens of 1B –/– mice (Shippenberg et al., 2000). These changes are likely to be related to the increased vulnerability to drugs of abuse, such as cocaine, reported in these mice (Rocha et al., 1998; Scearce-Levie et al., 1999). Such a greater sensitivity to cocaine has thus far been difficult to explain, since behavioral studies in rat and mouse have shown that 5-HT_{1B} stimulation reinforces the effects of cocaine, and that antagonism of the receptor can reverse this enhancement (Lucas et al., 1997; Parsons et al., 1998). The higher turnover of DA in the nucleus accumbens of 1B –/– mice might account for a constitutive state of sensitization resembling that of a wild-type mouse under a chronic cocaine regimen (Rocha et al., 1998).

There is considerable evidence to suggest that, in rodents, decreased brain 5-HT content is associated with aggressive behavior (Maas, 1962; Vergnes et al., 1986), whereas increased 5-HT reduces aggressiveness (Molina et al., 1987). The aggressiveness of 5-HT_{1B} knockouts has been shown to be greater than in wild-type mice in different behavioral tests, including territorial and maternal aggression (Saudou et al., 1994; for review, see Brunner and Hen, 1997). These altered behaviors in 5-HT_{1B} knockouts might be associated with some of the above described alterations in 5-HT and DA metabolism.

Conclusions

In many ways, the constitutive absence of 5-HT_{1A} or 5-HT_{1B} receptors in 129/Sv mice seems to have markedly different effects on the regional metabolism of monoamines in the CNS of these mice, which may entail opposite changes in their behavioral phenotypes (Zhuang et al., 1999). The lack of somatodendritic 5-HT_{1A} autoreceptors apparently enhances the tonic activity of the midbrain 5-HT neurons, which secondarily affects DA metabolism and NA content in several of territories of 5-HT innervation. Direct measurements of the activity of midbrain raphe neurons or of the release of 5-HT in some of these regions could be determinant in this regard, and test our hypothesis that increased tonic activity of the midbrain 5-HT neurons accounts for the anxious-like behavioral phenotype in these mice (see also Parks et al., 1998). The lack of 5-HT_{1B} autoreceptors is associated with reductions in 5-HT content in many territories of 5-HT innervation, presumed increases in 5-HT turnover and, notably, a similar pattern of changes for DA in the nucleus accumbens. It is likely that some of these changes account for the propensity of these mice to self-administer cocaine as well as for their increased aggressive behavior.

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FIGURE LEGENDS

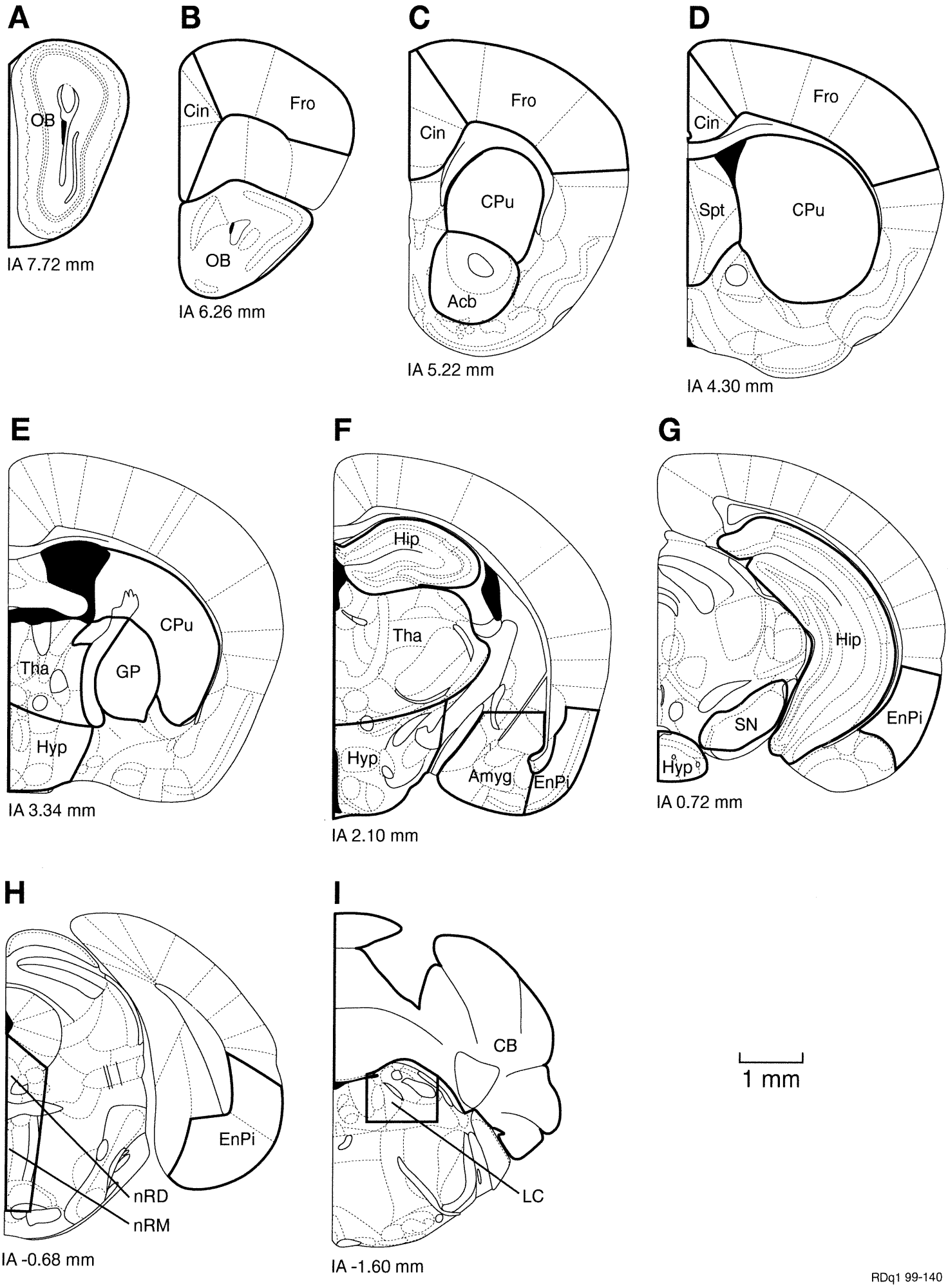
FIG. 1. Rostrocaudal transverse sections across a mouse cerebral hemisphere, illustrating the location of the specimens measured for their content in monoamines and metabolites (adapted from Franklin and Paxinos, 1997). The thick lines delineate the borders of the 16 areas selected. The spinal cord was dissected as a whole. The olfactory bulb (OB in **A** and **B**) was detached from the frontal pole. The cingulate cortex (Cin in **B–D**) included the prelimbic and cingulate areas, whereas the frontal cortex (Fro in **B-D**) comprised both primary and secondary motor cortices, as well as the primary somatosensory cortex. The nucleus accumbens (Acb in **C**) was sampled at the level of the rostral neostriatum and included the intranuclear portion of the anterior commissure. The septum (Spt in **D**) comprised the lateral dorsal, lateral intermediate, lateral ventral, and medial septal nuclei. The caudate-putamen (CPu in **C-E**) included tissue from its rostral and caudal halves, in front and behind the decussation of the anterior commissure. In dissecting the globus pallidus or lateral globus pallidus (GP in **E**), care was taken to exclude the internal capsule. The thalamic sample (Tha in **E** and **F**) comprised both anterior and posterior divisions, extending dorsally to the fimbria and dorsal hippocampus, laterally to the internal capsule and ventrally to the mammillothalamic tract. The hypothalamus (Hyp in **E–G**) began rostrally at the level of the optic chiasma and extended caudally up to the mammillary complex. The amygadala (Amyg in **F**) included mainly the basal, medial, anterior and posterior cortical nuclei. The sample

of entorhinal/piriform cortex (EnPi in **F–H**) comprised the ventral cortex lateral to the amygdala and the parasubiculum. The hippocampus (Hip in **F** and **G**) was separated from the midbrain and the overlying cerebral cortex, as outlined. In sampling the substantia nigra (SN in **G**), the ventral tegmental area was not included, nor the cerebral peduncle. The mesencephalic raphe nuclei (nRD/nRM in **H**) were contained in a trapezoidal sample from the caudal mesencephalon and rostral pons. The cerebellum (CB in **I**) was separated from the brainstem and included vermis and cerebellar hemispheres. The locus coeruleus (LC in **I**) was dissected as a rectangular sample, using the superior cerebellar peduncle and facial nerve root as landmarks.

FIG. 2. Chromatograms showing the separation of the different peaks for a standard solution (**A**) and specimens of dorsal/medial raphe nuclei (**B**), septum (**C**) and globus pallidus (**D**) from a +/+ 129/Sv mouse. The standard solution contained 0.5 ng each of the authentic compounds: (1) MHPG, (2) NA, (3) DOPAC, (4) 5-HTOL, (5) 5-HIAA, (6) DA, (7) HVA, (8) 5-HT, (9) 3-MT. The mobile phase consisted of 0.1*M* monochloroacetic acid (pH 3.3) containing 2.0 m*M* NA_2EDTA and 1.0 m*M* sodium octyl sulphate, and 10 % methanol. The flow rate was set at 0.5 ml/min, and the temperature of the column kept at 33–35°C. The samples were injected into a 3 μm particle-size analytical chromatographic column (100.0 x 4.6 mm; Adsorbosphere Catecholamine; Althech). The compounds were oxidized with a glassy carbon

electrode set at a potential of +680 m*V* relative to the reference electrode of the electrochemical detector (EE&G M-400, Princenton Applied Research), and the gain was usually 10 *n*A full scale. The indoleamine 5-HT (8), and its metabolites, 5-HIAA (5) and 5-HTOL (4), were highly concentrated in the three tissues shown, and 5-HTOL was present in huge amount in the dorsal/medial raphe nuclei compared to the septum and globus pallidus. The catecholamine DA (6), and its metabolites, DOPAC (3), HVA (7) and 3-MT (9), were found in decreasing concentration in globus pallidus, septum and dorsal/medial raphe nuclei.

FIG. 3. Autoradiographs of [^{3}H]8-OH-DPAT (**A–D**) and of [^{125}I]cyanopindolol (**E–H**) specific binding in the brain of wild-type (+/+; A,C,E and G), and 5-HT$_{1A}$ (1A –/–; B and D) and 5-HT$_{1B}$ (1B –/–; F and H) knockout 129/Sv mice. In the wild-type mouse, note the expected high density of labeling of [^{3}H]8-OH-DPAT to 5-HT$_{1A}$ receptors in the dorsal raphe nucleus (nRD in A) and hippocampus (Hip in C), and that of [^{125}I]cyanopindolol to 5-HT$_{1B}$ receptors in the globus pallidus (GP in E) and substantia nigra (SN in G). In contrast, there is a total lack of the respective binding sites in the corresponding null mutants. The nontransformed autoradiographic images were digitized, and exported as TIFF files that were resampled at a resolution of 300 x 300 dpi.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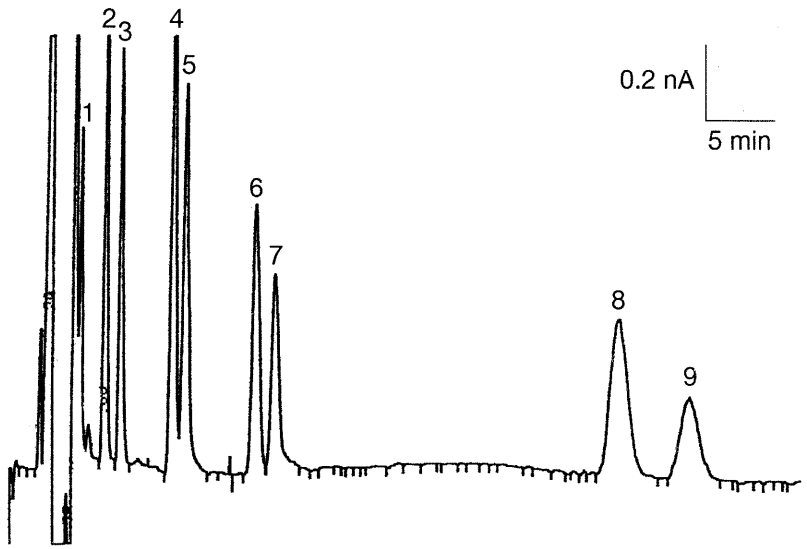
OB
IA 7.72 mm
B
Cin
Fro
OB
IA 6.26 mm
C
Fro
Cin
CPu
Acb
IA 5.22 mm
D
Fro
Cin
Spt
CPu
IA 4.30 mm
E
CPu
Tha
GP
Hyp
IA 3.34 mm
F
Hip
Tha
Hyp
Amyg
EnPi
IA 2.10 mm
G
Hip
SN
EnPi
Hyp
IA 0.72 mm
H
EnPi
nRD
nRM
IA -0.68 mm
I
CB
LC
IA -1.60 mm
1 mm
RDq1 99-140

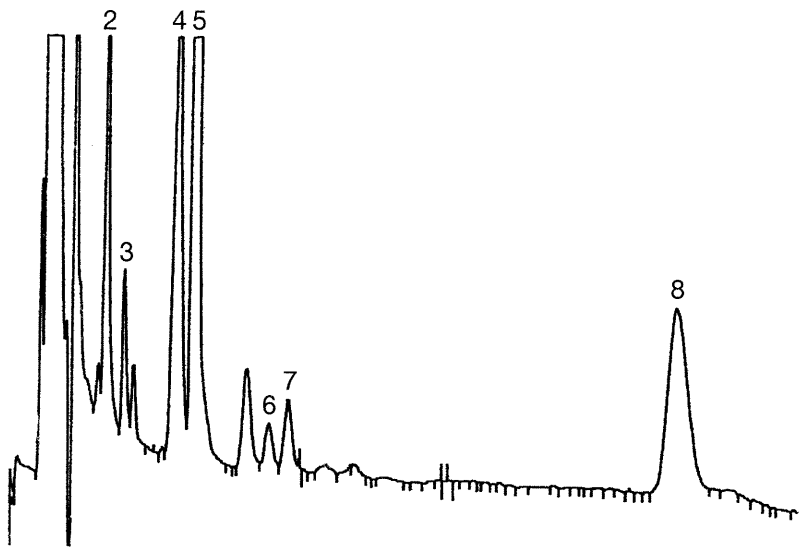
A

STANDARD



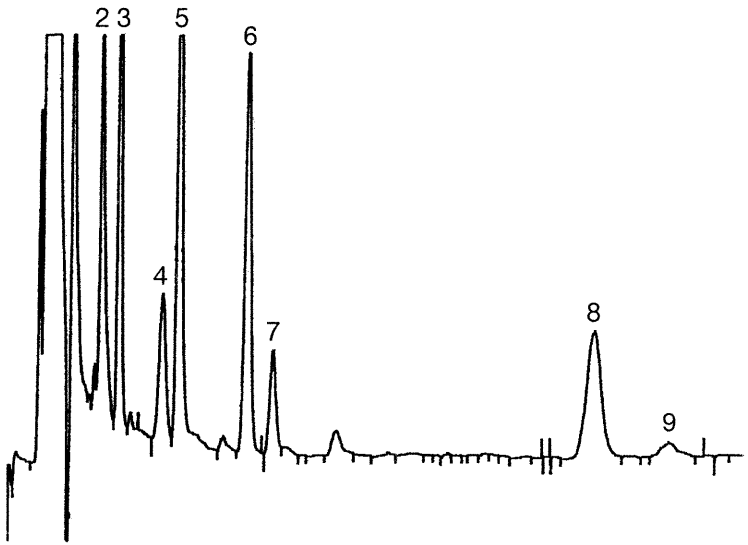
B

DORSAL/MEDIAL RAPHE NUCLEI



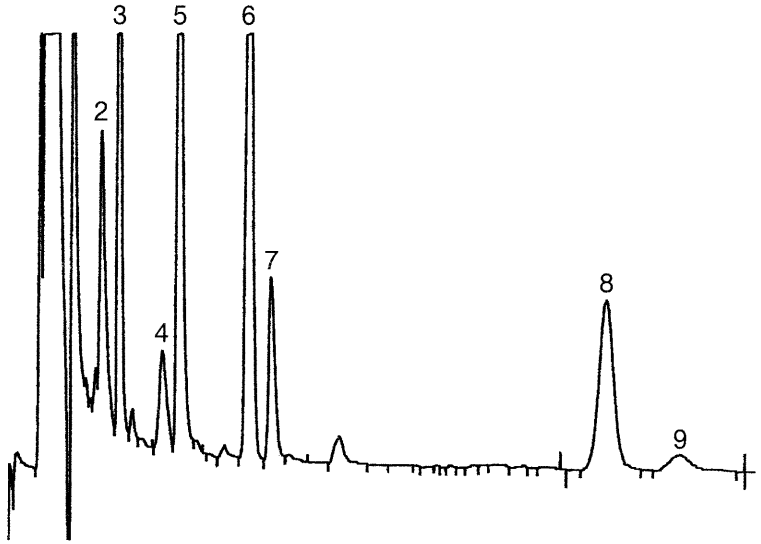
C

SEPTUM



D

GLOBUS PALLIDUS



RDq1 99-155-1

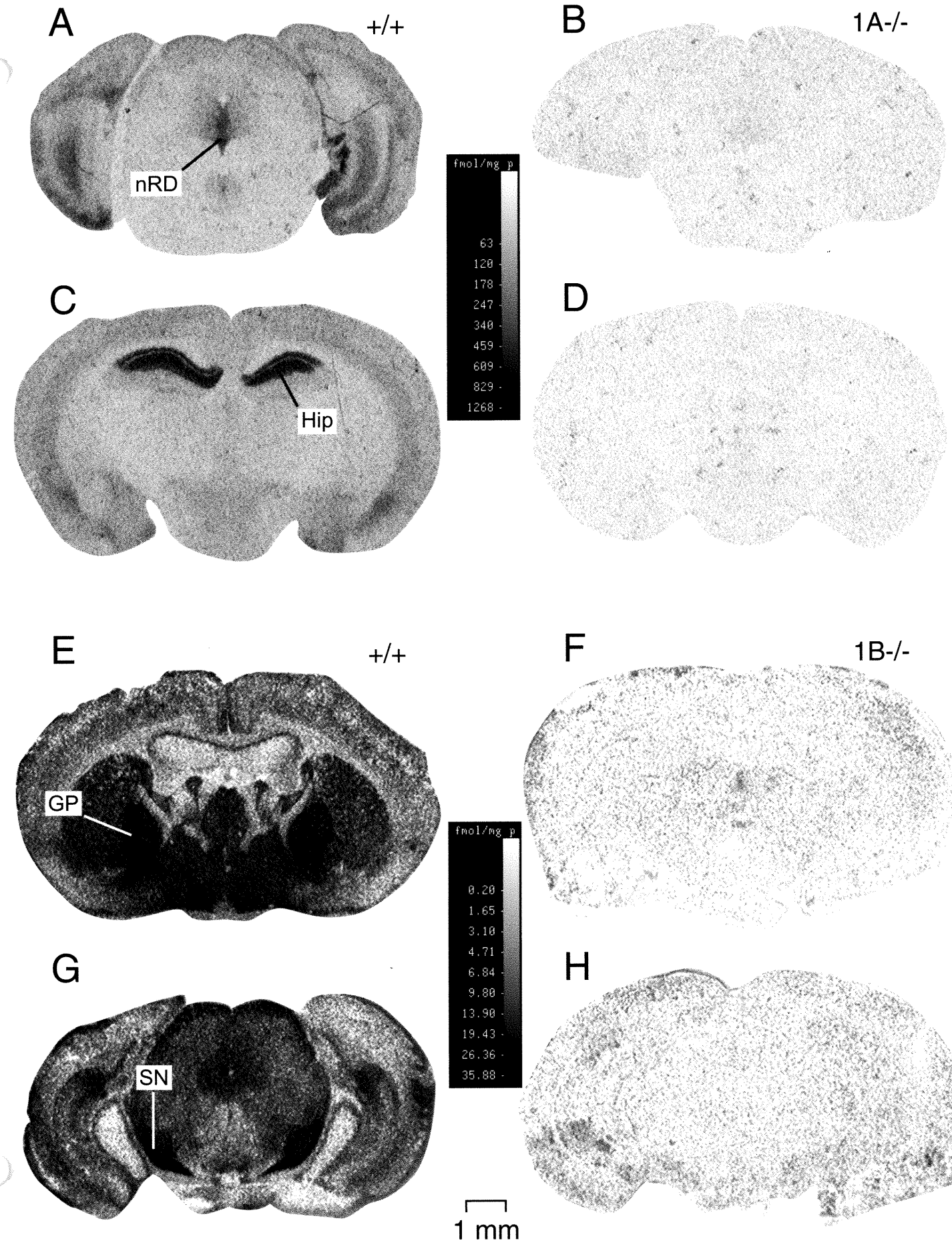

A
+/+
nRD
B
1A-/-
fmol/mg p
63
120
178
247
340
459
609
829
1268
C
Hip
D
E
+/+
GP
F
1B-/-
fmol/mg p
0.20
1.65
3.10
4.71
6.84
9.80
13.90
19.43
26.36
35.88
G
SN
H
1 mm

TABLE 1. *Tissue levels of serotonin (5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), 5-hydroxytryptophol (5-HTOL) and noradrenaline (NA) in wild type (+/+), 5-HT$_{1A}$ (1A -/-) and 5-HT$_{1B}$ (1B -/-) receptor knockout mice.*

