MONOAMINE OXIDASES AND AGGRESSIVE BEHAVIOUR: CLINICAL STUDIES AND ANIMAL MODELS

By

Jose Mejia

Department of Biology

McGill University, Montreal

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ABSTRACT

Monoamine oxidases (MAOs) are phylogenetically old enzymes which catalyze the deamination of monoamines. Interest in a relationship between MAO and aggressive behaviour derives from the report of a single family with a mutation which obliterates the activity of MAO A, as well as a long history of studies which substantiate a relationship between MAO activity and impulsive aggressive behaviour. The goals of this thesis were: (1) to examine the generalizability of the specific MAO mutation noted above; (2) to evaluate the relationship between platelet MAO activity and genetic polymorphisms in MAO genes, and (3) to extend knowledge regarding the developmental behavioural impact of MAO deficiency in mice treated pre- and perinatally with inhibitors of MAO.

In the first study we genotyped the C936T mutation in 100 subjects followed longitudinally and oversampled for aggressive behaviour. None of the subjects in our sample carried this mutation.

In the second study, we report the lack of association between platelet MAO activity and four intronic microsatellite polymorphisms of the MAO genes.

Studies of MAO knockout mice are at significant variance with clinical pharmacological experience using MAO inhibitors.Prompted by this and by other seminal basic experiments, we hypothesized that inhibition of MAO activity during the developmental period would have profound behavioural effects. MAO A and B inhibitors were administered, separately or in combination, to mice during gestation and lactation. Total prenatal MAO inhibition produced a severe pattern of behaviour, while MAO-B inhibited mice demonstrated a similar pattern with lower intensity. Aggression was elevated in MAO-A inhibited mice only after acute pharmacological challenges suggesting prenatal sensitization. Thus developmental inhibition of MAO activity engenders
behavioural effects which parallel those observed in animals devoid functional MAO. These data underscore the importance of neurochemical changes during development and provide a possible model for uninhibited aggression, common in clinical populations.
RÉSUMÉ

Les monoamines-oxydases (MAOs) sont des enzymes phylogénétiquement vieilles qui catalysent la désamination des monoamines. L'intérêt face à une éventuelle relation entre la MAO et le comportement agressif a été suscité par un rapport sur une famille unique présentant une mutation éliminant l'activité de la MAO A, de même que par une longue série d'études fournissant des preuves à l'appui d'une relation entre l'activité des MAO et un comportement impulsif et agressif.

La présente recherche avait trois buts: (1) évaluer la généralisabilité de la mutation spécifique éliminant l'activité de la MAO déjà mentionnée; (2) examiner la relation entre les niveaux plaquettaires de l'activité des MAO et les marqueurs génétiques des gènes MAO et (3) accroître nos connaissances sur l'impact comportemental d'une déficience en MAO, pendant la croissance, chez des souris traitées en période prénatale ou périnatale par des inhibiteurs de la MAO.

Dans la première étude nous avons cherché la mutation C936T dans cent adolescents suivie longitudinalement et sélectionnés pour ses comportements agressifs. Aucun des sujets de notre échantillon n'était porteur de la mutation.

Dans la seconde étude, nous rapportons l'absence d'association entre l'activité enzymatique de la MAO et quatre polymorphismes microsatellites introniques des gènes MAO.

Les études comportementales des souris transgéniques s'écartent considérablement de l'expérience clinique acquise sur les inhibiteurs pharmacologiques de la MAO. Ce constat ainsi que d'autres études fondamentales fécondes nous ont incités à poser l'hypothèse que l'inhibition de l'activité de la MAO durant la période de développement
aurait des effets profondes sur le comportement. Nous avons administré séparément ou en combinaison, des inhibiteurs de la MAO A et de la MAO B aux souris durant la gestation et la lactation. L'inhibition totale de la MAO A a généré un modèle comportemental grave alors que l'inhibition de la MAO B provoquait chez les souris un modèle semblable mais d'intensité moindre. Les souris chez qui on avait inhibé la MAO A avaient un taux d'agression élevé mais seulement après les épreuves pharmacologiques aiguës, ce qui laisse supposer une sensibilisation prénatale. L'inhibition en cours de développement de l'activité de la MAO provoque donc des effets comportementaux équivalents à ceux observés chez des animaux dont la fonction de la MAO a été génétiquement éliminée. Ces données mettent en évidence l'importance des changements neurochimiques durant la phase de développement et fournissent un modèle possible de l'agression par désinhibition, courante dans les populations cliniques.
CHAPTER I