Region	Mouse	5-HT	5-HIAA	5-HTOL	NA
Olfactory bulb	+/+	2.69 ± 0.37	2.67 ± 0.19	0.30 ± 0.02	1.15 ± 0.12
	1A -/-	3.15 ± 0.45	7.81 ± 0.97 **	0.32 ± 0.04	2.00 ± 0.16 **
	1B -/-	2.06 ± 0.33	2.42 ± 0.11	0.29 ± 0.02	1.17 ± 0.10
Cingulate cortex	+/+	3.08 ± 0.38	5.60 ± 0.39	0.37 ± 0.03	1.81 ± 0.17
	1A -/-	4.37 ± 0.17 *	7.67 ± 0.57	0.30 ± 0.03	2.59 ± 0.13
	1B -/-	2.98 ± 0.33	4.75 ± 0.83	0.35 ± 0.06	1.98 ± 0.32
Frontal cortex	+/+	5.06 ± 0.36	6.93 ± 0.55	0.40 ± 0.05	1.70 ± 0.11
	1A -/-	6.27 ± 0.82	9.69 ± 0.79	0.30 ± 0.02	2.04 ± 0.19
	1B -/-	5.71 ± 0.36	6.35 ± 1.02	0.40 ± 0.03	1.86 ± 0.09
Nucleus accumbens	+/+	8.17 ± 0.68	5.69 ± 0.41	1.07 ± 0.15	n.d.
	1A -/-	5.62 ± 0.40 *	6.14 ± 0.47	1.54 ± 0.16	n.d.
	1B -/-	4.37 ± 0.47 **	5.57 ± 0.78	0.80 ± 0.12	n.d.
Septum	+/+	6.83 ± 1.21	12.36 ± 1.70	1.13 ± 0.13	3.27 ± 0.31
	1A -/-	4.07 ± 0.16	10.79 ± 1.26	1.21 ± 0.05	3.91 ± 0.30
	1B -/-	3.91 ± 0.33	10.63 ± 1.39	1.21 ± 0.05	3.42 ± 0.39
Caudate-putamen	+/+	8.54 ± 0.97	6.25 ± 0.96	0.89 ± 0.09	n.d.
	1A -/-	8.61 ± 0.85	7.74 ± 0.72	1.08 ± 0.16	n.d.
	1B -/-	5.57 ± 0.59	6.09 ± 0.99	0.83 ± 0.07	n.d.
Globus pallidus	+/+	6.40 ± 0.62	9.26 ± 1.15	1.59 ± 0.09	2.52 ± 0.46
	1A -/-	5.53 ± 0.59	9.55 ± 1.16	1.85 ± 0.25	2.98 ± 0.28
	1B -/-	4.40 ± 0.21	9.11 ± 1.07	1.35 ± 0.09	2.45 ± 0.22
Thalamus	+/+	5.83 ± 0.74	15.56 ± 1.16	0.85 ± 0.14	5.08 ± 0.26
	1A -/-	6.57 ± 0.72	22.91 ± 1.59 *	0.65 ± 0.13	8.00 ± 0.75 *
	1B -/-	3.82 ± 0.39	15.47 ± 1.83	0.92 ± 0.13	4.81 ± 0.33
Hypothalamus	+/+	6.08 ± 0.51	15.74 ± 0.98	1.41 ± 0.20	10.89 ± 0.68
	1A -/-	5.23 ± 0.67	15.06 ± 1.01	0.94 ± 0.14	12.34 ± 0.71
	1B -/-	4.16 ± 0.16	15.08 ± 0.77	1.83 ± 0.21	12.55 ± 0.42

TABLE 1 (continued)

Region	Mouse	5-HT	5-HIAA	5-HTOL	NA
Amygdala	+/+	5.62 ± 0.43	11.27 ± 1.33	0.60 ± 0.05	2.13 ± 0.14
	1A -/-	5.50 ± 0.32	11.09 ± 1.78	0.51 ± 0.05	2.75 ± 0.11
	1B -/-	5.50 ± 0.64	12.55 ± 1.52	0.64 ± 0.06	2.47 ± 0.21
Hippocampus	+/+	5.08 ± 0.34	11.85 ± 0.53	0.41 ± 0.04	2.79 ± 0.27
	1A -/-	5.38 ± 0.40	12.92 ± 1.02	0.31 ± 0.03	3.53 ± 0.61
	1B -/-	4.04 ± 0.34	11.50 ± 0.93	0.52 ± 0.04	3.41 ± 0.40
Entorhinal/Piriform cortex	+/+	6.10 ± 0.51	11.29 ± 0.94	0.63 ± 0.08	1.84 ± 0.14
	1A -/-	5.40 ± 0.54	11.09 ± 0.80	0.33 ± 0.03	2.00 ± 0.14
	1B -/-	4.48 ± 0.41	9.00 ± 0.73	0.52 ± 0.05	1.98 ± 0.25
Substantia nigra	+/+	7.82 ± 1.17	6.76 ± 0.57	1.20 ± 0.09	2.15 ± 0.25
	1A -/-	7.66 ± 0.63	15.76 ± 1.44 **	2.69 ± 0.20 **	3.50 ± 0.40 *
	1B -/-	6.66 ± 0.47	6.77 ± 0.88	1.35 ± 0.08	2.69 ± 0.21
Dorsal and medial raphe nuclei	+/+	5.88 ± 0.80	11.38 ± 1.40	2.08 ± 0.12	9.39 ± 0.72
	1A -/-	6.70 ± 0.57	23.88 ± 2.02 **	4.27 ± 0.27 **	7.65 ± 0.98
	1B -/-	5.18 ± 0.48	10.26 ± 0.92	1.84 ± 0.15	9.05 ± 0.85
Cerebellum	+/+	0.94 ± 0.14	2.92 ± 0.35	0.20 ± 0.03	2.64 ± 0.33
	1A -/-	1.21 ± 0.12	3.19 ± 0.26	0.17 ± 0.01	4.16 ± 0.26 *
	1B -/-	0.96 ± 0.09	2.78 ± 0.46	0.21 ± 0.03	2.33 ± 0.16
Locus coeruleus	+/+	3.77 ± 0.43	4.98 ± 0.39	0.94 ± 0.09	10.20 ± 1.10
	1A -/-	3.03 ± 0.27	9.27 ± 0.89 **	1.51 ± 0.19	8.18 ± 1.16
	1B -/-	2.13 ± 0.17 *	3.50 ± 0.33	0.72 ± 0.13	9.60 ± 1.46
Spinal cord	+/+	5.29 ± 0.35	7.00 ± 0.52	0.54 ± 0.07	4.30 ± 0.24
	1A -/-	6.67 ± 0.26 *	6.61 ± 0.30	0.30 ± 0.01	4.61 ± 0.25
	1B -/-	3.29 ± 0.31 **	6.68 ± 0.71	0.64 ± 0.05	3.75 ± 0.25

The values are the means ± SEM (n = 6-8) in nanograms per milligram of protein (ng/mg protein). n.d. = not determined. Statistical comparisons were made by a one-way analysis of variance, followed by Fisher's *F*-distribution test and the probabilities were: *$p < 0.001$, *$p < 0.01$, compared to +/+ .

TABLE 2. *Tissue levels of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3-MT) in wild type (+/+), 5-HT_{1A} (1A -/-) and 5-HT_{1B} (1B -/-) receptor knockout mice.*

Region	Mouse	DA	DOPAC	HVA	3-MT
Olfactory bulb	+/+	0.81 ± 0.08	0.40 ± 0.04	0.76 ± 0.08	0.27 ± 0.05
	1A -/-	0.89 ± 0.11	0.88 ± 0.09 **	1.50 ± 0.22 *	0.40 ± 0.06
	1B -/-	0.79 ± 0.05	0.47 ± 0.04	0.71 ± 0.04	0.26 ± 0.04
Cingulate cortex	+/+	0.51 ± 0.07	0.89 ± 0.09	0.95 ± 0.09	0.28 ± 0.03
	1A -/-	0.48 ± 0.04	0.88 ± 0.09	1.15 ± 0.08	0.21 ± 0.03
	1B -/-	0.63 ± 0.12	0.95 ± 0.20	1.07 ± 0.19	0.28 ± 0.04
Frontal cortex	+/+	0.41 ± 0.05	1.19 ± 0.14	1.34 ± 0.13	0.33 ± 0.07
	1A -/-	0.63 ± 0.11	1.56 ± 0.19	1.83 ± 0.18	0.34 ± 0.07
	1B -/-	0.32 ± 0.04	0.90 ± 0.10	1.07 ± 0.09	0.40 ± 0.14
Nucleus accumbens	+/+	69.09 ± 4.16	55.18 ± 4.56	10.84 ± 1.11	4.45 ± 0.79
	1A -/-	45.44 ± 4.01 *	47.34 ± 3.49	8.91 ± 0.66	4.22 ± 0.46
	1B -/-	45.48 ± 4.40 *	45.06 ± 4.05	9.15 ± 1.13	4.96 ± 0.43
Septum	+/+	4.92 ± 0.77	14.55 ± 0.98	4.32 ± 0.48	0.77 ± 0.13
	1A -/-	4.01 ± 0.37	10.61 ± 1.43	3.72 ± 0.24	0.75 ± 0.08
	1B -/-	5.51 ± 0.55	12.28 ± 1.42	3.64 ± 0.46	1.00 ± 0.08
Caudate-Putamen	+/+	95.18 ± 5.72	63.09 ± 4.62	16.60 ± 1.74	8.60 ± 0.36
	1A -/-	147.08 ± 9.73 **	79.32 ± 3.93	19.85 ± 1.43	13.84 ± 1.03 *
	1B -/-	94.78 ± 5.75	52.93 ± 4.27	14.18 ± 1.60	11.96 ± 1.24
Globus pallidus	+/+	22.89 ± 2.47	18.47 ± 2.05	6.57 ± 0.60	1.66 ± 0.17
	1A -/-	20.84 ± 3.08	24.28 ± 1.36	8.43 ± 0.27	2.65 ± 0.23
	1B -/-	22.55 ± 2.81	16.10 ± 1.73	6.52 ± 0.52	2.33 ± 0.30
Thalamus	+/+	0.91 ± 0.10	3.49 ± 0.59	1.73 ± 0.20	0.38 ± 0.09
	1A -/-	1.03 ± 0.18	3.30 ± 0.51	2.32 ± 0.25	0.48 ± 0.04
	1B -/-	1.04 ± 0.10	3.78 ± 0.41	1.79 ± 0.13	0.38 ± 0.04
Hypothalamus	+/+	2.22 ± 0.30	3.74 ± 0.37	2.05 ± 0.16	0.52 ± 0.12
	1A -/-	1.32 ± 0.11 *	2.90 ± 0.15	1.90 ± 0.21	0.21 ± 0.00
	1B -/-	1.83 ± 0.08	4.35 ± 0.51	1.91 ± 0.19	0.48 ± 0.14

TABLE 2 (continued)

Region	Mouse	DA	DOPAC	HVA	3-MT
Amygdala	+/+	5.41 ± 0.49	7.13 ± 1.15	2.94 ± 0.37	0.66 ± 0.08
	1A -/-	4.07 ± 0.43	5.07 ± 0.62	2.91 ± 0.33	0.75 ± 0.10
	1B -/-	5.08 ± 0.50	6.36 ± 0.80	2.53 ± 0.30	0.81 ± 0.14
Hippocampus	+/+	0.47 ± 0.08	0.89 ± 0.07	0.68 ± 0.06	0.40 ± 0.00
	1A -/-	0.50 ± 0.09	0.75 ± 0.10	0.67 ± 0.06	0.43 ± 0.12
	1B -/-	0.36 ± 0.06	0.71 ± 0.06	0.57 ± 0.04	0.25 ± 0.08
Entorhinal/Piriform cortex	+/+	1.14 ± 0.08	1.79 ± 0.26	1.19 ± 0.19	0.45 ± 0.07
	1A -/-	0.68 ± 0.09	1.02 ± 0.11	0.85 ± 0.08	0.47 ± 0.07
	1B -/-	0.71 ± 0.07 *	1.07 ± 0.18	0.80 ± 0.10	0.28 ± 0.02
Substantia nigra	+/+	2.65 ± 0.23	2.21 ± 0.16	1.45 ± 0.13	0.66 ± 0.11
	1A -/-	2.49 ± 0.30	4.18 ± 0.50 *	2.30 ± 0.12 **	1.02 ± 0.15
	1B -/-	4.32 ± 0.58	3.00 ± 0.29	1.44 ± 0.13	0.48 ± 0.10
Dorsal and medial raphe nuclei	+/+	1.25 ± 0.09	1.76 ± 0.09	1.32 ± 0.12	0.64 ± 0.12
	1A -/-	0.77 ± 0.09 *	2.04 ± 0.18	1.55 ± 0.15	0.43 ± 0.04
	1B -/-	0.77 ± 0.09 *	1.45 ± 0.14	0.96 ± 0.09	0.55 ± 0.08
Cerebellum	+/+	0.07 ± 0.01	0.25 ± 0.02	0.20 ± 0.01	0.15 ± 0.04
	1A -/-	0.08 ± 0.01	0.21 ± 0.02	0.22 ± 0.01	0.18 ± 0.05
	1B -/-	0.08 ± 0.01	0.26 ± 0.03	0.24 ± 0.03	0.20 ± 0.04
Locus coeruleus	+/+	0.49 ± 0.04	0.77 ± 0.06	0.65 ± 0.08	0.41 ± 0.07
	1A -/-	0.38 ± 0.04	0.93 ± 0.09	0.54 ± 0.04	0.33 ± 0.09
	1B -/-	0.46 ± 0.05	0.60 ± 0.08	0.57 ± 0.07	0.46 ± 0.06
Spinal cord	+/+	0.32 ± 0.02	0.36 ± 0.02	0.14 ± 0.01	0.27 ± 0.04
	1A -/-	0.27 ± 0.01	0.22 ± 0.01	0.13 ± 0.01	0.37 ± 0.04
	1B -/-	0.27 ± 0.02	0.44 ± 0.04	0.20 ± 0.02	0.38 ± 0.06

The values are the means ± SEM (n = 6-8) in nanograms per milligram of protein (ng/mg protein). n.d. = not determined. Statistical comparisons were made by a one-way analysis of variance, followed by Fisher's *F*-distribution test and the probabilities were: $*p < 0.001$, $*p < 0.01$, compared to +/+ .

TABLE 3. *Indices of 5-HT and DA turnover in wild type (+/+), and 5-HT$_{1A}$ (1A -/-) and 5-HT$_{1B}$ (1B -/-) receptor knockout mice.*

Region	Mouse	5-HT turnover	DA turnover
Olfactory bulb	+/+	1.27 ± 0.08	1.81 ± 0.12
	1A -/-	2.38 ± 0.13 **	3.27 ± 0.30 **
	1B-/-	1.36 ± 0.08	1.85 ± 0.08
Cingulate cortex	+/+	1.92 ± 0.19	3.97 ± 0.11
	1A -/-	1.78 ± 0.13	4.21 ± 0.18
	1B -/-	1.68 ± 0.18	3.86 ± 0.15
Frontal cortex	+/+	1.19 ± 0.15	6.08 ± 0.50
	1A -/-	1.23 ± 0.06	5.91 ± 0.83
	1B -/-	1.00 ± 0.07	7.23 ± 0.34
Nucleus accumbens	+/+	1.30 ± 0.13	1.05 ± 0.05
	1A -/-	1.39 ± 0.04	1.36 ± 0.09 *
	1B -/-	1.80 ± 0.23	1.39 ± 0.05 *
Septum	+/+	1.91 ± 0.14	2.84 ± 0.28
	1A -/-	2.83 ± 0.25	2.96 ± 0.16
	1B -/-	2.92 ± 0.26 *	2.36 ± 0.24
Caudate-putamen	+/+	1.05 ± 0.06	0.90 ± 0.05
	1A -/-	1.11 ± 0.05	0.81 ± 0.03
	1B -/-	1.50 ± 0.16	0.81 ± 0.03
Globus pallidus	+/+	1.89 ± 0.16	1.07 ± 0.05
	1A -/-	2.06 ± 0.19	1.73 ± 0.18 *
	1B -/-	2.57 ± 0.21	1.27 ± 0.15
Thalamus	+/+	3.10 ± 0.26	5.52 ± 0.43
	1A -/-	3.82 ± 0.19	5.81 ± 0.43
	1B -/-	3.89 ± 0.14	5.69 ± 0.64
Hypothalamus	+/+	3.06 ± 0.25	3.65 ± 0.52
	1A -/-	3.37 ± 0.19	3.92 ± 0.90
	1B -/-	4.04 ± 0.41	3.70 ± 0.37

TABLE 3 (continued)

Region	Mouse	5-HT turnover	DA turnover
Amygdala	+/+	1.94 ± 0.17	2.04 ± 0.18
	1A -/-	2.05 ± 0.16	2.15 ± 0.14
	1B -/-	2.19 ± 0.26	1.86 ± 0.22
Hippocampus	+/+	2.48 ± 0.15	2.97 ± 0.46
	1A -/-	2.62 ± 0.10	3.75 ± 0.29
	1B -/-	2.81 ± 0.16	3.15 ± 0.44
Entorhinal/Piriform cortex	+/+	1.82 ± 0.14	3.01 ± 0.15
	1A -/-	1.77 ± 0.07	3.16 ± 0.17
	1B -/-	1.81 ± 0.11	2.67 ± 0.17
Substantia nigra	+/+	1.16 ± 0.10	1.78 ± 0.04
	1A -/-	2.76 ± 0.24 **	3.35 ± 0.22 **
	1B -/-	1.38 ± 0.10	1.36 ± 0.10
Dorsal and medial raphe nuclei	+/+	2.04 ± 0.13	2.87 ± 0.19
	1A -/-	3.66 ± 0.17 **	5.42 ± 0.26 **
	1B -/-	2.33 ± 0.15	3.15 ± 0.48
Cerebellum	+/+	3.20 ± 0.20	6.20 ± 0.47
	1A -/-	3.13 ± 0.20	6.67 ± 0.50
	1B -/-	2.58 ± 0.20	5.80 ± 0.64
Locus coeruleus	+/+	1.81 ± 0.10	4.17 ± 0.30
	1A -/-	3.68 ± 0.20 **	4.75 ± 0.44
	1B -/-	1.94 ± 0.12	3.96 ± 0.36
Spinal cord	+/+	1.57 ± 0.08	2.60 ± 0.12
	1A -/-	1.03 ± 0.04	2.87 ± 0.08
	1B -/-	2.54 ± 0.32 *	3.52 ± 0.31

The values are the means ± SEM (n = 6-8). Statistical comparisons were made by one-way analysis of variance, followed by Fisher's *F*-distribution test and the probabilities were : $^{**}p < 0.001$, $^{*}p < 0.01$, compared to +/+ .

Chapter III

Regional changes in density of serotonin transporter in the brain of 5-HT_{1A} or 5-HT_{1B} receptor knockout mice, and of serotonin innervation in the 5-HT_{1B} knockout

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As first author, I carried out most of the experiments, analyzed the data, and contributed in a significant way to all the steps toward the writing of the article.

Résumé — Les souris knockout (KO) 5-HT_{1A} présentent un phénotype de type anxieux, alors que les KO 5-HT_{1B} sont hyperagressives. Pour identifier les corrélats sérotoninergiques de ces comportements anormaux, nous avons visualisé et mesuré en autoradiographie les récepteurs 5 HT_{1A} and 5-HT_{1B} de la sérotonine (5-HT), ainsi que son transporteur membranaire, à l'aide des radioligands [^{3}H]8-OH-DPAT, [^{125}I]cyanopindolol et [^{3}H]citalopram, respectivement. Par comparaison aux souris sauvages (WT), la densité des récepteurs 5-HT_{1B} est apparue inchangée dans toutes les régions du cerveau des souris KO 5-HT_{1A}, de même que celle des récepteurs 5-HT_{1A} chez les KO 5-HT_{1B}. Par contre, une diminution de la densité de liaison au 5-HTT a été mesurées dans quelques régions chez les deux mutants. En outre, la densité de liaison au 5-HTT s'est avérée significativement augmentée dans le noyau amygdalo-hippocampique et l'hippocampus ventral du KO 5-HT_{1B}. Une quantification immunocytochimique de la longueur du réseau axonal immunoréactif pour la 5-HT et du nombre de ses varicosités a montré un accroissement proportionnel de la densité d'innervation 5-HT dans ces mêmes deux régions du KO 5-HT_{1B}, tandis qu'aucune des diminutions des sites de radioliaison au 5-HTT n'était associée à un changement de ce genre. Les conclusions qui suivent ont été tirées de ces résultats. (i) Les récepteurs 5-HT_{1B} ne font pas l'objet d'adaptation chez le mutant nul 5-HT_{1A}, et vice versa. (ii) Le 5-HTT subit une régulation à la baisse dans plusieurs régions cérébrales des deux mutants. (iii) Cette régulation à la baisse pourrait contribuer au phénotype de type anxieux du KO 5-HT_{1A}, en freinant la disparition (clearance) de la 5-HT dans divers territoires d'innervation 5-HT. (iv) L'hyperinnervation 5-HT du noyau amygdalo-hippocampique et de l'hippocampus ventral des KO 5-HT_{1B} pourrait contribuer à l'agressivité excessive de ces souris et expliquer leur performance améliorée lors de certaines épreuves de fonction cognitive. (v) De telles augmentation de densité d'innervation 5-HT sont la première indication d'un contrôle négatif de la croissance des neurones 5-HT exercé par l'entremise du récepteur 5-HT_{1B}.

REGIONAL CHANGES IN DENSITY OF SEROTONIN TRANSPORTER IN THE BRAIN OF 5-HT$_{1A}$ AND 5-HT$_{1B}$ KNOCKOUT MICE, AND OF SEROTONIN INNERVATION IN THE 5-HT$_{1B}$ KNOCKOUT

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Abbreviations: 5-HT, serotonin; 5-HTT, serotonin transporter; AHiPM, amygdalohippocampal posteromedial nucleus; BLP, basolateral posterior nucleus; KO, knockout; LD/LP, laterodorsal/lateroposterior thalamic nuclei; WT, wild-type.

Abstract — 5-HT_{1A} knockout (KO) mice display an anxious-like phenotype, whereas 5-HT_{1B} KOs are over-aggressive. To identify serotoninergic correlates of these altered behaviors, autoradiographic measurements of 5 HT_{1A} and 5-HT_{1B} serotonin (5-HT) receptors and transporter (5-HTT) were obtained using the radioligands [^{3}H]8-OH-DPAT, [^{125}I]cyanopindolol and [^{3}H]citalopram, respectively. By comparison to wild-type, density of 5-HT_{1B} receptors was unchanged throughout brain in 5-HT_{1A} KOs, and that of 5-HT_{1A} receptors in 5-HT_{1B} KOs. In contrast, decreases in density of 5-HTT binding were measured in several brain regions of both genotypes. Moreover, 5-HTT binding density was significantly increased in the amygdalo-hippocampal nucleus and ventral hippocampus of the 5-HT_{1B} KOs. Measurements of 5-HT axon length and number of axon varicosities by quantitative 5-HT immunocytochemistry revealed proportional increases in the density of 5-HT innervation in these two regions of 5-HT_{1B} KOs, whereas none of the decreases in 5-HTT binding sites were associated with any such changes. Several conclusions could be drawn from these results: (i) 5-HT_{1B} receptors do not adapt in 5-HT_{1A} KOs, nor do 5-HT_{1A} receptors in 5-HT_{1B} KOs. (ii) 5-HTT is down-regulated in several brain regions of 5-HT_{1A} and 5-HT_{1B} KO mice. (iii) This down-regulation could contribute to the anxious-like phenotype of the 5-HT_{1A} KOs, by reducing 5-HT clearance in several territories of 5-HT innervation. (iv) The 5-HT hyperinnervation in the amygdalo-hippocampal nucleus and ventral hippocampus of 5-HT_{1B} KOs could play a role in their increased aggressiveness, and might also explain their better performance in some cognitive tests. (v) These increases in density of 5-HT innervation provide the first evidence for a negative control of 5-HT neuron growth mediated by 5-HT_{1B} receptors.

Keywords: 5 HT transporter down-regulation – 5 HT_{1A} receptor – 5 HT_{1B} receptor – hyperinnervation

Running title: 5-HT_{1A} and 5-HT_{1B} knockout mice

INTRODUCTION

Imbalance in serotonin (5-hydroxytryptamine; 5-HT) neurotransmission-modulation in the CNS has been proposed as a common etiological factor in some of the most frequent psychiatric disorders, particularly anxiety, aggressiveness, depression and obsessive-compulsive disorders (e.g., Peroutka 1993; Lucki, 1998; Blier and de Montigny, 1999). Among the numerous 5-HT receptors identified to date, the 5-HT_{1A} and 5-HT_{1B} subtypes have received the most attention in this regard. Autoradiographic studies of mouse brain with selective radioligands have revealed high densities of 5-HT_{1A} binding sites in the raphe nuclei (5-HT_{1A} autoreceptors) and limbic-related structures (5-HT_{1A} heteroreceptors) (Laporte et al., 1994), as well as the presence of 5-HT_{1B} binding sites in the basal ganglia, dorsal subiculum and deep cerebellar nuclei, mostly as heteroreceptors (Boschert et al., 1994; Langlois et al., 1995). These localizations have been further documented and clarified in recent immunoelectron microscopic studies after immunogold labeling in the rat, which demonstrated the predilection of 5-HT_{1A} receptors for the plasma membrane of somata/dendrites in both the dorsal raphe nucleus and hippocampus, and that of 5-HT_{1B} receptors for preterminal axons in the globus pallidus and substantia nigra (Riad et al., 2000; see also Kia et al., 1996a,b and Sari et al., 1999).

It has long been known that 5-HT_{1A} autoreceptors located on the somata/dendrites of 5-HT neurons in the raphe nuclei inhibit the firing of these neurons via an hyperpolarization of their membrane (Sprouse and

Aghajanian, 1987; Dong et al., 1997), and hence the release of 5-HT. The 5-HT_{1B} autoreceptors appear to be mainly involved in local inhibitory controls of 5-HT release in territories of projection (Engel et al., 1986; Piñeyro et al., 1995). Both 5-HT_{1A} and 5-HT_{1B} receptor subtypes also act as heteroreceptors, controlling the firing and/or release of non 5-HT neurons (review in Barnes and Sharp, 1999).

The role of 5-HT_{1A} and 5-HT_{1B} receptors in animal behavior has been the subject of intense investigation, especially with the recent availability of genetically altered mice. 5-HT_{1A} knockout (KO) mice originating from three different strains were found to display a common phenotype, characterized by augmented anxious-like behavior in the elevated plus-maze, open field, and novel object tests, and reduced immobility in the forced-swim (Parks et al., 1998; Ramboz et al., 1998) or tail-suspension test (Heisler et al., 1998). 5-HT_{1B} KOs showed increased aggressiveness in the resident-intruder and maternal aggression tests (Saudou et al., 1994; see also Brunner and Hen, 1997), and increased exploratory activity in the open field (Ramboz et al., 1996) or when placed in a rich environment (Malleret et al., 1999).

A caveat of these experimental models is the likelihood of adaptive changes that might take place, particularly during development, to compensate for the constitutive inactivation of the gene of interest. For example, since both 5-HT_{1A} and 5-HT_{1B} receptors control 5-HT release, an upregulation of 5-HT_{1A} autoreceptors might have been expected in the 5-HT_{1B} KOs, even if recent electrophysiological and pharmacological evidence

do not support functional changes of 5-HT_{1B} receptors in 5-HT_{1A} KOs (Richer et al., 2000; but see Ramboz et al., 1998). In the present study, a detailed quantitative autoradiographic analysis was carried out in both mutant mice to investigate and either confirm or rule out possible changes in one of these receptors in the absence of the other. In view of regional changes in 5-HT metabolism recently reported in the brain of these mutants (Ase et al., 2000), and of previous data suggesting interactions between the plasma membrane 5-HT transporter (5-HTT) and 5-HT_{1B} receptors (Daws et al., 1999; 2000), it was also deemed of interest to similarly examine the fate of this transporter in both genotypes. Lastly, the study was completed by an immunocytochemical quantification of 5-HT innervation in those regions showing the greatest changes of 5-HTT binding sites.

MATERIALS AND METHODS

Animals

The 5-HT_{1A} KO and wild-type (WT) mice in the present study had exactly the same genetic background: 129/SvEvTac. These 5-HT_{1A} KOs were produced from 129/SvEvTac ES cells and the resulting chimeras were bred with 129/Sv/EvTac females (Ramboz et al., 1998). The 5-HT_{1B} KOs, in contrast, were initially produced on a distinct genetic background: 129/SvPas x 129/Sv-ter (Saudou et al., 1994). However, these mice were back-crossed for six generations with 129/SvEvTac mice (Phillips et al., 1999). As a result, the present 5-HT_{1B} KOs had a genetic background very

similar to the WT and 5-HT_{1A} KOs. The mice were housed four per cage in a colony room, and maintained on a 12-h light/dark cycle with food and water available *ad libitum*. They were all killed at 2–3 months of age. All animal procedures were in strict accordance with the Canadian Council on Animal Care, *Guide to the Care and Use of Experimental Animals* (2nd edition), and the protocols approved by the *Comité de déontologie pour l'expérimentation sur des animaux* at the Université de Montréal.

Quantitative autoradiography

Three groups of mice (3 males and 3 females in each group) were used: WT, 5-HT_{1A} and 5-HT_{1B} KO mice. In brief, the brain was quickly removed after decapitation, frozen in *N*-methylbutane cooled to –40°C, and stored at –80°C. Cryostat sections (20 µm-thick) were labeled as follow. For 5-HT_{1A} receptors, they were incubated with 2 nM of the tritiated agonist 8-hydroxy-2-(di-n-propylamino)tetralin ([^{3}H]8-OH-DPAT; 137 Ci/mmol; New England Nuclear/Du Pont, Boston, MA, USA), as described in Radja et al. (1991) and Ase et al. (1999). For 5-HT_{1B} receptors, the incubations were with 100 pM of the iodinated antagonist [^{125}I]cyanopindolol (2200 Ci/mmol; Amersham, Arlington Heights, IL, USA), in presence of 100 nM 8-OH-DPAT (RBI, Natick, MA, USA) as a 5-HT_{1A} blocker, and 30 µM isoproterenol (RBI) as b-adrenergic blocker (Boschert et al., 1994; Ase et al., 2000). The 5-HT transporters were labeled with 2 nM [^{3}H]citalopram (81.0 Ci/mmol; DuPont–NEN), according to D'Amato et al. (1987) and Strazielle et al. (1996).

The incubations were always carried out in triplicate: two slides were used for total binding and the third slide to determine nonspecific labeling, in the presence of 10 μM serotonin creatinine sulfate (RBI) for 5-HT receptors, or of 10 μM fluoxetine HCl (Eli Lilly Pharmaceuticals; Indianapolis, IN, USA) for 5-HT transporter. The autoradiographs were produced on Hyperfilm™ (Amersham). Exposures lasted 2 weeks ([^{3}H]) or 24 hours ([^{125}I]), and development was in Kodak D-19. Optical density measurements were obtained with an MCID™ image analysis system (Imaging Research, St-Catherines, ON, Canada). Specific binding was calculated by digital subtraction of nonspecific labeling from total binding. Standard curves generated from [^{3}H] or [^{125}I] microscales (Amersham) were used to convert optical densities into femtomoles per milligram of protein (Unnerstall et al., 1982; Reader and Strazielle, 1999). Multiple readings (10-100) were obtained from the regions of interest listed in Tables 1–3.

Slides stained with cresyl violet were used to aid in anatomical localization, and to determine eventual morphological alterations secondary to the 5-HT$_{1A}$ or 5-HT$_{1B}$ gene deletion. The nomenclature was that of the mice stereotaxic atlas of Franklin and Paxinos (1997).

Immunocytochemistry

In three separate experiments, the brain from a male WT, together with those from a 5-HT$_{1A}$ and a 5-HT$_{1B}$ KO mouse were processed for light microscopic 5-HT immunocytochemistry, as previously described in detail (Ase et al., 2000). In brief, each mouse was deeply anesthetized with sodium

pentobarbital (Somnotol™, 65 mg/kg, i.p.), perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer, and the brain postfixed overnight at 4°C in the same solution. Vibratome sections, 50 µm-thick, were incubated sequentially in: (i) rabbit antiserum against 5-HT (Incstar, Stillwater, MN, USA), diluted 1:1000 in phosphate buffer saline (PBS) solution containing 1% bovine serum albumin and 0.1% Triton X-100 (overnight); (ii) biotinylated goat anti-rabbit IgG's (Jackson, West Grove, PA, USA), diluted 1:1000 in PBS containing 0.1% Triton X-100 (1–2 h); (iii) avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector, Burlingame, CA, USA) (2 h), followed by a 0.05% solution of 3,3'-diaminobenzidine (DAB) containing 0.005% H_2O_2 in Tris buffered saline (1–2 min).

Quantification of serotonin innervation

In view of observed changes, the density of 5-HT innervation was quantified by comparison to WT in brain regions showing decreases in [^{3}H]citalopram binding in 5-HT_{1A} KO, and either decreases or increases in the 5-HT_{1B} KO mice.

In a first step, length measurements of 5-HT-immunostained axons were obtained from the parietal cortex, neostriatum (caudal) and substantia nigra of three 5-HT_{1A} KOs, the amygdala, hippocampus and thalamus of three 5-HT_{1B} KOs, and all these regions in three WT mice. The parietal cortex, substantia nigra and hippocampus were sampled at transverse levels respectively equivalent to A 4.06 mm, A 0.95 mm and A 0.95 interaural (Franklin and Paxinos, 1997). In the ventral hippocampus, the

measurements were obtained from three rows of sampling windows spanning across the entire region in its upper (I), middle (II) of lower third (III), as illustrated in Fig. 2. In amygdala, the examination was focused on nuclei amygdalo-hippocampal postero-medial (AHiPM) and basolateral posterior (BLP), and in the thalamus on the laterodorsal/ lateroposterior nuclei (LD/LP).

As described in Mechawar et al. (2000), using a microcomputer-based image analysis system, images of each region were digitized, printed and assembled as large photomontages, at a final magnification of 645 X. Series of square sampling windows, 50 μm in side, were drawn in each region, so as to fit 6 to 15 squares per region. All immunostained axon trajectories within the confines of these sampling windows were then drawn by hand on transparent film. These tracings were digitized, their lines reduced to a uniform width with the 'skeletonize' function of the program, all close outlines were cut open by removal of 1 pixel, and the network was measured in micrometers, based on a prior calibration of the system with a microscopic scale. The resulting values were expressed in μm of axon per mm^2 of tissue section.

In a second step, the number of axon varicosities per unit length of immunostained axon was determined in the parietal cortex and thalamus (LD/LP) of 5-HT_{1A} KOs, in the thalamus (LD/LP) and ventral hippocampus (middle level) of 5-HT_{1B} KOs, and in these same regions of WT mice. Varicosities were defined as axon dilations greater than 0.5 μm in transverse

diameter (Séguéla et al., 1989). In all animals, the number of such dilations was counted directly by light microscopy (X 1000) on 50 axon segments per region, for a total of 1050 counts.

Statistics

The autoradiographic results were tabulated as means ± S.E.M., in fmol/mG-protein, and compared between KO and WT mice by ANOVA followed by Fisher's *F*-distribution tests (Barlow, 1983; Frank and Althoen, 1994). The immunocytochemical results, expressed in number of axon varicosities per unit length of 5-HT-immunostained axon, and length of 5-HT immunostained axon per surface unit of section, were compared by paired *t*-tests. Only statistical differences with $p < 0.05$ were considered significant.

RESULTS

[^{125}I]Cyanopindolol binding to 5-HT$_{1B}$ receptor in wild-type and 5-HT$_{1A}$ knockout mice

The measurements of 5-HT$_{1B}$ receptor densities in WT and 5-HT$_{1A}$ KO mice are shown in Table 1. As in WT, the highest densities of [^{125}I]cyanopindolol binding in 5-HT$_{1A}$ KOs were found in substantia nigra, ventral pallidum and lateral globus pallidus. High levels were also measured in dorsal subiculum, superior colliculus, periaqueductal grey matter, medial globus pallidus and deep cerebellar nuclei. In hippocampus, there was moderate labeling in stratum oriens and stratum lacunosum-moleculare of

CA1. Moderate and low levels were found in the remaining brain regions. Among the 26 regions examined, none showed statistically significant differences in density of [^{125}I]cyanopindolol binding between the two genotypes.

[^{3}H]8-OH-DPAT binding to 5-HT$_{1A}$ receptor in wild-type and 5-HT$_{1B}$ knockout mice

Table 2 lists the measurements of 5-HT$_{1A}$ receptor binding in WT and 5-HT$_{1B}$ KO mice. In both genotypes, the highest densities of [^{3}H]8-OH-DPAT binding were measured in discrete forebrain and brainstem regions, notably in Ammon's horn of hippocampus, dorsal raphe nucleus, medial entorhinal cortex and interpeduncular nucleus, while the remaining brain regions showed moderate or low levels of labeling. As in the case of 5-HT$_{1B}$ receptor in 5-HT$_{1A}$ KOs, there were no statistically significant differences between WT and 5-HT$_{1B}$ KO mice in any of the 18 regions examined for [^{3}H]8-OH-DPAT binding.

[^{3}H]Citalopram binding to 5-HT transporter in wild-type, 5-HT$_{1A}$ and 5-HT$_{1B}$ knockout mice

Sites of specific [^{3}H]citalopram binding were heterogeneously distributed throughout the brain of WT mice. The highest density was measured in dorsal raphe nucleus (Table 3), followed by substantia nigra and paraventricular thalamic nucleus. Most regions fell in an intermediate (35-50%) range, when compared to the dorsal raphe nucleus. The cortical

areas had the lowest densities (20%), yet higher in the superficial (I-IV) than deep layers (V-VI; data not shown). Among limbic structures, the amygdaloid nuclei showed the highest density. In hippocampus, the labeling was much denser in Ammon's horn than in the ventral (temporal) or dorsal (septal) regions measured as a whole. The neostriatum showed no apparent difference between its rostral and caudal halves. In thalamus, two increasing gradients encompassing the different thalamic nuclei were noticeable, i.e.: from lateral to medial, and ventral to dorsal. In brainstem, the density in the median raphe nucleus was approximately half of that measured in the dorsal raphe nucleus.

Compared to WT, 5-HT_{1A} KO mice showed significantly lower densities of [^{3}H]citalopram binding in ten of the thirty-one regions examined (Table 3; see also Figure 1). As a percent of WT values, these changes were slight (< 20%) to moderate (> 20%): parietal cortex (–26%), lateral septum (–17%), AHiPM (amygdala) nucleus (–14%), rostral (–16%) and caudal (–18%) neostriatum, lateral globus pallidus (–18%), paraventricular thalamic nucleus (–21%), LD/LP thalamic nuclei (–26%), lateral geniculate nuclei (–15%) and substantia nigra (–22%). In the 5-HT_{1B} KOs, five regions showed statistically significant differences in [^{3}H]citalopram binding (Table 3; see also Figure 1). There were slight decreases in rostral (–13%) and caudal (–16%) neostriatum, a moderate decrease in the LD/LP thalamic nuclei (–27%), and highly significant increases in the AHiPM (amygdala) nucleus (+24%) and the ventral hippocampus (+25%).

Immunocytochemical quantification of 5-HT innervation

The quantitative evaluation of 5-HT innervation density was focused on those regions which displayed the greatest changes in [^{3}H]citalopram binding (Table 4). In the 5-HT$_{1A}$ KOs, the density of 5-HT axons was not different from WT in the three brain regions showing the greatest reductions of [^{3}H]citalopram binding: parietal cortex, neostriatum and substantia nigra. Similarly, in the 5-HT$_{1B}$ KOs, the density of 5-HT axons was not significantly different from WT in the LD/LP thalamic nuclei, one of the two regions showing a decreased density of [^{3}H]citalopram binding in this mutant. In contrast, the two regions in which the density of [^{3}H]citalopram binding was increased in the 5-HT$_{1B}$ KOs, AHiPM (amygdala) nucleus and ventral hippocampus, displayed significant and parallel increases in 5-HT axonal densities (+27% and +39%, respectively). As illustrated in Fig. 2, the increased density of 5-HT innervation in the ventral hippocampus spanned across all its layers. The density of 5-HT innervation was unchanged in the BLP nucleus of amygdala, adjacent to the AHiPM, in which [^{3}H]citalopram binding was also comparable to WT.

In all regions examined to quantify the number of 5-HT varicosities per unit length of 5-HT axon (Table 5), averages of 4.4 and 4.5 varicosities per 10 μm of axon were measured in the KO and the WT mice, respectively. Thus, while there were no significant differences in the number of varicosities per unit length of 5-HT axon between genotypes, the actual number of 5-HT varicosities was obviously increased in the regions of 5-HT$_{1B}$ KOs displaying

concomitant increases in [^{3}H]citalopram binding sites and density of 5-HT axons.

DISCUSSION

The present study demonstrates significant changes in density of 5-HTT binding in several brain regions of 5-HT$_{1A}$ and 5-HT$_{1B}$ KO mice, in the absence of any change in 5-HT$_{1B}$ or 5-HT$_{1A}$ receptors, respectively. In the 5-HT$_{1A}$ KOs, only decreases in the density of 5-HTT were measured, whereas in the 5-HT$_{1B}$ KOs, there were also increases, in the amygdalo-hippocampal area and ventral hippocampus. These latter increases were tightly correlated with increases in 5-HT innervation density.

Lack of compensatory changes in 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors

The regional densities of 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors measured in the present study were consistent with abundant ligand binding autoradiographic data previously obtained from mouse brain (Boschert et al., 1994; Laporte et al., 1994; Langlois et al., 1995). In the 5-HT$_{1A}$ KOs, the lack of any compensatory changes in 5-HT$_{1B}$ receptor density was consistent with the recent demonstration by Richer et al. (2000) of equivalent inhibitory effects of sumatriptan or CP 93129 (5-HT$_{1B}$ agonists) on the electrically-evoked release of [^{3}H]5-HT from preloaded brain slices. It was also in keeping with our earlier biochemical demonstration of an increased 5-HT turnover in the dorsal/medial raphe nuclei and several territories of 5-HT innervation of

these mice (Ase et al., 2000), now known to result from a doubling of the spontaneous firing rate of 5-HT neurons (Richer et al., 2000). Altogether, these data would not seem to favor compensatory changes in the efficacy of 5-HT_{1B} receptors in the 5-HT_{1A} KOs, as previously suggested (Ramboz et al., 1998).

The lack of changes in 5-HT_{1A} receptor density in the 5-HT_{1B} KOs also came as a surprise, since both these receptor subtypes, albeit differentially located on 5-HT neurons, ultimately control 5-HT release. It was however, consistent with the results of Evrard et al. (1999), who found equivalent dose-dependent inhibition of 5-HT neuron firing by the 5-HT_{1A} agonist 8-OH-DPAT in WT and 5-HT_{1B} KO mice. It remains to be determined whether the efficacy of 5-HT_{1A} receptors is similarly unchanged in territories of 5-HT innervation, in which these receptors act as heteroreceptors and display markedly different pharmacological properties (e.g., Dong et al., 1997; Le Poul et al., 2000).