MONOAMINE OXIDASES
Historical antecedents

The deamination of amines through oxidative processes was first demonstrated in 1877 by Schmiedeberg in a group of animal and human tissue extracts (Berry et al., 1994). In 1924, Mary Hare extracted and identified an enzyme with amine deaminating activity and called it tyramine oxidase (Hare, 1928; Weyler et al., 1990; Berry et al., 1994). In 1937, two other amine oxidases were described and named aliphatic amine oxidase and adrenaline oxidase (Pugh & Quastel, 1937). Shortly after, Blashko et al. demonstrated that the three proteins were nothing but the same enzyme, which they called amine oxidase (Blashko et al., 1937; Berry et al., 1994).

The debate about one vs. several different forms of the enzyme continued almost three decades later when Hardegg and Heilbron discovered that monoamine oxidase (MAO) inhibitors such as iproniazid have different effects over substrates like serotonin and tyramine (Hardegg & Heilbron, 1961). In 1968, Johnston (Johnston, 1968) noticed that such selectivity is dose dependent. In Johnston's experiments, high concentrations of the enzyme inhibitor clorgyline not only stopped serotonin oxidation when administered to a tissue preparation, but also blocked the metabolism of benzylamine and phenylethylamine; meanwhile, low doses of the drug are capable of inhibiting serotonin catabolism exclusively. Four years later, Knoll and Magyar (1972) substantiated the former finding by demonstrating that small amounts of deprenyl, also a MAO inhibitor, blocked selectively the metabolism of benzylamine and phenylethylamine, but high doses of the drug administered to the same preparation were capable of stopping serotonin metabolism as well. Consequently, the selective affinity for substrate here described reinforced the concept that two forms of the enzyme do exist. The clorgyline sensitive enzyme
was named MAO A, while the deprenyl sensitive enzyme was called MAO B (reviewed in Singer, 1995).

Over the past twenty years alternative explanations to the concept of two different MAOs have been proposed. One suggested that MAO was a single protein with two different active sites, as could be inferred from the observation that MAO B naturally exists as a dimer in some tissues. This argument was put to rest in 1989, when Weyler (Weyler, 1989) demonstrated that both forms of the enzyme exist as dimers. Each has two identical subunits with identical catalytic sites. The second theory derived from assays in which a change in substrate sensitivity was detected after the elimination of membrane phospholipids from the experimental preparations. This led to the belief that MAO's catabolic activity changes according to the lipid contents in which the enzyme is working. This hypothesis was negated by purification of MAO B from bovine kidney and MAO A from human placenta, showing that it is possible to completely eliminate phospholipids from the reaction's environment without loss of activity or affinity for specific substrates (Singer, 1995).

Starting in 1973, immunological studies by McCauley and Racker added evidence in favour of the existence of two distinct forms of the enzyme (McCauley & Racker, 1973). Polyclonal anti-MAO antibodies applied to bovine brain labeled MAO B in cultured cells, but failed to label preparations containing only MAO A. Furthermore, the production of specific monoclonal antibodies against MAO B from human platelets and anti-MAO A from human placenta showed that these two forms of the enzyme are also immunologically distinct (Weyler et al., 1990; Berry et al., 1994; Singer, 1995).

Notwithstanding the accumulated evidence, the structural and genetic characterization of both isozymes has been the most important evidence in favour of the existence of two forms of MAO. In
1979, Breakefield demonstrated that the purified forms of the putative MAO A and MAO B enzymes must have been originated from two different peptides (reviewed in Singer, 1995). Finally, in 1988, Bach et al. isolated cDNA clones for each form of the enzyme. Amino acid sequencing demonstrated that only 70% of such sequences are identical between both iso-enzymes (Bach et al., 1988; Weyler et al., 1990; Berry et al., 1994; Singer, 1995). Both genes, one for each form of the enzyme, lie together in the X chromosome in a head to head disposition (Chen et al., 1992). Interestingly, differential gene expression could be the product of independent mechanisms of regulation, suggesting that each enzyme has specific roles in different tissues at different moments in the life of those that possess the enzyme (Weyler et al., 1990; Berry et al., 1994; Singer, 1995).