The 5-HTT is down-regulated in the 5-HT_{1A} and 5-HT_{1B} knockout mice

In both mutant mice, region-specific decreases in the density of [^{3}H]citalopram binding could be indicative of a reduction of 5-HTT at the transcriptional or post-transcriptional level, in the absence of measurable changes in 5-HT innervation density. In the 5-HT_{1A} KOs, 5-HTT gene expression was probably not reduced, since the density of 5-HTT binding was unchanged in the raphe nuclei, i.e. in 5-HT somata/dendrites. Moreover, in rat raphe nuclei, chronic and sustained administration of paroxetine, a

5-HTT blocker, was shown to reduce 5-HTT binding without alteration of its mRNA levels (Benmansour et al., 1999; see also Neumaier et al., 1996; Koed and Linnet, 1997). The decreases in density of 5-HTT here measured in parts of the cerebral cortex, limbic system, thalamus and basal ganglia of the 5-HT_{1A} KOs were therefore interpreted as post-transcriptional effects. In these territories of 5-HT innervation, the reduction in 5-HTT density might decrease 5-HT clearance and thus contribute to enhanced 5-HT neurotransmission. Interestingly, reduced 5-HT clearance as well as reduced density of 5-HTT binding, without significant effects on tissue levels of 5-HT or 5-HIAA, have been documented in rat dorsal/medial raphe nuclei, hippocampus and/or cerebral cortex after long-term administration of 5-HT uptake inhibitors (Kovachich et al., 1992; Piñeyro et al., 1994; Benmansour et al., 1999). Similarly, in the 5-HT_{1A} KOs, our earlier biochemical measurements showed no significant changes of 5-HT and its metabolites in most of the brain regions here displaying a decreased density of 5-HTT (Ase et al., 2000).

In the 5-HT_{1B} KOs, the decreases in 5-HTT density were even more restricted than in the 5-HT_{1A} KOs. A link between 5-HT_{1B} receptors and 5-HTT has been recently suggested by the amperometric study of Daws et al. (1999), providing evidence of a decreased 5-HTT efficacy upon acute administration of 5-HT_{1B} receptor antagonists in normal rat hippocampus. In the constitutive absence of 5-HT_{1B} receptors throughout brain, 5-HTT transporter could be down-regulated as in the 5-HT_{1A} KOs. However, it then

remains to be explained why such a phenomenon would involve only the neostriatum and LD/LP of thalamus.

Increased density of 5-HTT is due to 5-HT hyperinnervation in the 5-HT_{1B} knockouts

There was unequivocal evidence that the increases in [^{3}H]citalopram binding sites measured in the amygdalo-hippocampal area (AHiPM) and ventral hippocampus of the 5-HT_{1B} KOs represented a 5-HT hyperinnervation. In both regions, 5-HT axon density was increased proportionally to the increases in [^{3}H]citalopram binding sites, while remaining normal in the adjacent BLP nucleus of amygdala and LD/LP nuclei of thalamus, in which [^{3}H]citalopram binding was normal or decreased, respectively. There was no obvious explanation for the regional selectivity of this hyperinnervation. However, its existence was a likely explanation for the recent results of Malagié et al (2001), who reported an excessive increase of the microdialysate levels of 5-HT in the ventral hippocampus, but not frontal cortex, of 5-HT_{1B} KOs, after a single dose of the 5-HT reuptake inhibitor paroxetine (see also de Groote et al., 2000).

The region-specific increases in 5-HT axon length and arborization demonstrated in the present study provided the first indication of an inhibitory, or negative, control of 5-HT axon growth mediated by 5-HT_{1B} receptors. Interestingly, in prior studies, 5-HT_{1B}-mediated stimulatory effects of 5-HT on neuritic outgrowth have been demonstrated, at least in primary cultures of thalamic neurons (Lotto et al., 1999; see also Salichon et al.,

2001). In both these studies, the receptors activated were obviously hetero- and not autoreceptors. The only prior report describing effects of 5-HT on the growth of 5-HT fibers, after specific damage of 5-HT neurons by 5,7-dihydroxytryptamine, incriminated astrocytic 5-HT_{1A} receptors mediating a stimulatory effect on the glial protein S-100 (Whitaker-Azmitia et al., 1990). The present data did not reveal the precise localization of the proposed 5-HT_{1B}-mediated inhibitory control of 5-HT axon growth. A direct effect on 5-HT terminals would be consistent with the preterminal localization of 5-HT_{1B} autoreceptors (Riad et al., 2000), but an effect normally exerted through 5-HT_{1B} heteroreceptors located on the preterminals of neurons containing other transmitters could not be excluded. For example, 5-HT_{1B} receptors are known to be normally present on the terminals of cholinergic neurons innervating the hippocampus (Maura et al., 1989), one of the two regions showing the 5-HT hyperinnervation in the 5-HT_{1B} KOs.

In this context, the lack of any change in the average number of varicosities per unit length of 5-HT axons was of interest. It indicated that the absolute number of 5-HT varicosities was indeed increased in the two regions, but also that the increased density of innervation was primarily due to an increased length, and presumably branching, of the 5-HT axon network. It has already been pointed out that the lengthening and branching of central axons is likely to depend on local factors within the territories of innervation, more than on inherent properties of the neurons of origin (e.g., Aznavour et al., in press; Mechawar et al., in press). 5-HT_{1B} receptors,

located on the terminal branches of 5-HT or of other neurons, might well be part of the signaling cascade for such growth regulatory signals.

Behavioral implications

How the localized decreases in 5-HTT might affect the behavior of 5-HT_{1A} KOs remains a matter of speculation. The reduced number of uptake sites, particularly in limbic regions, might result in increased 5-HT transmission and therefore enhance anxious-like behavior, or antidepressant-like responsiveness, through the activation of different 5-HT receptor subtypes (Heisler et al., 1998). In the 5-HT_{1B} KOs, however, the 5-HTT decreases were restricted to two brain regions not hitherto implicated in aggressive behavior (neostriatum and LD/LP of thalamus). It is therefore likely that the behavioral phenotype of this mutant (Saudou et al., 1994; see also Zhuang et al., 1999) was related to the 5-HT hyperinnervations in amygdala (AHiPM) and ventral hippocampus, in addition to the constitutive absence of 5-HT_{1B} receptors.

The amygdala supports various forms of aggressive behavior through its connections with the hypothalamus and periaqueductal gray matter (e.g., Hen, 1996). It has already been shown that, in amygdala, 5-HT might enhance conditioned fear (Graeff et al., 1997) through 5-HT_{1A}, 5-$HT_{2A/2C}$ and/or 5-HT_3 receptors on both projection and interneurons (Stein et al., 2000). The present results ruled out changes at least of 5-HT_{1A} receptors in the 5-HT_{1B} KOs. In basal conditions, increased release resulting from a 5-HT hyperinnervation should be compensated by increased uptake of 5-HT

(Jackson and Abercrombie, 1992; see also Romero et al., 1998). In the absence of 5-HT_{1B}-mediated inhibitory control of 5-HT release, however, stimulated release of 5-HT under specific environmental stimuli could have a major impact in the 5-HT-hyperinnervated amygdala of the 5-HT_{1B} KOs.

A similar situation could prevail in the hyperinnervated hippocampus, although with quite different behavioral consequences. In normal rats, the administration into hippocampus of buspirone or 8-OH-DPAT, a partial and full 5-HT_{1A} agonist, respectively, has been shown to increase the time spent exploring the open arms of the elevated plus maze, and the number of central entries in the open field (Kostowski et al., 1989; Menard and Treit, 1998). Similarly, the 5-HT_{1B} KO mice show increased exploration in the elevated plus maze (Brunner et al., 1999), and a higher locomotor/exploratory behavior compared to WT when exposed to a novel object and/or a structurally rich environment (Malleret et al., 1999). This could be due to excessive activation of 5-HT_{1A} receptors in the 5-HT-hyperinnervated hippocampus, especially since, contrary to 5-HT_{1A} autoreceptors, hippocampal 5-HT_{1A} heteroreceptors do not desensitize upon chronic activation by 5-HT or 5-HT_{1A} agonists (Blier and de Montigny, 1987; Dong et al., 1997). Furthermore, both 5-HT_{1A}- (Izumi et al., 1994; Fujii et al., 1997) and 5-HT_4-mediated (Consolo et al., 1994; Fontana et al., 1997; Letty et al., 1997; Marchetti-Gauthier et al., 1997) facilitatory effects of 5-HT on acetylcholine release should be exaggerated, at least under conditions of behavioral activation in the 5-HT_{1B} KOs. This could explain some of the

recent results of Malleret et al. (1999) indicating that, in the spatial version of the Morris water maze, this mutant performs better than WT mice in both acquisition and transfer tests.

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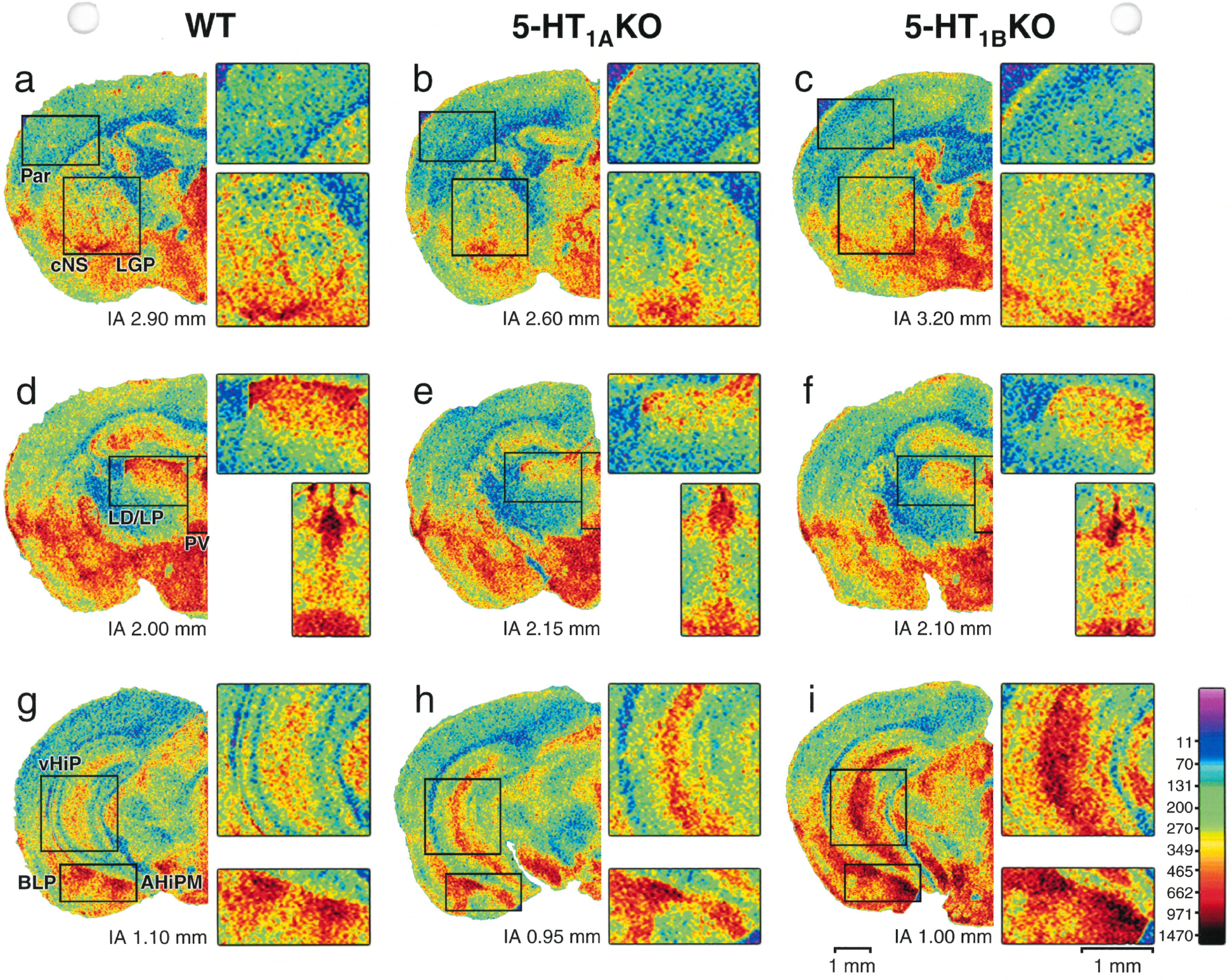
Figure legends

Figure 1. Color-coded autoradiographs of [^{3}H]citalopram binding to 5-HTT in different brain regions from WT (**a**,**d**,**g**), 5-HT$_{1A}$ (**b**,**e**,**h**) and 5-HT$_{1B}$ (**c**,**f**,**i**) KO mice. The corresponding stereotaxic planes are in accordance to Franklin and Paxinos (1997). Inserts beside every hemisection focus on the regions showing significant differences in binding densities (see also Table 3). In **a**,**b**,**c**, note the weaker labeling in the parietal cortex (Par), neostriatum (cNS) and lateral globus pallidus (LGP) of 5-HT$_{1A}$ KOs (**b**), and in the neostriatum of 5-HT$_{1B}$ KOs (**c**), compared to WT mice (**a**). In **d**,**e**,**f**, the labeling is markedly reduced in the thalamus (LD/LP) of both 5-HT$_{1A}$ (**e**) and 5-HT$_{1B}$ KO mice (**f**), and also in the periventricular nucleus (PV) of the 5-HT$_{1A}$ (**e**) but not 5-HT$_{1B}$ (**f**) KOs. In **g**,**h**,**i**, there is a restricted decrease in the amygdalo-hippocampal nucleus (AHiPM) in the 5-HT$_{1A}$ KOs (**h**), and a marked increase in the 5-HT$_{1B}$ KOs (**i**), whereas the adjacent basolateral posterior nucleus (BLP) of amygdala remains unchanged. Also note the increased labeling in the ventral hippocampus (vHip) of the 5-HT$_{1B}$ KOs (**i**), contrasting with the absence of any significant change in this region of the 5-HT$_{1A}$ KOs (**h**). The non-transformed autoradiographic images were digitized and exported as TIFF files, and re-sampled at a 300 x 300 dpi resolution. The calibration scale runs from 11 to 1470 fmol per mg of protein.

Figure 2. Two series of digitized light microscopic images from a WT mouse (**a**,**c** and **e**) and a 5-HT$_{1B}$ KO (**b**,**d** and **f**), illustrating the sampling technique used for the quantification of the 5-HT innervation, and one of its main results: the demonstration of a 5-HT hyperinnervation in the ventral hippocampus of the 5-HT$_{1B}$ KO. In the low power views on top (**a** and **B**), the three rows of white squares labeled I, II and III represent the small sampling windows, 50 μm in side, from which length measurements of the 5-HT-immunostained axon network were obtained, as described in Materials and methods. In (**c**) and (**d**), one of the squares from each of the upper rows, designated by the asterix in (**a**) and (**b**), is reproduced at a medium magnification (x 170), sufficient to distinguish the individual 5-HT-immunostained fibers pervading the region, and giving rise to its layered pattern of 5-HT innervation. The greater density of 5-HT innervation can already be appreciated at this magnification, particularly in the richly 5-HT-innervated stratum lacunosum moleculare of CA1, in which the sampling window is outlined in black. A greater enlargement (x 1700) of these same squares in (**e**) and (**f**) further illustrates the morphological features of the fine varicose 5-HT axons as well as their increased number in the 5-HT$_{1B}$ KO mouse.


WT
5-HT1AKO
5-HT1BKO
a
b
c
Par
cNS
LGP
IA 2.90 mm
IA 2.60 mm
IA 3.20 mm
d
e
f
LD/LP
PV
IA 2.00 mm
IA 2.15 mm
IA 2.10 mm
g
h
i
vHiP
BLP
AHiPM
IA 1.10 mm
IA 0.95 mm
IA 1.00 mm
11
70
131
200
270
349
465
662
971
1470
1 mm
1 mm

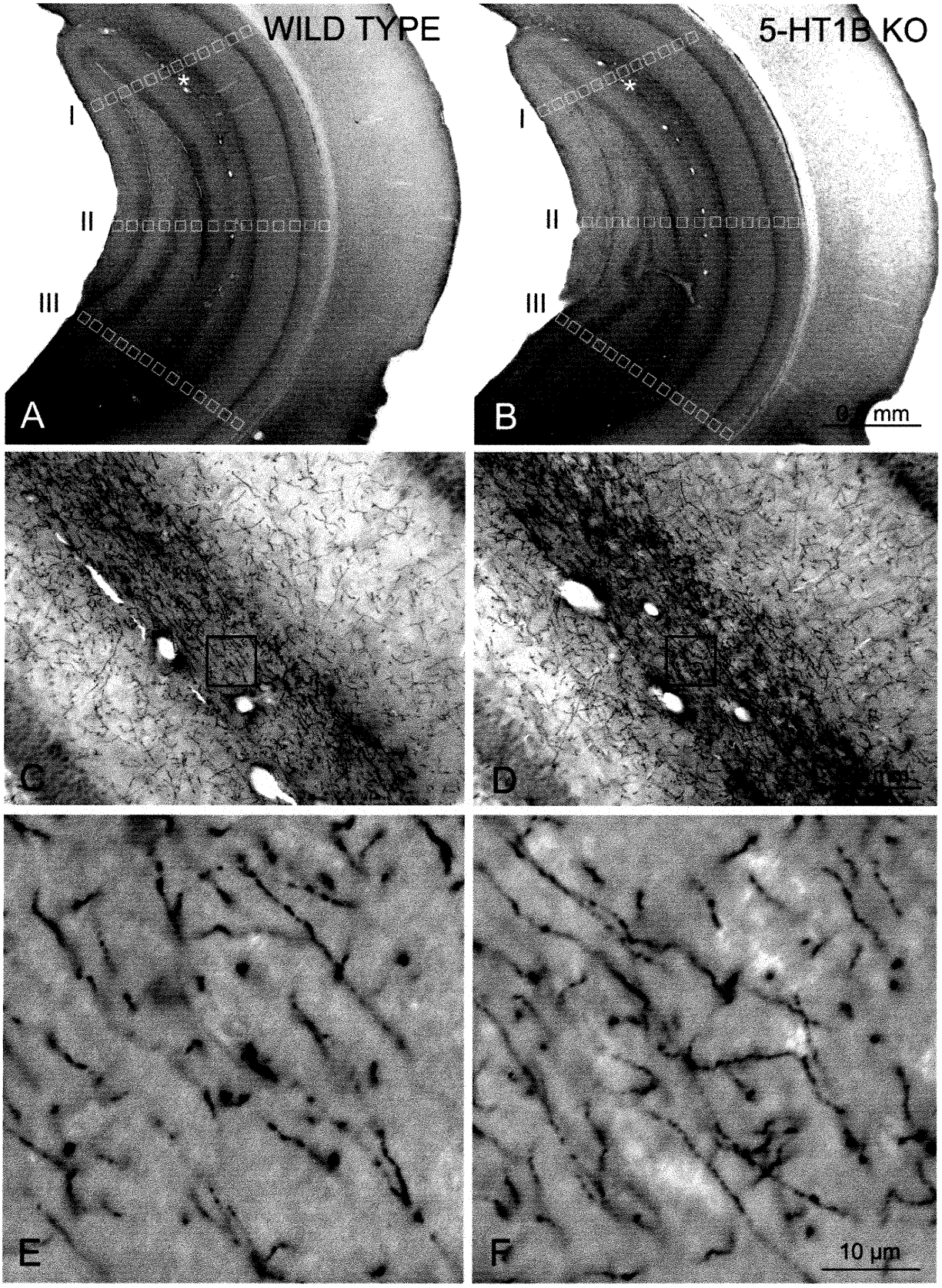

WILD TYPE
5-HT1B KO
I
II
III
A
B
0.5 mm
C
D
E
F
10 µm

Table 1

[^{125}I]CYANOPINDOLOL BINDING TO 5-HT$_{1B}$ RECEPTORS IN THE BRAIN OF WILD-TYPE (WT) AND 5-HT$_{1A}$ KNOCKOUT (KO) MICE

		WT	5-HT$_{1A}$ KO
Cortex			
Cingulate		27.9 ± 1.2	29.4 ± 1.8
Frontal		23.6 ± 1.2	21.0 ± 1.7
Limbic Areas			
Nucleus accumbens		41.7 ± 1.9	46.4 ± 4.6
Lateral septum		41.4 ± 2.6	40.8 ± 1.6
Amygdala		39.2 ± 2.1	37.2 ± 2.5
Dorsal subiculum		89.0 ± 8.7	108.9 ± 8.7
Hippocampus (CA1),	lac. moleculare	47.9 ± 3.3	45.0 ± 3.4
	oriens	52.5 ± 2.5	51.8 ± 2.4
Basal Ganglia			
Caudate-putamen		32.7 ± 1.6	36.3 ± 2.4
Ventral pallidus		100.1 ± 4.1	101.1 ± 5.6
Lateral globus pallidus		91.0 ± 5.5	100.0 ± 7.9
Medial globus pallidus		86.4 ± 1.6	84.3 ± 8.3
Substantia nigra		109.6 ± 7.5	106.5 ± 9.4
Thalamus			
Geniculate nuclei, DLG		47.3 ± 2.1	42.9 ± 4.0
	VLGMC	87.6 ± 9.3	84.7 ± 6.5
	VLGPC	35.5 ± 2.4	32.4 ± 2.8

Table 1 (continued)

	WT	5-HT$_{1A}$ KO
Hypothalamus		
Medial hypothalamus nuclei	47.7 ± 1.9	47.2 ± 4.1
Lateral hypothalamus nucleus	46.5 ± 2.2	51.0 ± 5.7
Brainstem		
Ventral tegmental area	38.9 ± 2.6	36.8 ± 2.7
Periaqueductal grey matter	88.7 ± 8.6	108.9 ± 8.7
Superior colliculus	84.9 ± 8.4	82.2 ± 6.2
Inferior colliculus	53.1 ± 1.4	47.4 ± 3.3
Dorsal raphe nucleus	50.5 ± 3.8	48.2 ± 5.2
Locus coeruleus	64.6 ± 3.4	57.7 ± 2.8
Parabrachial nuclei	66.9 ± 2.6	59.1 ± 4.3
Cerebellum deep nuclei	72.1 ± 3.6	75.8 ± 5.9

Mean ± SEM in femtomoles per milligram of protein. Data from 6 mice in each group.

Table 2

[^{3}H]8-OH-DPAT BINDING TO 5-HT$_{1A}$ RECEPTORS IN THE BRAIN OF WILD-TYPE (WT) AND 5-HT$_{1B}$ KNOCKOUT (KO) MICE

	WT	5-HT$_{1B}$ KO
Cortex		
Cingulate	127.0 ± 10.1	123.6 ± 4.3
Frontal	125.3 ± 10.0	120.3 ± 6.7
Parietal	110.7 ± 9.8	95.8 ± 5.9
Claustrum	214.1 ± 19.7	216.4 ± 15.3
Endopiriform nucleus	244.9 ± 21.5	231.6 ± 10.7
Lateral entorhinal	157.6 ± 12.4	136.5 ± 5.3
Medial entorhinal	302.5 ± 27.7	272.7 ± 13.4
Limbic Areas		
Septum	224.5 ± 17.7	235.4 ± 10.2
Diagonal band (Broca)	187.1 ± 18.9	198.1 ± 23.5
Amygdala	167.8 ± 8.5	164.9 ± 7.6
Hippocampus, dorsal	285.2 ± 27.1	267.7 ± 15.7
ventral	251.4 ± 30.8	231.6 ± 10.0
Ammon' s horn	438.6 ± 41.8	410.8 ± 17.7
Brainstem		
Interpeduncular nucleus	318.8 ± 19.1	378.1 ± 31.7
Superior colliculus	116.0 ± 10.3	119.2 ± 5.4
Inferior colliculus	109.9 ± 14.4	91.4 ± 10.6
Dorsal raphe nucleus	374.8 ± 39.5	358.0 ± 19.0
Medial raphe nucleus	171.4 ± 11.6	161.3 ± 9.3

Mean ± SEM in femtomoles per milligram of protein. Data from 6 mice in each group.

Table 3

[^{3}H]CITALOPRAM BINDING TO 5-HT TRANSPORTERS IN THE BRAIN OF WILD-TYPE (WT), 5-HT$_{1A}$ AND 5-HT$_{1B}$ KNOCKOUT (KO) MICE

	WT	5-HT$_{1A}$ KO	5-HT$_{1B}$ KO
Cortex			
Cingulate	265.2 ± 16.2	219.3 ± 10.4	296.9 ± 23.0
Frontal	200.8 ± 11.7	165.1 ± 12.5	215.3 ± 13.8
Parietal	168.6 ± 9.6	124.6 ± 5.6 **	151.0 ± 6.4
Limbic area			
Olfactory tubercle	546.3 ± 36.0	534.2 ± 35.8	571.3 ± 25.6
Nucleus accumbens	331.8 ± 7.9	326.0 ± 30.1	326.5 ± 11.2
Lateral septum	467.5 ± 15.2	387.4 ± 10.7 *	508.6 ± 27.9
Amygdala	481.2 ± 28.2	451.7 ± 18.2	515.6 ± 23.5
Basolateral amygdaloid nucleus	748.1 ± 36.4	701.2 ± 57.9	776.4 ± 29.5
Amygdalohippocampal area	697.0 ± 21.1	600.4 ± 13.4 *	866.7 ± 27.2 ***
Hippocampus, dorsal	227.4 ± 10.3	215.6 ± 9.6	228.6 ± 8.5
ventral	294.6 ± 10.1	295.3 ± 5.3	368.7 ± 9.6 ***
Ammon' s horn	482.1 ± 19.9	475.5 ± 21.3	559.8 ± 13.5 *
Basal ganglia			
Caudate-putamen, rostral	267.9 ± 11.2	225.5 ± 11.7 *	233.7 ± 9.4 *
caudal	291.9 ± 5.2	240.0 ± 13.6 **	244.5 ± 14.2 *
Lateral globus pallidus	478.0 ± 24.2	390.3 ± 25.4 *	440.5 ± 19.9
Ventral pallidum	539.4 ± 24.3	496.1 ± 19.8	560.0 ± 27.9
Subthalamic nucleus	573.4 ± 22.6	499.1 ± 33.3	605.2 ± 38.5
Substantia nigra	1006.9 ± 79.1	786.0 ± 34.8 *	990.6 ± 77.9

Table 3 (continued)

	WT	5-HT$_{1A}$ KO	5-HT$_{1B}$ KO
Thalamus			
Paraventricular nucleus	918.7 ± 53.5	722.3 ± 56.8 *	896.0 ± 51.4
Anterior ventromedial	607.5 ± 40.8	559.3 ± 36.3	652.4 ± 21.0
Lateral dorsal/posterior nuclei	454.9 ± 38.7	334.9 ± 15.7 *	333.5 ± 5.9 *
Lateral geniculate nuclei	443.5 ± 16.0	375.5 ± 21.8 *	412.2 ± 27.7
Hypothalamus			
Medial nuclei	521.7 ± 21.0	509.3 ± 48.6	578.5 ± 28.2
Lateral nucleus	507.2 ± 28.1	470.7 ± 35.1	544.2 ± 25.8
Mammillary bodies	414.5 ± 31.8	411.2 ± 28.7	479.4 ± 25.5
Brainstem			
Ventral tegmental area	591.8 ± 44.6	515.3 ± 39.1	546.8 ± 36.1
Interpeduncular nucleus	547.7 ± 36.1	515.1 ± 24.9	598.8 ± 33.9
Periaqueductal grey	508.0 ± 18.2	458.3 ± 18.4	545.7 ± 27.2
Superior colliculus	473.6 ± 23.4	426.4 ± 29.2	502.0 ± 35.6
Dorsal raphe nucleus	1241.2 ± 75.2	1161.0 ± 89.6	1272.5 ± 52.4
Median raphe nucleus	757.8 ± 28.9	748.5 ± 61.1	742.9 ± 26.7
Locus coeruleus	713.9 ± 60.0	646.4 ± 38.2	708.4 ± 22.9
Parabrachial nuclei	6.17 ± 46.1	607.3 ± 26.4	687.0 ± 21.4

Mean ± SEM in femtomoles per milligram of protein. Data from 6 mice in each group.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, by comparison to WT mice.

Table 4

DENSITY OF 5-HT INNERVATION IN WILD TYPE (WT), 5-HT_{1A} AND 5-HT_{1B} KNOCKOUT (KO) MICE

	WT	5-HT_{1A} KO	5-HT_{1B} KO	% change	
Parietal cortex	57.6 ± 6.2	59.1 ± 7.9	—	nil	(- 26)
Amygdala					
AHiPM	140.3 ± 21.5	—	177.8 ± 23.1 *	+ 27	(+ 24)
BLP	151.2 ± 11.0	—	154.4 ± 6.6	nil	(nil)
Hippocampus, ventral					
I	72.1 ± 24.9	—	99.2 ± 24.6 ***	+ 37	
II	75.6 ± 22.0	—	107.1 ± 16.3 *	+ 42	(+ 25)
III	108.6 ± 22.8	—	128.6 ± 17.2	+ 18	
Caudate putamen, caudal	102.6 ± 5.1	105.6 ± 6.1	—	nil	(- 18)
Substantia nigra	130.1 ± 5.0	136.6 ± 4.4	—	nil	(- 22)
Thalamus (LD/LP)	157.9 ± 7.2	—	166.6 ± 4.8	nil	(- 27)

Mean ± SEM in μm of axons per mm^2 of tissue section, from 3 mice in each group. Measurements were carried out in 6-15 sampling windows per region, and in equal number between WT and KO mice. In hippocampus (ventral), I, II, III designate windows that were respectively located in its upper, middle and lower third. LD/LP are for the latero-dorsal/latero-posterior thalamic nuclei. % change refers to the comparison between KO and WT mice. The adjacent values in brackets are the corresponding changes in [^{3}H]citalopram binding (data from Table 2). —, not measured. * $P < 0.05$, *** $P < 0.001$, compared to WT mice.

Table 5

NUMBER OF VARICOSITIES PER UNIT LENGTH OF 5-HT AXON IN WILD-TYPE (WT), 5-HT_{1A} AND 5-HT_{1B} KNOCKOUT (KO) MICE

	WT	5-HT_{1A} KO	5-HT_{1B} KO
Parietal cortex	4.57 ± 0.14	4.61 ± 0.10	—
Hippocampus, ventral	4.55 ± 0.14	—	4.33 ± 0.05
Thalamus (LD/LP)	4.62 ± 0.03	4.55 ± 0.30	4.56 ± 0.01

Mean ± SEM for the number of varicosities per 10 μm of 5-HT axon, as explained in Materials and Methods. Data from 3 mice in each group. —, not measured.

Chapter IV

Decreased G-protein coupling of serotonin 5-HT_{1A} receptors in the brain of 5-HT_{1B} knockout mice

Ariel R. Ase, Jacques Sénécal, Tomás A. Reader, René Hen and Laurent Descarries

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As first author, I carried out most of the experiments, analyzed the data, and contributed in a significant way to all the steps toward the writing of the article.

Résumé

L'activité des neurones à sérotonine (5-HT) du SNC et leur capacité de libération de la 5-HT font l'objet d'un autocontrôle par l'entremise de récepteurs 5-HT_{1A} et 5-HT_{1B} respectivement situés sur les corps cellulaires et dendrites (autorécepteurs 5-HT_{1A}) et les arborisations axonales terminales (autorécepteurs 5-HT_{1B}) de ces neurones. Afin de mieux caractériser les capacités d'adaptation mutuelles de ces deux sous-types de récepteur en l'absence de l'un d'entre eux, nous avons mesuré par radioautographie leur couplage respectif aux protéines G chez des souris sauvages (WT) et knockout (KO) homozygotes pour les récepteurs 5-HT_{1A} ou 5-HT_{1B}. Tel que prévu, chez les WT, l'activation par un agoniste non sélectif des récepteurs 5-$HT_{1A/1B}$, la 5-carboxytryptamine (5-CT) a stimulé l'incorporation de guanosine 5'-O-(γ-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) dans la plupart des régions du cerveau munies de l'un et/ou l'autre récepteur. Chez les WT et les KO respectifs, aucune stimulation n'a été observée dans les régions connues pour exprimer uniquement ou majoritairement le récepteur délété. Chez les KO 5-HT_{1A}, l'amplitude de la stimulation par la 5-CT dans les régions munies de récepteurs 5-HT_{1B} est apparue inchangée par rapport au WT. Chez les KO 5-HT_{1B}, l'activation par la 5-CT était de même amplitude que chez les WT dans toutes les régions contenant le récepteur 5-HT_{1A}, sauf l'amygdale, où elle est apparue significativement plus faible, même s'il s'agissait d'une des régions les plus fortement activées chez les WT. Un résultat semblable a été obtenu dans l'amygdale des KO 5-HT_{1B} après activation par l'agoniste 5-HT_{1A} sélectif 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT). Dans ces conditions cependant, l'incorporation de [^{35}S]GTPγS stimulée par le 8-OH-DPAT (mais non son incorporation basale) est apparue significativement plus faible que chez le WT dans toutes les régions munies de récepteurs 5-HT_{1A} receptors, incluant le noyau raphé dorsalis. Ainsi, même si la liaison au récepteur 5-HT_{1A} comme au récepteur 5-HT_{1B} est inchangée dans tous le cerveau des KO réciproques, une diminution compensatoire de l'efficacité de couplage aux protéines G du récepteur 5-HT_{1A} s'est développée chez le mutant nul 5-HT_{1B}. Ceci pourrait représenter la première indication d'une interaction locale (cross-talk) entre ces deux sous-types réceptoriels 5-HT, du moins dans les régions cérébrales où ces deux récepteurs sont co-localisés dans les mêmes neurones.

Decreased G-protein coupling of serotonin 5-HT_{1A} receptors in the brain of 5-HT_{1B} knockout mouse

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Keywords : G-protein coupling; Serotonin receptors; 5-HT_{1A} and 5-HT_{1B} null mutants; [^{35}S]GTPγS autoradiography; 5-Carboxyamidotryptamine (5-CT); 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT)

Abstract

The firing of central serotonin (5-hydroxytryptamine, 5-HT) neurons and their capacity to release 5-HT are subjected to a receptor-mediated auto-control via 5-HT_{1A} and 5-HT_{1B} receptors respectively located on the somata/dendites (5-HT_{1A} autoreceptors) and preterminal axon arborizations (5-HT_{1B} autoreceptors) of these neurons. To further characterize mutual adaptations of these two receptor subtypes in the absence of one of them, activation of G-protein coupling by agonist was measured and compared to wild-type (WT) in 5-HT_{1A} and 5-HT_{1B} homozygous knockout (KO) mice. As expected, in WT, the non selective 5-$HT_{1A/1B}$ receptor agonist 5-carboxyamidotryptamine (5-CT) stimulated guanosine 5'-*O*-(γ-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) incorporation in many brain regions endowed with one and/or the other receptor. In the respective KOs, no stimulation was measured in regions known to express only or mainly the deleted receptor. In the 5-HT_{1A} KOs, the amplitude of G-protein activation in regions endowed with 5-HT_{1B} receptors was unchanged by comparison to WT. In the 5-HT_{1B} KOs, the magnitude of the 5-CT stimulation was the same as WT in all regions containing 5-HT_{1A} receptors, except in the amygdala, where it was significantly lower, even if this region was one of the most strongly activated in the WT. A similar result was obtained in the amygdala of 5-HT_{1B} KOs after activation by the selective 5-HT_{1A} receptor agonist *R*-(+)8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT). Under these conditions, however, there was in addition a significant lowering of the stimulated (but not basal) [^{35}S]GTPγS incorporation by comparison to WT in all regions endowed with 5-HT_{1A} receptors, including the dorsal raphe nucleus. Thus, even though agonist radioligand binding to either 5-HT_{1A} or 5-HT_{1B} receptors is unchanged in the reciprocal KOs, it appears that a compensatory decrease in the efficiency of G-protein coupling to 5-HT_{1A} receptors has developed in the 5-HT_{1B} mutant. This could represent the first indication of a crosstalk between these two 5-HT receptor subtypes, at least in brain regions where they are colocalized in the same neurons.

Introduction

The serotonin (5-hydroxytryptamine, 5-HT) receptors, 5-HT_{1A} and 5-HT_{1B}, control both the firing and the release of 5-HT neurons as autoreceptors, and thus play a key role in regulating 5-HT transmission in brain (Barnes and Sharp, 1999). Central 5-HT transmission has been associated with anxiety, depression, as well as a group of impulse-related disorders, including aggression and substance abuse (Lucki, 1998; Blier and de Montigny, 1999). In recent years, 5-HT_{1A} and 5-HT_{1B} knockout (KO) mice have proved useful for characterizing the implication of 5-HT and its 5-HT_{1A} and 5-HT_{1B} receptors in mood and behavior (Zhuang et al., 1999; Bonasera and Tecott, 2000; Gingrich and Hen, 2001). 5-HT_{1A} KO mice from three different strains have been shown to display an anxious-like phenotype in several behavioral tests, such as the elevated plus and zero mazes, the light/dark choice, the open field and the forced-swimming test (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). In 5-HT_{1B} KO mice, a high vulnerability to cocaine was observed, as well as an enhancement of aggressive behavior in the resident-intruder and isolation-induced or maternal aggression tests (Saudou et al., 1994; Rocha et al., 1998).

In addition to changes directly imputable to the absence of these receptors, the 5-HT_{1A} and 5-HT_{1B} KO mice have provided an opportunity to search for mutual adaptations of these two G-protein-coupled autoreceptors in the constitutive absence of one another. Several electrophysiological and pharmacological studies failed to reveal such compensatory changes (Evrard

et al., 1999). Accordingly, in a detailed ligand binding autoradiographic study, we did not detect any difference from WT in the density of 5-HT_{1B} and 5-HT_{1A} receptors throughout the brain of 5-HT_{1A} and 5-HT_{1B} mutants, respectively (Ase et al., 2001). However, a recent microdialysis study has demonstrated an enhanced sensitivity of 5-HT_{1B} receptors in the striatum but not ventral hippocampus of the 5-HT_{1A} KOs, as well as a decreased 5-HT_{1A} agonist response in the ventral hippocampus but not the striatum of 5-HT_{1B} KO (Knobelman et al., 2001).

To further investigate eventual reciprocal adaptations in the functioning of 5-HT_{1A} and 5-HT_{1B} receptors, we examined the coupling of both receptors to their G-protein in selected brain regions of 5-HT_{1A} and 5-HT_{1B} KO mice by comparison to WT. The first step in the signaling cascade following activation of G-protein-coupled receptors is an exchange of GDP for GTP on the a-subunit of the interacting G-protein. Agonist-induced incorporation of guanosine 5'-*O*-(γ-[^{35}S]thio)triphosphate ([^{35}S]GTPγS), a non-hydrolyzable analogue of GTP, provides a mean to measure the amount of receptor coupling to G-protein and thus receptor efficacy. Combined with autoradiography, this method has already provided anatomically specific information on the function of a large variety of receptors in brain (Sim et al., 1995; reviewed by Sóvágó et al., 2001).

As stimulation of [^{35}S]GTPγS incorporation could not be achieved with selective 5-HT_{1B} receptor agonists (Dupuis et al., 1998), we used the non-selective 5-$HT_{1A/1B}$ agonist 5-carboxyamidotryptamine (5-CT) to derive 5-HT_{1B}

stimulation from results obtained in 5-HT_{1A} mutants. For the activation of 5-HT_{1A}-mediated coupling, the selective agonist *R*-(+)8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) was available. The receptor specificity of the measurements with both 5-CT and 8-OH-DPAT was established by demonstrating the virtual absence of stimulated incorporation of [^{35}S]GTPγS in brain regions of KOs known to contain only one or the other subtype of receptors.

2. Materials and methods

2.1. Animals

The 5-HT_{1A} KO and WT mice in the present study had exactly the same genetic background: 129/SvEvTac. The 5-HT_{1A} KOs were produced from 129/SvEvTac ES cells, and the resulting chimeras were bred with 129/Sv/EvTac females (Ramboz et al., 1998). The 5-HT_{1B} KOs, in contrast, were initially produced on a distinct genetic background: 129/SvPas x 129/Sv-ter (Saudou et al., 1994). However, these mice were backcrossed for six generations with 129/SvEvTac mice (Phillips et al., 1999). As a result, the present 5-HT_{1B} KOs had a genetic background very similar to the WT and 5-HT_{1A} KO mice. All animal procedures were in strict accordance with the Canadian Council on Animal Care, *Guide to the Care and Use of Experimental Animals* (2nd edition), and the protocols approved by the *Comité de déontologie pour l'expérimentation sur des animaux* at the Université de Montréal. The mice were housed four per cage in a colony room, and

maintained on a 12-h light/dark cycle with food and water available *ad libitum*. They were all killed at 3 months of age, and the absence of the deleted receptor in the KOs was verified by ligand binding autoradiography, as described in a companion study carried out with these same mice (Ase et al., 2000).

2.2. Basal and agonist-stimulated [^{35}S]GTPγS autoradiography

The brains were quickly removed after decapitation, frozen in 2-methylbutane cooled to – 40°C with liquid nitrogen, and stored at – 80°C. Cryostat sections (20 μm-thick) were thaw-mounted onto gelatin-coated slides, and kept at – 80°C until used. Autoradiography of agonist-stimulated [^{35}S]GTPγS (1250 Ci/mmol; Perkin-Elmer, Boston, MA) incorporation was performed as previously described (Sim et al., 1997; Waeber and Moskowitz, 1997; Dupuis et al., 1998) with slight modifications. Adjacent sections were soaked for 20 min at 25°C in Tris-HCl 50 mM (pH 7.4) for removal of endogenous 5-HT, and then preincubated for 15 min in Tris-Cl 50 mM (pH 7.4) supplemented with 100 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 2 mM GDP and 10 μM DPCPX (Sigma, St-Louis, MO); the latter is an adenosine A1 receptor antagonist and was used to block [^{35}S]GTPγS incorporation mediated by these receptors (Laitinen and Jokinen, 1998). Thereafter, sections were incubated for 2 h at 25°C in the same solution containing 40 pM [^{35}S]GTPγS, with (stimulation condition) or without (basal condition) 10 μM of the 5-$HT_{1A/1B}$ receptor agonist 5-CT (Sigma), or 10 μM of the selective 5-HT_{1A} receptor agonist 8-OH-DPAT (RBI, Natick, MA). Non-specific labeling

(background) was determined on autoradiographs from adjacent sections incubated with 10 μM of unlabeled GTPγS. Sections were rinsed twice (2 min each) in 50 mM Tris-Cl buffer, once (30 sec) in distilled water to remove the buffer salts, and air-dried. The autoradiographs were produced on Kodak Biomax MR™ film. Exposures lasted 48-72 h and development was in Kodak GBX.

2.3. Data analysis

Optical density readings were obtained with an MCID™ image analysis system (Imaging Research, St-Catherines, ON, Canada). Standard curves were generated from [^{14}C]-microscales (Amersham, Arlington Heights, IL) and used to convert optical densities into femtomoles per milligram of protein. Multiple readings (10-100) were obtained from the cingulate cortex (Cin), frontal cortex (Fro), lateral septum (LS), claustrum (Cl), endopiriform nucleus (En), nucleus accumbens (Acb), caudate-putamen (CPu), lateral globus pallidus (LGP), substantia nigra (SN), whole hippocampus (Hip), CA1 of hippocampus (CA1), amygdala (Amyg) and its central (Ce) basomedial (BM), medial (Me) and amygdalo-hippocampal (AHi) nuclei, interpeduncular nucleus (IP) and dorsal raphe nucleus (DRN). The anatomical nomenclature was that of the mice stereotaxic atlas of Franklin and Paxinos (1997). Slides stained with cresyl violet (Nissl) were used to aid in anatomical localization.