**Distribution**

**Species distribution.** Monoamine oxidases are found in all mammal and several non-mammal species (i.e. fish, birds, reptiles, amphibians, mollusca, echinoderm, arthropoda and parasitic worms). In almost all species MAO enzymes resemble the affinity for substrates and inhibitors of human A and B forms. Some species of fish, though, show a mix of enzymatic affinities. Moreover, starfish has a form of MAO that is completely dissimilar to the mammalian A or B. It has been suggested that such differences are obvious only between aquatic and terrestrial beings. In fact, MAO B is required only in terrestrial species in which it is so important that it takes on the main role of catabolism in some tissues such as human brain. The meaning of this distinction is not well understood, but it suggests that MAO B is a phylogenetic development that appeared only after the evolution of amphibians (Berry et al., 1994)
Over the course of the lifespan, several species experience variation in the levels of MAO activity, perhaps as a consequence of adaptations following environmental demands and/or regulatory mechanisms of expression. Also interesting is the fact that eggs and embryos have a decline in enzymatic activity that precedes important episodes in neural development. Later, the level of catabolism returns and may even surpass previous levels of function. This not only demonstrates the existence of an active mechanism of regulation during embryological development of the central nervous system, but also presents the possibility of MAO activity being involved in regulating levels of monoamines during critical central nervous system developmental periods (Weyler et al., 1990).

**Tissue distribution.** In mammals, MAO is present in all tissues except erythrocytes. Both isozymes co-exist in the same tissues and in the same cells. It is not known, though, if both forms could be expressed in the same mitochondria. A few tissues express mainly one form of the enzyme. For instance, pig’s liver, human platelets and lymphocytes, and bovine kidney and liver, all express mainly MAO B. Meanwhile, bovine thyroid, human fibroblasts, intestines and placenta, all express almost exclusively MAO A.

Activity amongst tissues in the same individual, also shows high variability. The highest levels of activity in humans are those found in lungs, liver, skeletal muscle and aorta, while the lowest levels of activity occur in spleen, skin and cerebral microvessels. Although these differences are broadly accepted, caution should be exercised when interpreting them due to discrepancies demonstrated in results obtained with different techniques used to measure MAO activity. Other non-mammalian species tend to have similar distributions of enzymatic activity amongst tissues. Nonetheless, slight differences in
the activity for the same tissue are to be expected from one species to another (Weyler et al., 1990; Berry et al., 1994).

Enzymatic activity is also influenced by the changes inherent to each tissue. For example, human fallopian tubes show a peak of activity during the secretory phase, with levels being lower during the other phases of the menstrual cycle (Weyler et al., 1990; Berry et al., 1994).

**Nervous system distribution.** MAO distribution is not homogeneous in the human central nervous system. Taking into account differences related to the techniques employed to measure MAO quantities, it is accepted that the total amount of MAO B is 2 to 8 times greater than that of MAO A. Different regions in the brain also show differential distributions. Roughly, in the brain stem and limbic areas the proportion of MAO A to B approaches 1:1, while in basal ganglia, cortex and other structures the amount of MAO B is much higher compared to the A form, going up to 4:1 in some areas. Something particular to the cortical regions is that there are interlaminar differences as well, with lamina I having the highest concentration of MAO, and laminae IV and V the lowest concentration (Berry et al., 1994; Saura et al., 1996). Less than 10% of total MAO activity occurs in neurons, while the rest is mainly concentrated in glial cells (Gorkin, 1983).

Central nervous system distribution in other species has shown to be inconsistent as well. According to some reports in the literature reviewed by Berry et al. (1994), MAO A accounts for up to 85% of total MAOs in rat brain. However, in a recent study by Inoue et al. (1999), various non-selective substrates were used in combination with deprenyl or clorgyline to measure the activity of both forms of MAO in
liver and brain of several species (cow, rat, rabbit, dog, cynomologus monkey, baboon, mice C57BL/6 and ICR, and human). The results showed that all of them have a higher proportion of MAO B activity compared to that of MAO A. The amount of MAO B activity could reach up to 98% of the total MAO activity as was the case in monkeys, but it was never less than 75% in rat’s brain. Comparatively, MAO A activity varies from being undetectable in primates to a 25% of total MAO activity in the rat, mice and man. A third way of measuring MAO activity using antibodies or titration techniques, shows equal concentrations of both enzymes in similar tissue preparations (O’Carrol et al., 1989).