Basal and agonist-stimulated [^{35}S]GTPγS incorporation were measured in all mice. Background was not subtracted from these values, as it was uniformly low (± 0.25 fmol/mg protein) in every region. In each genotype,

differences between regions were analyzed by one-way ANOVA with repeated measures, followed by Bonferroni's multiple comparison test, and differences between basal and stimulated values by Student's *t* test. Differences between genotypes were analyzed by two-way ANOVA (GraphPad, Prism Software Inc., San Diego, CA). Differences with $p < 0.05$ were considered significant.

3. Results

3.1. Basal and agonist-stimulated [^{35}S]GTPγS incorporation in wild-type mice

Fig. 1A and B illustrates the autoradiographic visualization of basal and 5-CT-stimulated incorporation of [^{35}S]GTPγS in the brain of WT mice. The levels of basal, 5-CT-stimulated and 8-OH-DPAT-stimulated incorporation of [^{35}S]GTPγS in different regions of WT mice brain known for their content in 5-HT$_{1A}$ and/or 5-HT$_{1B}$ receptors are shown in Fig. 1C and Fig. 3A. Basal incorporation varied between regions ($p < 0.001$), the highest values being found in Amyg. After 5-CT activation, there were only two regions, CPu and IP, which showed no statistically significant difference between basal and agonist-stimulated [^{35}S]GTPγS incorporation (Fig. 1C). 5-CT-stimulation was significantly greater in En and Amyg than in Acb, LGP, SN and DRN, and also in En than LS, and CA1 than LGP and DRN. After 8-OH-DPAT activation (Fig. 3A), significant stimulation was detected in Cin, Fro, Cl, LS, En, Me, AHi and whole Amyg, entire Hip, CA1 and DRN, whereas there was

no statistically significant activation in Ce and BM of Amyg and IP, and no hint of any activation in CPu, LGP, SN.

3.2. Basal and 5-CT-stimulated [^{35}S]GTPγS incorporation in 5-HT$_{1A}$ and 5-HT$_{1B}$ KO mice

Fig. 2A and B are examples of the basal and 5-CT-stimulated [^{35}S]GTPγS incorporation in the brain of 5-HT$_{1A}$ and 5-HT$_{1B}$ KO mice, respectively. As shown in Fig. 2C and D, there were no significant differences from WT in the basal incorporation of [^{35}S]GTPγS, in all regions of both KO mice examined after 5-CT activation. As expected, in the 5-HT$_{1A}$ KOs (Fig. 2C), the 6 regions known to be normally endowed with 5-HT$_{1A}$ receptor displayed virtually no 5-CT-stimulated incorporation, whereas the levels of 5-CT-stimulated incorporation were similar to WT in all 4 regions containing 5-HT$_{1B}$ receptors. Conversely, in the 5-HT$_{1B}$ KOs (Fig. 2D), there was no stimulated incorporation in all regions known to contain 5-HT$_{1B}$ receptors in the WT (Acb, CPu, LGP and SN). In 5-HT$_{1A}$ rich regions, the 5-CT stimulation was the greatest in En and CA1, as in the WT, and there was no activation in IP. At variance with the WT, however, there was no significant activation in whole Amyg. This latter change also resulted in a significant difference from WT, when comparing 5-CT-stimulated [^{35}S]GTPγS incorporation between WT and KO mice ($p < 0.01$).

3.3. Basal and 8-OH-DPAT-stimulated [^{35}S]GTPγS incorporation in 5-HT$_{1B}$ KO mice

Similar values of basal [^{35}S]GTPγS incorporation as in WT were measured in all regions of 5-HT$_{1B}$ KO mice examined after 8-OH-DPAT activation (Fig. 3A and B). As expected, there was no 8-OH-DPAT activation of [^{35}S]GTPγS incorporation in regions of 5-HT$_{1B}$ KO brain known to contain mainly 5-HT$_{1B}$ receptors in WT (Fig. 3B). In most regions containing 5-HT$_{1A}$ receptors (Cin, Fro, En, Cl, LS, Amyg, Me, AHi, Hip, CA1, DRN), stimulation of [^{35}S]GTPγS incorporation was now lower than in WT ($p < 0.001$), or than after 5-CT activation ($p < 0.01$). In the latter two regions, however, there seemed to be little change by comparison to WT.

4. Discussion

4.1. G-protein activation in WT mice: methodological considerations

To our knowledge, this is the first study in which autoradiography of [^{35}S]GTPγS incorporation was used to determine the anatomical location and degree of regional activation of 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor coupling to G-proteins in mice lacking 5-HT receptor subtypes. Parallel experiments of agonist-stimulated [^{35}S]GTPγS incorporation for other G-protein coupled receptors were carried out in brain slices from the same animals to standardize the method (positive controls). For this, [^{35}S]GTPγS incorporation was measured in the presence of the μ-opioid agonist DAMGO (10 μM) or GABA$_B$ agonist baclofen (300 μM), yielding the strong signals and

characteristic labeling patterns previously reported by Sim et al. (1995; 2000) (data not shown). Because there an ubiquitous endogenous incorporation of [^{35}S]GTPγS was known to depend on adenosine A1 receptors (Laitinen and Jokinen, 1998), the measurements were systematically obtained in the presence of a specific A1 antagonist to facilitate the detection of eventual changes in incorporation due to the activation of other receptor subtypes. Because basal incorporation depends on the intrinsic activity of a variety of receptors, it could have been affected by adaptive processes secondary to the deletion of the 5-HT$_{1A}$ or 5-HT$_{1B}$ receptors. Reliable comparison of receptor efficacy between WT and KO mice therefore required that basal as well as agonist-stimulated incorporation be measured in each genotype.

Regional variations in basal [^{35}S]GTPγS incorporation did not appear to be accounted for by differences in densities of 5-HT$_{1A}$ or 5-HT$_{1B}$ receptors. Indeed, the profiles of basal activity in both knockouts were not significantly different from WT. In contrast, regional variations in agonist-stimulated incorporation appeared to be entirely explained by activation of one and/or the other of the targeted receptors, since regions known to contain mainly or exclusively one of them no longer displayed any significant activation in the respective knockouts. In principle, regional or genotypic differences in stimulated activity could represent differences in affinity and/or number of the targeted receptors, as well as in nature and content in G-proteins or other determinants of the efficacy of coupling (e.g., Weaber and Moskovitz,

1997; Dupuis et al., 1998). Here however, the receptor changes could be ruled out as accounting for differences between genotypes, since no measurable changes in regional density of either 5-HT_{1A} or 5-HT_{1B} receptors were reported in these null mutants (Ase et al., 2001). In the absence of significant activation within a region known to contain a significant amount of either receptor, one had to assume that these existed in an inactive form. Changes in the efficacy of coupling were indeed likely to account for all regional differences in stimulated incorporation that were not proportional to the respective density of the activated receptor. A good example was the considerably greater 5-HT_{1A}-mediated G-protein activation in En than LS of WT in the face of comparable densities of 5-HT_{1A} receptors (Ase et al., 2001).

When using the potent but non selective 5-$HT_{1A/1B}$ agonist 5-CT to stimulate [^{35}S]GTPγS incorporation, care was taken to include anatomical regions known to contain mainly, or exclusively, 5-HT_{1A} or 5-HT_{1B} receptors. This strategy allowed to measure 5-HT_{1B} dependent G-protein activation in regions known to be deprived of 5-HT_{1A} receptors even in the absence of sufficiently powerful selective 5-HT_{1B} agonists (Dupuis et al., 1998). In brain regions of WT mice rich in 5-HT_{1B} binding sites, we had indeed found negligible activation by incubation with 10 µM CGS-12066A, a specific 5-HT_{1B} agonist (data not shown). The increases in [^{35}S]GTPγS incorporation induced by 5-CT in 5-HT_{1A}-rich regions of WT mouse (DRN and hippocampus) were somewhat lower than previously reported under different experimental conditions in mouse with a different genetic background (Fabre

et al., 2000; Frogier et al., 2001). The 5-HT_{1B}-mediated increases were in keeping with earlier observations in mouse or rat substantia nigra after activation by CP 93129 (Fabre et al., 2000) or 5-CT, respectively. The lack of stimulation by 5-CT in basal ganglia structures of 5-HT_{1B} KO mice provided unequivocal evidence of an effect mediated by these receptors in the WT or 5-HT_{1A} KO mice (Fig. 2A and B).

To activate 5-HT_{1A} receptors, the agonist 8-OH-DPAT was chosen for its recognized selectivity, which was verified in the present study. 8-OH-DPAT-stimulated [^{35}S]GTPγS incorporation was absent in mice lacking 5-HT_{1A} receptors. Also, the anatomical distribution of 8-OH-DPAT-stimulated [^{35}S]GTPγS incorporation correlated with that of 5-HT_{1A} receptor binding (Laporte et al., 1994; Ase et al., 2001), with high levels of both receptors and receptor-activated G-proteins in Hip, Amyg, LS and DRN of the WT mice. These increases of [^{35}S]GTPγS incorporation appeared to be of the same order of magnitude as previously measured in CD-1 mouse brain (Li et al., 2000). The generally lower increases after 8-OH-DPAT than 5-CT suggested a lesser efficacy of the selective agonist in activating in activating 5-HT_{1A} receptors (Pauwels et al., 1997).

4.2. G-protein activation in 5-HT_{1A} knockout mice

In the 5-HT_{1A} KOs, the similar levels of 5-CT-stimulated [^{35}S]GTPγS incorporation as in WT in all four regions known to contain 5-HT_{1B} receptors ruled out compensatory changes in the efficacy of coupling of these receptors to G-protein. The observation that in all regions known to contain 5-HT_{1A}

but not 5-HT_{1B} receptors in WT, 5-CT-stimulated [^{35}S]GTPγS incorporation was absent in the 5-HT_{1A} KO, confirmed that this agonist acts mainly on these two 5-HT receptor subtypes. It was in keeping with previous observations indicating that, in regions rich in 5-HT_{1A} receptors, [^{35}S]GTPγS incorporation stimulated by 5-CT is abolished by the addition of the highly selective 5-HT_{1A} antagonist NAN-190 and WAY 100635, in guinea pig and rat brain respectively (Waeber and Moskowitz, 1997; Fabre et al., 2000; Froger et al., 2001).

4.3. G-protein activation in 5-HT_{1B} knockout mice

A similar level of 5-HT_{1A}-mediated, 5-CT-stimulated [^{35}S]GTPγS incorporation as in WT was observed in all brain regions of 5-HT_{1B} KOs known to contain 5-HT_{1A} receptors, except amygdala. In most forebrain regions, these receptors act as heteroreceptors, i.e. receptors located on non-5-HT neurons, whereas, in the DRN, they act as autoreceptors, on the 5-HT neurons themselves (Kia et al., 1996a,b and Riad et al., 2000). Thus, the efficacy of 5-HT_{1A} receptor coupling to G-protein did not appear to be tightly correlated with the markedly different pharmacological properties of 5-HT_{1A} receptors acting as auto- versus heteroreceptors (Dong et al., 1997; Le Poul et al., 2000), and notably with their capacity to internalize (and desensitize) as auto- but not heteroreceptors upon acute treatment by agonist (Riad et al., 2001). In whole amygdala, the lack of any significant 5-CT activation suggested some dependence on 5-HT_{1A} receptors. Since a previous autoradiographic study, carried out with [^{3}H]8-OH-DPAT (Ase et al., 2001),

had found no change in the density of 5-HT_{1A} receptor binding sites in amygdala as in other brain regions of 5-HT_{1B} KO mice, such lowered activation had to be interpreted as a reduced efficacy of coupling.

The reduced activation of 5-HT_{1A}-mediated [^{35}S]GTPγS incorporation in amygdala was also observed with the selective 5-HT_{1A} agonist 8-OH-DPAT, and more precise measurements indicated that it mainly involved two subdivisions of this structure, the Me and AHi nuclei, and less clearly Ce and BM. In addition, stimulated increases were no longer apparent (e.g., Cin, Fro, LS and DRN), or were of lesser magnitude (En, Cl) in several brain regions known to contain 5-HT_{1A} receptors. Why such decreases had not been detected with 5-CT could reflect the lesser efficacy of 8-OH-DPAT than 5-CT toward the 5-HT_{1A} receptor, as suggested by the lower activations measured in 5-HT_{1A}-rich regions of WT mice. Since our measurements involved the first step in the complex signaling cascade induced by agonist stimulation, the difference could hardly be attributed to multiple versus single pathways of biochemical-response (Fargin et al., 1991; Liu and Albert, 1991). Differences in agonist efficacy have also been imputed to their ability to promote ternary complex formation (agonist–receptor–G-protein) (Matesic et al., 1991; for review, see Kenakin, 1995). However, such data is still lacking for the 5-CT and 8-OH-DPAT agonists.

The results obtained with 8-OH-DPAT indicated a generalized, compensatory decrease in G-protein coupling to 5-HT_{1A} receptors in the 5-HT_{1B} mutant. This could be viewed as an indication of cross-talk between

these two receptors, at least in regions where they are known to coexist as autoreceptors in the same neurons, such as the DRN (Bruinvels et al., 1994; Hopwood and Stamford, 2001). Previous evidence of receptor cross-talk involving 5-$HT_{1B/1D}$ receptors has only been reported in peripheral blood vessels, for $G_{q/11}$-coupled receptors such as thromboxane A_2 or angiotensin AT_1, in which case very low efficacy or even silent $G_{i/o}$-coupled 5-$HT_{1B/1D}$ receptors became profoundly active (for review, see Martin et al., 1998). To our knowledge, this type of data is not yet available regarding central 5-HT_{1B} or 5-HT_{1A} receptors.

The reduced efficacy of 5-HT_{1A} receptor coupling to G-protein in the DRN could well explain the decreased responsiveness to 5-HT_{1A} receptor agonist (8-OH-DPAT) observed by Knobelman et al. (2001) in the hippocampus of the 5-HT_{1B} KO mice, and imputed to a partial desensitization of the 5-HT_{1A} autoreceptors in the raphe nuclei. In non-5-HT neurons, it might have a significant impact on the firing and release of various transmitters, i.e., acetylcholine, noradrenaline and glutamate, which are known to be modulated by 5-HT via 5-HT_{1A} receptors (Bianchi et al., 1990; Done and Sharp, 1994; Kia et al., 1996c; Hajós-Korcsok and Sharp, 1999). All these adaptive changes, direct or indirect, could contribute to the behavioral phenotype of the 5-HT_{1B} KO mutant.

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Figure legends

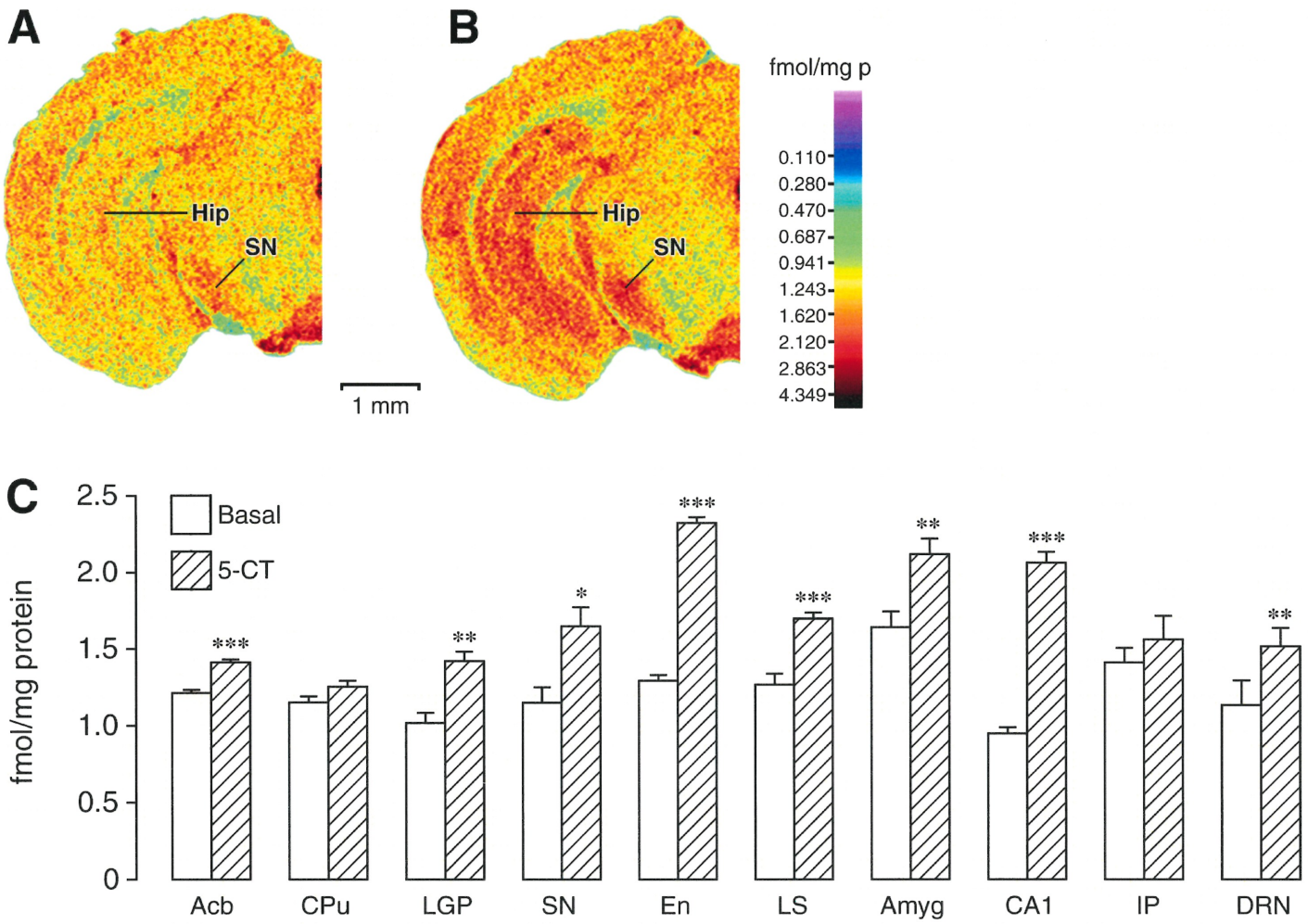
Figure 1. Basal and 5-CT-stimulated of [^{35}S]GTPγS incorporation in the brain of WT mice. The color-coded autoradiographs illustrate the [^{35}S]GTPγS incorporation in regions containing 5-HT$_{1A}$ (e.g. hippocampus, Hip) and 5-HT$_{1B}$ receptors (e.g., substantia nigra, SN), under basal (**A**) and 5-CT-stimulated (**B**) conditions. In both Hip and SN, note the virtual absence of labeling under the basal condition (**A**). In contrast, there is a strong [^{35}S]GTPγS incorporation in the same regions after 5-CT stimulation (**B**). The histogram (**C**) displays the values of basal and 5-CT-stimulated [^{35}S]GTPγS incorporation in ten brain regions, the first four of which are known to contain mainly 5-HT$_{1B}$, and the last six, 5-HT$_{1A}$ receptors. Most regions show statistically significant stimulation by comparison to basal, as denoted by asterisks: Acb, LGP, SN, En, LS, Amyg, CA1 and DRN. Only caudate nucleus (CPu) and interpeduncular nucleus (IP) do not show a significant increase in [^{35}S]GTPγS incorporation. Means ± SEM from six mice. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by Student's *t* test.

Figure 2. [^{35}S]GTPγS incorporation in the brain of 5-HT$_{1A}$ and 5-HT$_{1B}$ knockout (KO) mice. The color-coded autoradiographs (**A** and **B**) illustrate 5-CT-stimulated incorporation in the respective KOs. In the 5-HT$_{1A}$ KOs (**A**), 5-CT-stimulated incorporation is apparent in SN, but not in Hip nor Amyg (compare with Fig. 1A). On the contrary, in the 5-HT$_{1B}$ KOs (**B**), strong stimulation is visible in Hip and Amyg but not in SN (compare with Fig. 1B).