**Cellular distribution.** Distribution of neurotransmitters seems to influence MAO distribution as well. Serotoninergic cell bodies contain mainly MAO B, while catecholaminergic cell bodies contain predominantly MAO A and almost undetectable MAO B activity. Noradrenergic cell bodies, on the other hand, have both (Berry et al., 1994). All neurons that have high levels of tyroxine hydroxylase activity also have high levels of MAO A activity (Weyler et al., 1990). Saura et al. (1996) reported that MAO A is mainly located in noradrenergic and adrenergic neurons, and probably some glial cells, while MAO B is located in serotoninergic neurons of the median, paramedian and dorsal raphé nuclei, and histaminergic neurons of the lateral and tuberomammillary hypothalamic nuclei. An anatomical analysis using in situ hybridization of radiolabeled oligonucleotides demonstrated location of the mRNAs of MAOs in noradrenergic neurons in the case of MAO A, with moderate transcript expression and protein in serotoninergic neurons and low positive reaction for Dopaminergic neurons. Non-aminergic neurons gave a very strong hybridization signal. MAO B transcripts were very abundant in
serotonergic and histaminergic neurons, Bergmann glial cells and ependyma, while they were weakly manifested in non-aminergic neurons (Luque et al., 1995). According to histochemical techniques, astrocytes located in regions with high monoaminergic innervation express mainly MAO B activity; however, immunochemical techniques have demonstrated equal concentrations of both isozymes (Berry et al., 1994). The former observation raises the possibility of a different metabolic role for MAO B activity.

**Biochemical characteristics**

**Chemical and kinetic mechanisms.** The catabolic action of MAO is a two step oxidative deamination. The first step is the oxidation of substrates and reduction of flavin adenin dinucleotide into hydroxiquinone, as happens in the case of primary aromatic amines, primary aliphatic, secondary and tertiary amines. During the second step the oxidized intermediate product, an imine or hydroxylamine, is spontaneously hydrolyzed by water to its catabolites after being released from the surface of the enzyme, while molecular oxygen reacts with reduced flavin adenin dinucleotide, re-oxidizing it with the release of \( \text{H}_2\text{O}_2 \) (Williams & Walker, 1984; Weyler et al., 1990; Berry et al., 1994).

Historically, the kinetic mechanism of this reaction was thought to follow either a ping-pong pathway, as is the case for \( 2\)-phenylethylamine, tryptamine and tyramine (Husain et al., 1982; Weyler et al., 1990), or the formation of ternary intermediates, as occurs during the metabolism of benzylamine (Pearce & Roth, 1985; Weyler et al., 1990). Contrary to this idea, Ramsay (1987) demonstrated that, depending on the substrate, the reduced flavin adenin dinucleotide-product complex is not only reoxidized as
originally thought; it could instead be combined with substrate and
O₂, resulting in a complex mechanism that coexists with the regular
oxidative mechanisms of the same complex. Furthermore, the
formation of ternary complexes with substrate or product seems to
play a crucial role in regulating the rate at which the oxidative portion
of the reaction occurs (Weyler et al., 1990; Singer, 1995).

**Structure and MAO isozymes.** As detailed in the first section of
this chapter, Hardegg & Heilbron suspected the presence of two
isoforms of the enzyme in 1961. Yet, it was not until 1968 that
Johnston characterized the form A of the enzyme as being sensitive to
inhibition by low doses of clorgyline, and the B form as being sensitive
to low doses of deprenyl (Johnston, 1968). In 1973, McCauley and
Rocker demonstrated that the two forms of the enzyme were also
antigenically distinct (Singer, 1995), which was confirmed by the
subsequent isolation of monoclonal antibodies anti-MAO A and anti-
MAO B that do not cross-react (Berry et al, 1994). The dual existence
of MAO became better understood in 1979 when Brakefield presented
the results of proteolytic analysis in which dissimilar peptides were
the outcome of the digestion of the two isozymes (referred by Cawthon
et al., 1981 and Singer, 1995). Simultaneously, liver MAO B and
placental MAO A were available in highly purified preparations
without prejudice to their enzymatic activities (Zeller et al.,
1979; Salach & Detmer, 1979; Singer, 1995). The isolation of cDNA
clones, the deduced amino acid sequences, and the discovery of only
70% homology between the two forms of the enzyme were the final
blocks in building up the argument in favour of the existence of two
isozymes (Bach et al., 1988).

MAO is synthesized in the cytosol by free ribosomes. It may be
transported by mitochondria to the places of activity such as axons in
neurons, given that both enzymes exist in the outer membrane of these organelles. Both isozymes are relatively resistant to proteolysis (Weyler et al., 1990; Berry et al., 1994).