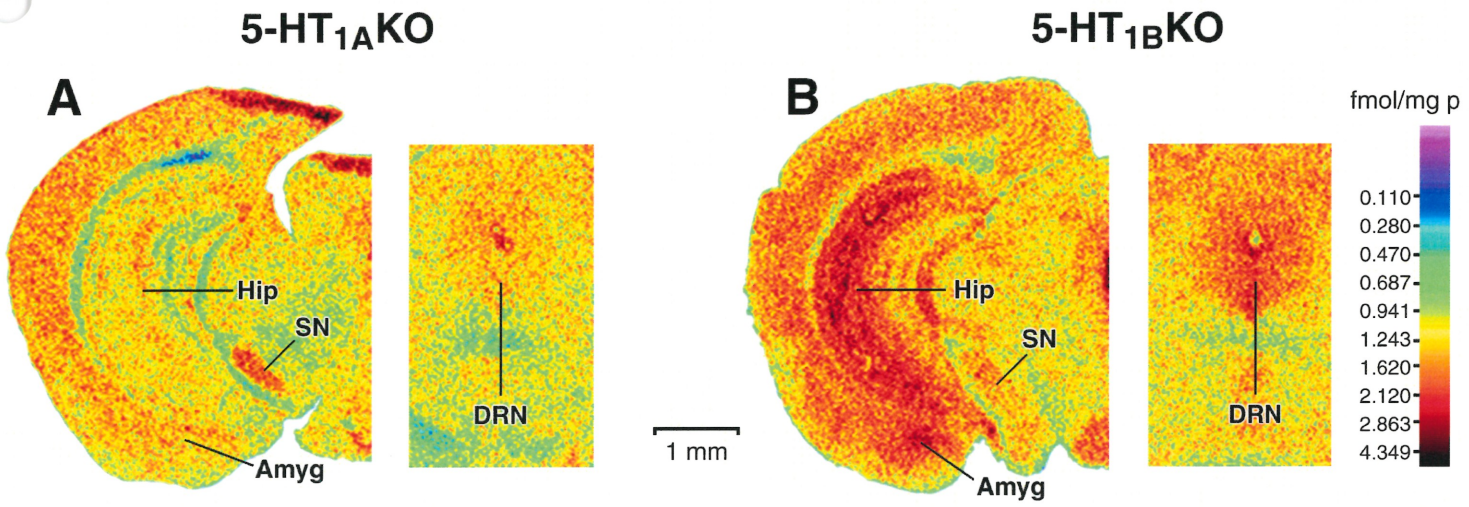
The histograms (**C** and **D**) display the levels of basal and 5-CT-stimulated incorporation in the two KOs. As expected, in the 5-HT_{1A} KOs (**C**), there is significant 5-CT stimulation in most regions containing 5-HT_{1B} receptors (Acb, LGP and SN), whereas, in the 5-HT_{1B} KO (**D**), 5-CT stimulation is observed in all regions rich in 5-HT_{1A} receptors and showing stimulation in the WT, except amygdala. Same abbreviations as in Fig. 1. Means ± SEM from 6 mice in both groups. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by Student's *t* test.

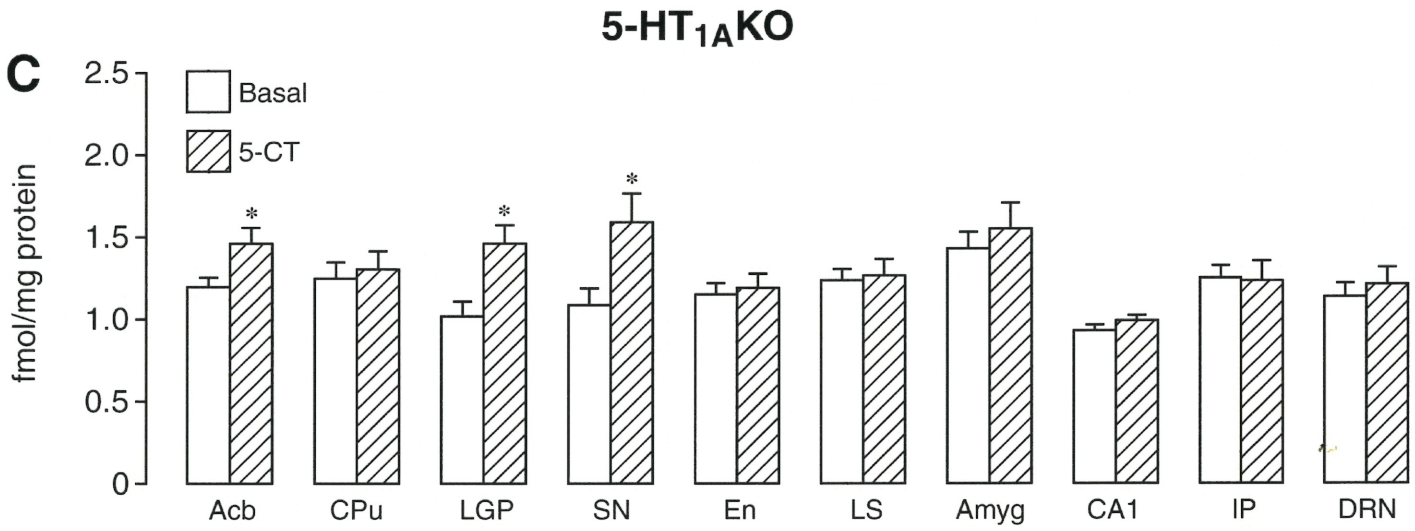
Figure 3. Basal and 8-OH-DPAT-stimulated [^{35}S]GTPγS incorporation in the brain of WT (**A**) and 5-HT_{1B} KO (**B**) mice. Means ± SEM from the same mice as in Fig. 1 (**A**) and Fig. 2D (**B**). As shown in **A,** 8-OH-DPAT-stimulated [^{35}S]GTPγS incorporation is measured in most regions of WT mice known to contain 5-HT_{1A} receptors, and not in the CPu, LGP and SN, which contain mainly 5-HT_{1B} receptors in the WT. In **B,** 8-OH-DPAT-stimulated [^{35}S]GTPγS incorporation is no longer significant in Cin and Fro, LS, Amyg, Me (medial nucleus of amygdala) and DRN, and less significant than WT in En, Cl, AHi (amygdalo-hippocampal nucleus), Hip and CA1 (Student's *t* test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The global comparison of regions containing 5-HT_{1A} receptors by two-way ANOVA with repeated measures confirms the lower levels of stimulation in the 5-HT_{1B} KOs ($p < 0.01$). Additional abbreviations: Ce, central and BM, baso-medial nucleus of amygdala.

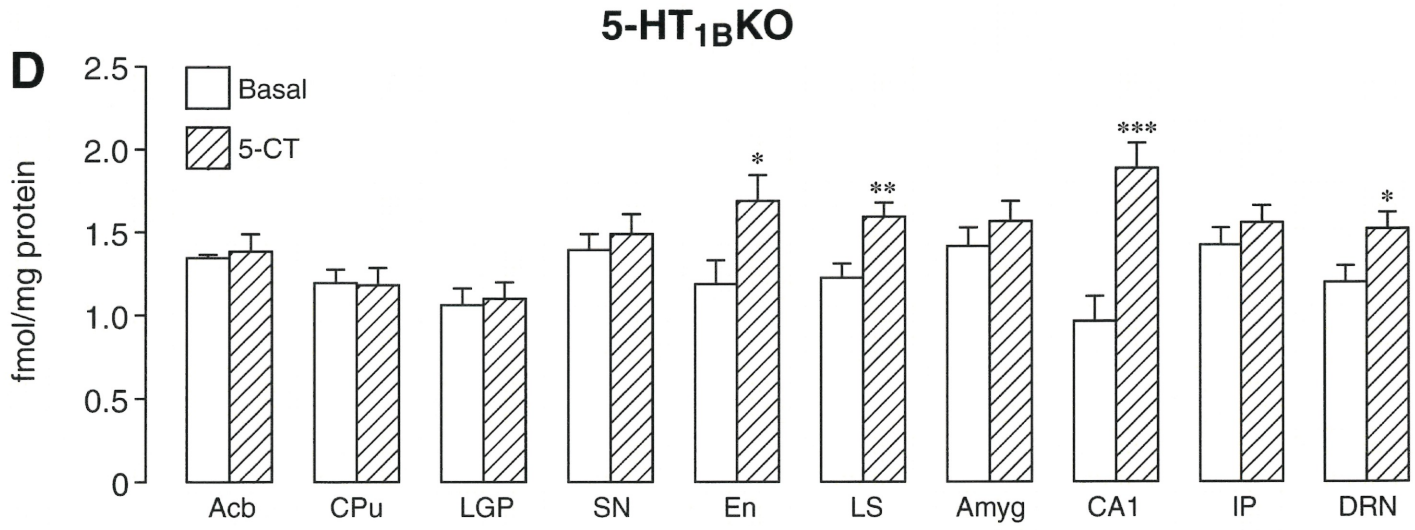
Basal and 5-CT-stimulated [^{35}S]GTPγS incorporation in wild-type mice



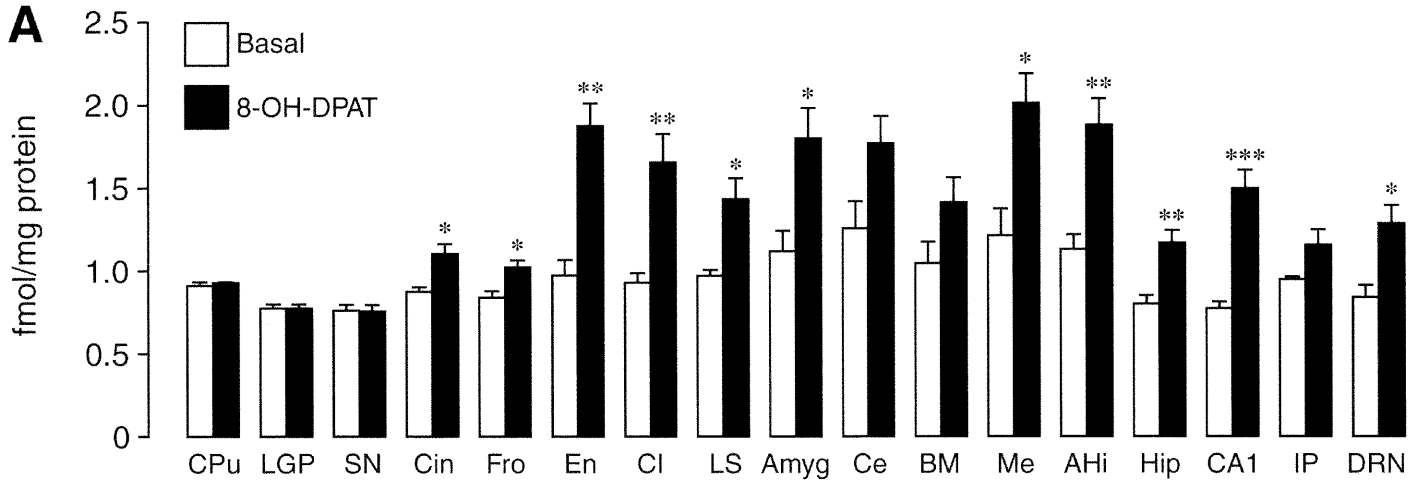
5-CT-stimulated [^{35}S]GTPγS incorporation in knockout mice



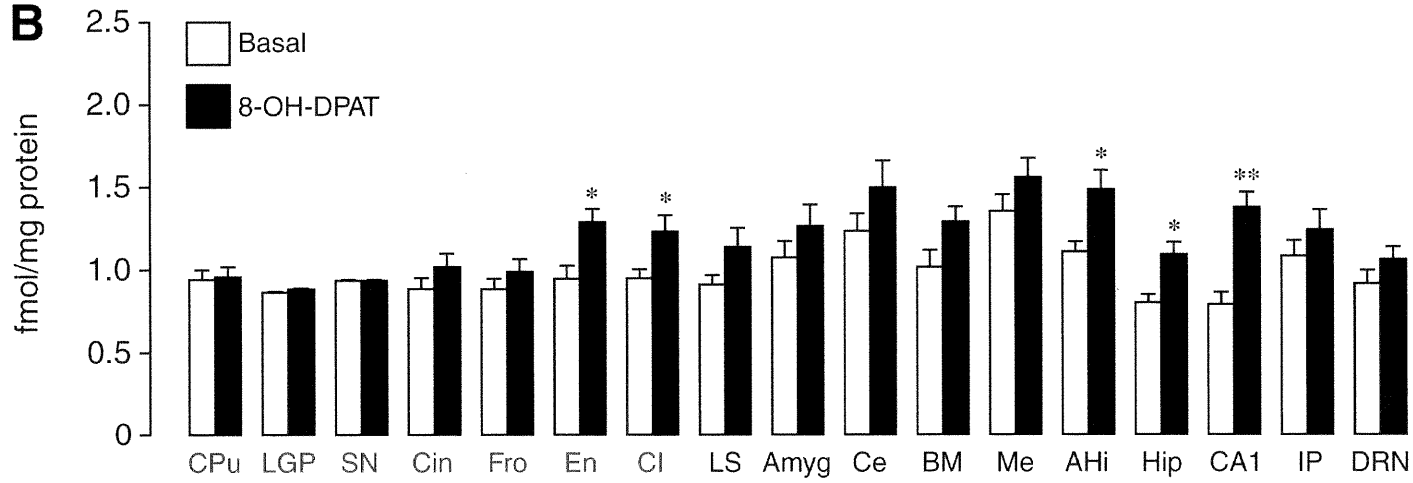




Basal and 8-OH-DPAT-stimulated [^{35}S]GTPγS incorporation in wild-type mice



Basal and 8-OH-DPAT-stimulated [^{35}S]GTPγS incorporation in 5-HT$_{1B}$KO mice



Chapter V

GENERAL DISCUSSION

V. 1 THE 5-HT$_{1A}$ RECEPTOR KNOCKOUT MOUSE

V.1.1 Increased 5-HT turnover

Our first study (Chapter II) revealed major changes in monoamine metabolism in discrete CNS regions of both homozygous 5-HT$_{1A}$ and 5-HT$_{1B}$ KO mice of the 129/Sv strain. In the 5-HT$_{1A}$ KOs, 5-HT concentration was decreased, compared to wild-type mice, in brain regions showing no changes in 5-HT metabolites, i.e., nucleus accumbens, locus coeruleus and spinal cord. However, the higher [5-HT metabolites]/[5-HT] ratios in dorsal/medial raphe nuclei, olfactory bulb, substantia nigra and locus coeruleus indicate an augmented 5-HT turnover and thus, presumably, increased 5-HT release (Shannon et al. 1986; Rollema et al. 1996; Ross and Stenfors, 1997; Stenfors et al., 1999). As these increases involved the nuclei of origin of 5-HT neurons (dorsal/medial raphe nuclei) as well as three of their territories of innervation, it can be assumed that they reflect increases in both the somatodendritic (Héry et al., 1982) and axon terminal release of 5-HT.

It is well established that somatodendritic 5-HT$_{1A}$ autoreceptors mediate an inhibition of the firing and release by 5-HT neurons (Hjorth and Magnusson, 1988; Hamon et al., 1990). This inhibitory effect has been demonstrated to be present under basal conditions, as reflected by increases in the spontaneous firing of 5-HT neurons *in vivo* or *in vitro* after systemic administration or superfusion of the highly selective 5-HT$_{1A}$ antagonists, WAY 100635 (Fornal et al., 1996; Corradeti et al., 1996) or p-MPPI (Bjorvatn et al., 1998). In the constitutive absence of the 5-HT$_{1A}$ receptor, increased

5-HT turnover in the nuclei of origin and territories of projection was therefore likely to result from an enhanced tonic activity (lack of autoinhibition) of 5-HT neurons. It is noteworthy that a very recently report by Richer et al. (2002) has confirmed that the spontaneous firing of dorsal raphe neurons is considerably increased by about 90% in 5-HT1A KO compared to WT mice.

The increased 5-HT turnover in olfactory bulb could be explained on this basis, since its 5-HT innervation has been shown to originate from both the dorsal and median raphe nuclei, in mouse as well as in rat (Araneda et al., 1980ab; McLean and Shipley, 1987). Studies in neonate rats have indicated that this 5-HT input is important for the acquisition or expression of olfactory-based learned behavior and thus facilitates conditioned olfactory learning induced by NA (McLean et al., 1993; Price et al., 1998). Whether these behaviors are affected in the 5-HT_{1A} KO mice remains to be determined. If they are not, it would be of interest to identify the adaptive mechanism responsible for the takeover of function.

In substantia nigra pars reticulata, a double-labeling immunoelectron microscopic study has shown many 5-HT terminals to be in synaptic contact with tyrosine hydroxylase-positive as well as -negative, somata and dendrites (Corvaja et al., 1993). Facilitation of basal DA release by 5-HT itself, or by 5-HT agonists, has been repeatedly demonstrated in various subcortical areas, both *in vitro* and *in vivo* (Jiang et al., 1990; Benloucif and Galloway, 1991; Galloway et al., 1993; Parsons and Justice, 1993; Iyer and Bradberry,

1996). Also, in substantia nigra pars reticulata 5-HT has been shown to inhibit GABA release acting through 5-HT_{1B} receptors (Johnson et al., 1992; Stanford and Lacey, 1996) located on the preterminal axons of striatonigral neurons (Boschert et al., 1994; Doucet et al., 1995; Riad et al., 2000). The enhanced tonic activity of midbrain 5-HT neurons in 5-HT_{1A} KO mice should therefore entail both a direct facilitation and a concomitant disinhibition of the nigral DA neurons. The ensuing disinhibition of the nigral DA neurons could in turn account for the elevation of DA, or its metabolites in the caudate-putamen as well as in other DA-innervated brain regions. The higher DA turnover in olfactory bulb and dorsal/medial raphe nuclei might result from similar mechanisms operating within these regions, in which DA neurons are present, albeit in smaller number than in the substantia nigra (Descarries et al., 1982; Björklund and Lindvall, 1984).

The presumed increase of 5-HT turnover in the locus coeruleus was also of interest in relation with the anxious-like behavior of 5-HT_{1A} KO mice. In rodents as well as in primates, anxious-like behaviors and increased responses to stressful stimuli have generally been associated with increased activity in this noradrenergic nucleus (review in Charney et al., 1995). The NA concentration was unchanged in the locus coeruleus, but it showed an increase in olfactory bulb, thalamus, substantia nigra and cerebellum, these regions are innervated by the locus coeruleus. Even if the interpretation of these data was somewhat hampered by the lack of measurement of NA metabolites, it was likely that such changes reflected a diminished activity of

NA neurons in locus coeruleus, secondary to the constitutive increase in serotoninergic tone (Haddjeri et al., 1997; Kaehler et al., 1999).

V.1.2 Lack of compensatory changes in 5-HT$_{1B}$ receptor density

The values of regional densities of 5-HT$_{1B}$ receptors measured in the present study of WT and KO 129/Sv mice were consistent with earlier autoradiographic data obtained in other mouse strains (Laporte et al., 1994; Langlois et al., 1995). In the 5-HT$_{1A}$ KOs, the lack of any compensatory changes in 5-HT$_{1B}$ receptor densities was consistent with the recent demonstration by Richer et al. (2000) of equivalent inhibitory effects of sumatriptan or CP 93129 (5-HT$_{1B}$ agonists) on the electrically-evoked release of [^{3}H]5-HT from preloaded brain slices. It was also in keeping with our earlier biochemical demonstration of an increased 5-HT turnover in the dorsal/medial raphe nuclei and several territories of 5-HT innervation of these mice (Ase et al., 2000a), now known to result from a doubling of the spontaneous firing rate of 5-HT neurons (Richer et al., 2002). Altogether, these data would not seem to favor compensatory changes in the efficacy of 5-HT$_{1B}$ receptors in the 5-HT$_{1A}$ KOs, as previously suggested (Ramboz et al., 1998).

V.1.3 Down-regulation of the plasma membrane 5-HT transporter

In this mutant, region-specific decreases in the density of [^{3}H]citalopram binding could be indicative of a reduction of 5-HTTs at the transcriptional or post-transcriptional level, in the absence of measurable

changes in 5-HT innervation density. 5-HTT gene expression was probably not reduced, since the density of 5-HTT binding was unchanged in the raphe nuclei, i.e. in 5-HT somata/dendrites. Moreover, in rat raphe nuclei the chronic and sustained administration of the 5-HTT blocker paroxetine was shown to reduce 5-HTT binding without alteration of its mRNA levels (Benmansour et al., 1999; see also Neumaier et al., 1996; Koed and Linnet, 1997). Therefore, decreases in densities of 5-HTTs here measured in parts of the cerebral cortex, limbic system, thalamus and basal ganglia of the 5-HT_{1A} KOs were interpreted as post-transcriptional effects. In these territories of 5-HT innervation, the reduction in 5-HTT densities might decrease 5-HT clearance and thus contribute to enhanced 5-HT neurotransmission. Interestingly, reduced 5-HT clearance as well as reduced densities of 5-HTT binding, without significant effects on tissue levels of 5-HT or 5-HIAA, have been documented in rat dorsal/medial raphe nuclei, hippocampus and/or cerebral cortex after long-term administration of 5-HT uptake inhibitors (Kovachich et al., 1992; Piñeyro et al., 1994; Benmansour et al., 1999). Similarly, our earlier biochemical measurements in the 5-HT_{1A} KOs showed no significant changes of 5-HT and its metabolites in most of the brain regions here displaying a decreased density of 5-HTT (Ase et al., 2000a).

V.1.4 Lack of compensatory changes in the efficacy of 5-HT_{1B} receptor coupling to G-protein

To our knowledge, ours was the first study in which autoradiography of [^{35}S]GTPγS incorporation was used to determine the anatomical location and

degree of regional activation of 5-HT_{1A} and 5-HT_{1B} receptor coupling to G-proteins in mice lacking 5-HT receptor subtypes. Our previous study (Chapter III) had shown that regional variations in basal [^{35}S]GTPγS incorporation could not be accounted for by differences in densities of 5-HT_{1A} or 5-HT_{1B} receptors. In this context, it was all the more interesting to investigate G-protein coupling since eventual changes would indeed be indicative of a true lack in efficiency of coupling, independent of the affinity or number of the receptors in question. The similar profiles of basal [^{35}S]GTPγS incorporation in WT and KO mice could have been viewed as suggesting a lack of 5-HT receptor change in the KOs, but this conclusion could not be drawn since the regional profiles rather indicated that neither 5-HT_{1A} nor 5-HT_{1B} receptors significantly contribute to this basal, or endogenous activity. In contrast, regional variations in agonist-stimulated incorporation appeared to be entirely imputable to activation of one or the other 5-HT, since regions known to contain mainly or exclusively one of them no longer displayed any significant activation in the respective KOs. However, changes in the efficacy of coupling were likely to account for all regional differences in stimulated incorporation that were not proportional to the respective density of the activated receptor. A good example was the considerably greater 5-HT_{1A}-mediated G-protein activation in endopiriform nuclei than lateral septum of WT mice in the face of comparable densities of 5-HT_{1A} receptors (Ase et al., 2001). In the absence of significant activation

within a region known to contain a significant amount of either receptor, one had to assume that these existed in an inactive form.

The similar levels, between WT and 5-HT_{1A} KO mice, of 5-CT-stimulated [^{35}S]GTPγS incorporation in regions known to contain 5-HT_{1B} receptors ruled out compensatory changes in the efficacy of coupling of these receptors to G-protein. This lack of evidence for any change in 5-HT_{1B} receptor efficacy in 5-HT_{1A} KO mice when using the potent but non selective 5-$HT_{1A/1B}$ agonist 5-CT to stimulate [^{35}S]GTPγS incorporation was somewhat puzzling. It might have been expected that, at least in regions with an excessive 5-HT innervation (amygdala and hippocampus), an increase in G-protein coupling would have resulted from the increased number of 5-HT axon terminals endowed with 5-HT_{1B} autoreceptors. A likely explanation for this apparent lack of effect might be that the proportion of 5-HT_{1B} receptors actually located on 5-HT terminals as opposed to other axon terminals in both these regions is to small to induce a detectable change.

The agonist 8-OH-DPAT was chosen to activate 5-HT_{1A} receptors specifically, because of its recognized selectivity and efficacy toward this receptor. 8-OH-DPAT-stimulated [^{35}S]GTPγS incorporation was indeed absent in mice lacking 5-HT_{1A} receptors, while no stimulation was observed in 5-HT_{1B} containing brain regions of WT as well as 5-HT_{1A} KO mice. In general (but see above), the regional profile of 8-OH-DPAT-activation of 5-HT_{1A} receptor coupling in WT mice was consistent with the presumed amounts of 5-HT_{1A} receptor in the regions of interest normally endowed with this

receptor subtype (Laporte et al., 1994; Ase et al., 2001). For example, levels of both 8-OH-DPAT binding and 8-OH-DPAT-stimulated [^{35}S]GTPγS incorporation were high in hippocampus, amygdala, lateral septum and DRN of the WT mice. In terms of efficacy, however, the lesser amounts of G-protein activation by 8-OH-DPAT than 5-CT suggested that this selective agonist might not be as powerful as 5-CT in activating 5-HT$_{1A}$ receptors (Pauwels et al., 1997).

In any event, the results in both WT and the 5-HT$_{1A}$ KO mice altogether ruled out the possibility that compensatory changes in the efficacy of 5-HT$_{1B}$ receptor coupling to G-protein might be playing a significant role in some aspect of the behavioral phenotype of this mutant.

V.1.5 Behavioral implications

In rodents, it is generally assumed that activation of 5-HT$_{1A}$ autoreceptors produces its anxiolytic-like effects via the suppression of 5-HT neuron activity and consequent decrease in 5-HT release (Sprouse and Aghajanian, 1987; Andrews et al., 1994; De Vry, 1995). The converse enhancement of anxiety observed in 5-HT$_{1A}$ KO mice might be secondary to the increased activity of 5-HT neurons in the dorsal/medial raphe nuclei. Surprisingly, increased firing rate of 5-HT neurons and selective desensitization of 5-HT$_{1A}$ autoreceptors were measured in neurokinin 1 receptor (NK1 -/-) KO mice, a mutant displaying decreased anxiety-related behaviors (Santarelli et al., 2001). The associated increases in DA and NA contents, and particularly in DA turnover, might also contribute to the

abnormal behavioral phenotype of these mice, through mechanisms that remain to be identified and characterized. How the localized decreases in 5-HTTs might affect the behavior of 5-HT_{1A} KOs remains for now a matter of speculation. The reduced number of uptake sites, particularly in limbic regions, might also result in increased 5-HT transmission and therefore enhance anxious-like behavior, or antidepressant-like responsiveness, through the activation of different 5-HT receptor subtypes (Heisler et al., 1998).

V.2. THE 5-HT_{1B} RECEPTOR KNOCKOUT MOUSE

V.2.1 Altered monoamine metabolism

In 5-HT_{1B} KO mice, the decreased 5-HT concentration in spinal cord, nucleus accumbens, locus coeruleus, and presumably other territories of 5-HT innervation was associated also with normal 5-HT metabolite levels. This suggested a dysregulation of 5-HT biosynthesis at the level of the nerve terminals in the absence of 5-HT_{1B} autoreceptors. Other regions of 5-HT projection did not show such changes, in keeping with the results of a microdialysis study (Trillat et al., 1997), which did not demonstrate significant differences in the basal or potassium-evoked release of 5-HT in frontal cortex and ventral hippocampus of the 5-HT_{1B} KO. The lack of altered 5-HT turnover in the raphe nuclei was also consistent with recent electrophysiological findings of Evrard et al. (1999), who found no changes in

the spontaneous firing of the raphe 5-HT neurons, nor in the potency of the agonist 8-OH-DPAT to inhibit their firing, in 5-HT_{1B} KO mice.

The few alterations in DA metabolism observed in 5-HT_{1B} KO were not as readily explained as in 5-HT_{1A} KO mice. The decreased DA concentration in nucleus accumbens was associated with an increased DA turnover, perhaps as a consequence of the augmented 5-HT turnover in this region. Indeed, concordant microdialysis studies have demonstrated a 5-HT-induced increase in extracellular DA in rat nucleus accumbens upon local application of 5-HT, or following *in vivo* stimulation of the dorsal raphe nucleus (Parsons and Justice, 1993; De Deurwaerdere and Spampinato, 1999).

V.2.2 Lack of compensatory changes in 5-HT_{1A} receptor density

As in the case of the 5-HT_{1B} receptor, the regional densities of 8-OH-DPAT binding to 5-HT_{1A} receptors measured throughout the brain of WT and KO mice were in good agreement with earlier autoradiographic data obtained from other mouse species (Boschert et al., 1994). The lack of changes in 5-HT_{1A} receptor densities in the 5-HT_{1B} KOs came as a surprise, since both these receptor subtypes, albeit differentially located on 5-HT neurons, ultimately control 5-HT release. In the absence of the 5-HT_{1B} autoreceptor, an increase in 5-HT_{1A} receptor density was expected, at least in the raphe nuclei. These apparently negative findings were, however, in keeping with the results of Evrard et al. (1999), who reported equivalent dose-dependent inhibition of 5-HT neuron firing by the 5-HT_{1A} agonist 8-OH-DPAT in WT and 5-HT_{1B} KO mice. It has not yet been determined

whether in 5-HT$_{1B}$ KO mice neuronal firing is similarly unaffected by agonist treatment in territories of 5-HT innervation, where these receptors are known to act as heteroreceptors and display markedly different pharmacological properties (e.g., Dong et al., 1997; Le Poul et al., 2000; see also Riad et al., 2001).

V.2.3 Down-regulation of the plasma membrane 5-HT transporter

The region-specific decreases in density of [^{3}H]citalopram binding detected in 5-HT$_{1B}$ KO mice were interpreted as a reduction of 5-HTTs at the transcriptional or post-transcriptional level, in the absence of measurable changes in 5-HT innervation density. These decreases in 5-HTT densities were even more restricted than in the 5-HT$_{1A}$ KOs. A link between 5-HT$_{1B}$ receptors and 5-HTTs has been recently suggested by an amperometric study, providing evidence of decreased 5-HTT efficacy upon acute administration of 5-HT$_{1B}$ receptor antagonists in normal rat hippocampus Daws et al. (1999). In the constitutive absence of 5-HT$_{1B}$ receptors throughout brain, 5-HTTs might be down-regulated as in the 5-HT$_{1A}$ KOs. However, it remains to be explained why such a phenomenon was seemingly restricted to the neostriatum and two thalamic nuclei.

V.2.4 5-HT hyperinnervation accounts for localized increases in 5-HT transporters

There was unequivocal evidence that the increases in [^{3}H]citalopram binding sites measured in the amygdalo-hippocampal area (AHiPM) and

ventral hippocampus of the 5-HT_{1B} KOs represented a 5-HT hyperinnervation. In both regions, 5-HT axon densities were increased proportionally to the increases in [^{3}H]citalopram binding sites, while remaining normal in the adjacent basolateral posterior (BLP) nucleus of amygdala and laterodorsal/lateroposterior (LD/LP) nuclei of thalamus, where [^{3}H]citalopram binding was normal or decreased, respectively. There was no obvious explanation for the regional selectivity of this hyperinnervation. However, its existence could explain recent results showing an excessive increase of 5-HT microdialysate levels in the ventral hippocampus, but not frontal cortex, of 5-HT_{1B} KOs, after a single dose of the 5-HT uptake inhibitor paroxetine (Malagié et al., 2001; see also de Groote et al., 2000).

The existence of such 5-HT hyperinnervations in the 5-HT_{1B} KO mouse provided the first indication of an inhibitory, or negative, control of 5-HT axon growth mediated by 5-HT_{1B} receptors. The only prior report describing effects of 5-HT on the growth of 5-HT fibers incriminated astrocytic 5-HT_{1A} receptors mediating a stimulatory effect on the glial protein S-100, following lesioning of 5-HT neurons with 5,7-dihydroxytryptamine (Whitaker-Azmitia et al., 1990). Interestingly, in previous studies, the 5-HT_{1B}-mediated effects of 5-HT on neuritic outgrowth had also been found to be stimulatory, at least in primary cultures of thalamic neurons (Lotto et al., 1999; see also Salichon et al., 2001). In both these latter studies, however, the receptors activated were obviously hetero- and not auto-5-HT_{1B} receptors.

The present data did not reveal the precise localization of the proposed 5-HT_{1B}-mediated inhibitory control of 5-HT axon growth. A direct effect on 5-HT terminals would be consistent with the preterminal localization of 5-HT_{1B} autoreceptors (Riad et al., 2000), but an effect normally exerted through 5-HT_{1B} heteroreceptors located on the preterminals of neurons containing other transmitters could not be excluded. For example, 5-HT_{1B} receptors are known to be normally present on the terminals of cholinergic neurons innervating the hippocampus (Maura et al., 1989), one of the two regions showing the 5-HT hyperinnervation in the 5-HT_{1B} KOs.

In this context, the lack of any change in the average number of varicosities per unit length of 5-HT axons was also of interest. It indicated that the absolute number of 5-HT varicosities was indeed increased in the two regions, and also that the increased density of innervation was primarily due to an increased length, and presumably branching, of the 5-HT axon network. It has already been pointed out that the lengthening and branching of central axons is likely to depend on local factors within the territories of innervation, more than on inherent properties of the neurons of origin (e.g., Aznavour et al., 2001; Mechawar et al., 2001). 5-HT_{1B} receptors, located on the terminal branches of 5-HT or of other neurons, might well be part of the signaling cascade for such growth regulatory signals.

V.2.5 Decreased coupling of 5-HT_{1A} receptors to G-protein

The levels of 5-HT_{1A}-mediated, 5-CT-stimulated [^{35}S]GTPγS incorporation measured in all brain regions of 5-HT_{1B} KO mice known to contain 5-HT_{1A}

receptors were not significantly different from WT, except in amygdala, in which there was no significant activation, in contrast with the strong effect measured in WT. In view of the previous autoradiographic results showing no change in [^{3}H]8-OH-DPAT binding in this as well as in other brain regions of 5-HT$_{1B}$ KOs (see Chapter IV), such a difference from WT could only be interpreted as reflecting a change in the efficacy of G-protein coupling, independent of the amount of ligand recognition sites of the receptor complex. It was tempting to consider this decreased efficacy of 5-HT$_{1A}$ receptors as a compensatory response to the increased 5-HT innervation observed in this region. However, a similar effect was not observed in the hippocampus, even in the presence of 5-HT hyperinnervation.

Moreover, while reduced activation of 5-HT$_{1A}$-mediated [^{35}S]GTPγS incorporation was also observed in amygdala with the selective 5-HT$_{1A}$ agonist 8-OH-DPAT, more precise measurements indicated that it mainly involved two subdivisions of the amygdalar complex, the medial (Me) and amygdalo-hippocampal (AHi) nuclei, and less clearly the central (Ce) and basomedial (BM) nuclei. In addition, with 8-OH-DPAT, stimulated increases were no longer statistically different from basal levels (e.g., cingulate and frontal cortex, lateral septum and DRN), or were of lesser magnitude (endopiriform nucleus and clastrum) in all brain regions known to contain 5-HT$_{1A}$ receptors and to be activated by 8-OH-DPAT in WT mice. In addition, a two-way ANOVA with repeated measures, aimed at assessing the overall difference between the regional levels of 8-OH-DPAT-activated [^{35}S]GTPγS

incorporation in 5-HT_{1B} KOs versus WT mice clearly indicated a statistically significant lowering in the KOs, in the absence of any difference in basal levels between the two groups. Why such a generalized decrease had not been detected with 5-CT could not be ascertained. Since our measurements involved the first step in the complex signaling cascade induced by agonist stimulation, such a difference could hardly be attributed to multiple versus single pathways of biochemical-response (Fargin et al., 1991; Liu and Albert, 1991). It could reflect a lesser potency of 8-OH-DPAT than 5-CT in spite of its greater selectivity as a full agonist and not partial of the receptor, as suggested by the apparently lower levels of activation measured with 8-OH-DPAT than 5-CT in the 5-HT_{1A}-rich regions of WT mice. Differences in agonist efficacy have already been imputed to their ability to promote ternary complex formation (agonist–receptor-G protein) (Matesic et al., 1991; for review, see Kenakin, 1995). However, such data is still lacking for both the 5-CT and 8-OH-DPAT agonists.

The generalized, compensatory decrease in G-protein coupling to 5-HT_{1A} receptors in the 5-HT_{1B} mutant could be viewed as an indication of cross-talk between these two receptors, at least in regions where they are known to coexist as autoreceptors in the same neurons, such as the DRN (Bruinvels et al., 1994; Hopwood and Stamford, 2001). The reduced efficacy of 5-HT_{1A} receptor coupling to G-protein in the DRN could well explain the decreased responsiveness to the 5-HT_{1A} receptor agonist 8-OH-DPAT observed in the hippocampus of the 5-HT_{1B} KO mice, and imputed to a partial desensitization

of 5-HT_{1A} autoreceptors in the raphe nuclei (Knobelman et al., 2001). In non 5-HT neurons, it might have a significant impact on the firing and release of other transmitters, i.e., acetylcholine, noradrenaline and glutamate, known to be modulated by 5-HT via 5-HT_{1A} receptors (Bianchi et al., 1990; Done and Sharp, 1994; Kia et al., 1996c; Hajós-Korcsok and Sharp, 1999).

V.2.6 Behavioral implications

There is considerable evidence indicating that decreased brain 5-HT content in rodents is associated with aggressive behavior (Maas, 1962; Vergnes et al., 1986), whereas increased 5-HT reduces aggressiveness (Molina et al., 1987). The aggressiveness of 5-HT_{1B} KOs has been shown to be greater than in wild-type mice in different behavioral tests, including territorial and maternal aggression (Saudou et al., 1994; for review, see Brunner and Hen, 1997). These altered behaviors in 5-HT_{1B} knockouts might be associated with some of the above-described alterations in 5-HT and DA metabolism.

Other KO mice showing aggressive phenotype have also been reported. For example, in nitric oxidase synthase (nNOS -/-), reduced 5-HT metabolism and 5-HT_{1A} and 5-HT_{1B} receptor function have also been measured, accompanied by spared catecholamine levels (Chiavegatto et al., 2001). Although the nNOS and 5-HT_{1B} KO mice seem to display a similar biochemical phenotype, aggressiveness has also been reported in the monoamine oxidase A (MAO-A) KO mouse in which the biochemical parameters are in someway different. In these mice, increased 5-HT and NA

levels and decreased 5-HT metabolites have been measured throughout brain, associated with a down-regulation of 5-HT_{1A} receptors in the dorsal raphe nucleus but not hippocampus (Cases et al., 1995; Mannoury la Cour et al., 2001; see also Holschneider et al., 2001). It is clear from these data that the regional distribution of the metabolic changes observed in the various KO models must be taken into consideration in trying to account for the behavioral abnormalities.

Recently, both basal extracellular and cocaine-evoked DA levels were shown to be increased in nucleus accumbens of 5-HT_{1B} KO mice (Shippenberg et al., 2000). These changes are likely to be related to the increased vulnerability to drugs of abuse, namely cocaine, reported in these mice (Rocha et al., 1998b; Scearce-Levie et al., 1999). Such a greater sensitivity to cocaine has thus far been difficult to explain, since behavioral studies in rat and mouse have shown that 5-HT_{1B} stimulation reinforces the effects of cocaine, while blockade of the receptor can reverse this enhancement (Lucas et al., 1997; Parsons et al., 1998). The higher DA turnover in nucleus accumbens of 5-HT_{1B} KO mice could well account for a constitutive state of sensitization, resembling that of a wild-type mouse under a chronic cocaine regimen (Rocha et al., 1998b).

In addition to the above metabolic changes, 5-HT_{1B} KO mice displayed 5-HTT decreases restricted to two brain regions not hitherto implicated in aggressive behavior (neostriatum and LD/LP of thalamus), but also increases

in 5-HTT densities associated with a 5-HT hyperinnervation in amygdala (AHiPM) and ventral hippocampus.

The amygdala supports various forms of aggressive behavior through its connections with the hypothalamus and periaqueductal gray matter (e.g., Hen, 1996). It has already been shown that in amygdala, 5-HT might enhance conditioned fear (Graeff et al., 1997) through 5-HT_{1A}, 5-$HT_{2A/2C}$ and/or $5HT_3$ receptors on both projection and interneurons (Stein et al., 2000). In basal conditions, increased release resulting from a 5-HT hyperinnervation might be compensated by increased uptake of 5-HT (Jackson and Abercrombie, 1992; see also Romero et al., 1998). However, in the absence of 5-HT_{1B}-mediated inhibitory control, 5-HT release induced by specific environmental stimuli could have a greater impact in the 5-HT-hyperinnervated amygdala of the animal.

A similar situation could prevail in the 5-HT-hyperinnervated hippocampus, although with quite different behavioral consequences. In normal rats, the administration into hippocampus of buspirone or 8-OH-DPAT, i.e. a partial and full 5-HT_{1A} agonist, respectively, has been shown to increase the time spent by wild mice in exploring the open arms of the elevated plus maze, and the number of central entries in the open field (Kostowski et al., 1989; Menard and Treit, 1998). Similarly, the 5-HT_{1B} KO mice show increased exploration in the elevated plus maze (Brunner et al., 1999) and a higher locomotor/exploratory behavior compared to WT when exposed to a novel object and/or a structurally rich environment (Malleret et

al., 1999). This could be due to excessive activation of 5-HT_{1A} receptors in the 5-HT- hyperinnervated hippocampus, especially since, and contrary to the fate of 5-HT_{1A} autoreceptors, hippocampal 5-HT_{1A} heteroreceptors do not desensitize upon chronic activation by 5-HT or 5-HT_{1A} agonists (Blier and de Montigny, 1987; Dong et al., 1997). Furthermore, both 5-HT_{1A}- (Izumi et al., 1994; Fujii et al., 1997) and 5-HT_4-mediated (Consolo et al., 1994; Fontana et al., 1997; Letty et al., 1997; Marchetti-Gauthier et al., 1997) facilitatory effects of 5-HT on acetylcholine release should be exaggerated, at least under conditions of behavioral activation in the 5-HT_{1B} KOs. This could explain some of the recent results of Malleret et al. (1999) showing that in the spatial version of the Morris water maze this mutant performs better than WT mice in both acquisition and transfer tests.

The question arises as to how the decreased efficiency of 5-HT_{1A} receptor coupling to G-protein demonstrated throughout the brain of the 5-HT_{1B} KO might also contribute to the behavioral phenotype of this mutant. It should be recalled that, since this receptor is negatively coupled to adenylate cyclase the net effect of its reduced coupling to G-protein should lead to an increased production of cyclic AMP upon activation, and this irrespective of the region examined (auto- or heteroreceptors). The consequences of such a change in terms of inhibition by 5-HT of the firing and release by 5-HT neurons and by other chemically-defined neuronal types remain have not yet been investigated. They should, however, be amenable to future research,

that might also help to understand how they result in some aspects of the abnormal behavior of the 5-HT_{1B} mouse.

V.3 CONCLUDING REMARKS

Although some pharmacological agents classically designated as 5-HT_{1A} or 5-HT_{1B} antagonists may be highly specific, their effects on behavior sometimes differ from those observed in the corresponding KOs, thus emphasizing some of the limitations of the KO technology in the study of receptor function in relation with behavior. As already mentioned, a main drawback is that the absence of a gene coding for a specific receptor during development may lead to compensatory changes in other components of the same and/or heterologous chemical systems that are normally regulated by the deleted receptor. Interestingly, we have been able to demonstrate that such changes may occur, at least in the 5-HT_{1B} KO, in addition to the changes directly caused by the absence of the receptor, and may contribute to the behavioral phenotype of the animal.

While inducible and rescue mice will be soon available for investigation, the classic KOs remain, as pointed out by Gingrich and Hen (2000), a powerful tool to model genetic human disorders rather than a tool to study the effects of acute blockade of a receptor (Zhuang et al., 1999). For example, mutant mice for 5-HT_1-like receptor subtypes have already proved relevant to human neuropsychiatric conditions, such as anxiety (5-HT_{1A} KO) and substance abuse (5-HT_{1B} KO). Moreover, by detecting neurochemical or developmental compensatory adaptations in these mice, we may soon be in a

position to begin elucidating downstream potential genes whose altered function, at both the transcriptional and posttranscriptional levels, might influence behavior. The down-regulation of 5-HTTs in 5-HT_{1A} KO mice, as well the reduced G-protein coupling to 5-HT_{1A} receptors in the absence of changes in receptor density in 5-HT_{1B} KO mice, might well provide for such an opportunity.

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