MAO A protein spans 527 amino acids with a molecular mass of 59.7 kDa. It has consistently shown to be 3-5 kilo Daltons (kDa) bigger than MAO B. Transcription results in two mRNAs, one 4.4-5.4 kilo bases (kb) and a second one of 2.1 kb. Comparatively, MAO B is 520 amino acids long with a molecular mass of 58.8 kDa. Transcription renders only one mRNA of 2.8-3.1 kb as seen in human and bovine tissues (Hsu et al., 1989; Berry et al., 1994).

Protein sequences have demonstrated up to 70% homology within species with almost identical hydropatic plots (Bach et al., 1988). Protein homology is up to 90% identical amongst several species studied in certain conserved areas such as:

• An adenosine diphosphate binding site near the N terminal (residues 15-45 of MAO A), which is also probably needed for flavin adenin dinucleotide binding in the active site of the enzyme;

• a region close to the C-terminal (residues 389 for MAO B and 406 MAO A) that contains a covalent cystein attachment site for flavin adenin dinucleotide;

• a region (residues 187-230 MAO A) that has an unknown function and

• a very hydrophobic sequence (residues 504-521 MAO A) that sits near the C terminal surrounded by positively charged amino acids. Due to its similarity to a guiding sequence near the N terminal of the yeast protein, it has been suggested that it directs MAO to its cellular location.

MAO molecules exist in the external membrane of mitochondria anchored by the last sequence. The small proportion of hydrophobic
regions in their protein structure suggests that MAO enzymes are not integral membrane proteins (Hsu et al., 1989; Weyler et al., 1990).

Studying bovine and human MAO, it was originally proposed that MAO molecules were constituted by two protein sub-units that shared a single flavin-adenin dinucleotide or flavin adenin dinucleotide (Minamiura & Yasunobu, 1978; Weyler et al., 1990). However, Weyler (1989) proved that the ratio of flavin adenin dinucleotide/MAO A was 1:63 kDa of protein. Even when it was still unclear if MAO exists in vivo as a monomer or as a dimer, Weyler's finding established that the minimum catalytic unit must include at least one molecule of MAO (55-60 kDa in size) per each flavin adenin dinucleotide co-factor (Weyler, 1989; Weyler et al., 1990; Berry et al., 1994). The binding of flavin adenin dinucleotide cofactor takes place between the cystein residue of the sequence Ser-Gly-Gly-Cys-Tyr in the protein, and the 8αCH₂ element of flavin adenin dinucleotide (Weyler et al., 1990; Berry et al., 1994).

**Substrate and inhibitor specificity.** MAO catabolic activities are mainly directed to serotonin, adrenaline, dopamine, norepinephrine, 2-phenylethylamine and tyramine. Other amines (aliphatic, aromatic, primary, secondary and tertiary) could also serve as substrates. The characterization of the two forms of MAO has been always related to their specificity with regard to substrates and inhibitors. However, it is also known that such specificity is directly related to concentration, affinity and turnover rate of the substrate or inhibitor (Weyler et al., 1990). Specificity also tends to differ from in vitro preparations to in vivo systems (Berry et al., 1994). For example MAO A preferentially degrades 65 to 85% of dopamine in in vitro preparations of rat's brain. If recapture is allowed in the preparation, this proportion could be as high as 100% (Oreland et al., 1983). Comparatively, MAO B takes part
in dopamine degradation only if recapture mechanisms are blocked (Liccione & Azzaro, 1988). Similarly, inhibitors may show non-selective effects depending on their concentrations as is the case for deprenyl and clorglyline (Singer, 1995).

Despite these considerations, it is well accepted that MAO A has a higher affinity for serotonin as substrate and is selectively inhibited by clorglyline, moclobemide, harmaline, brofaromine and other experimental compounds. MAO B, on the other hand, is related to the metabolism of substrates such as 2[phenylethylamine], benzylamine and aliphatic amines, while it is mainly inhibited by deprenyl, parglyline and other experimental compounds. Substrates such as norepinephrine, dopamine, tyramine and kynuramine are catabolized in similar proportion by both enzymes. Inhibitors such as tranylcypromine, isocarboxazid, phenelzine, iproniazid and nialamide exercise their effects upon MAO in a non-selective fashion (Berry et al., 1994).

Several experiments have rendered different results in determining which parts of the MAO structures play an important role in catalytic activity and in selectivity to specific substrates and inhibitors. Chen and Shih (1998) reported that substitution of Cys 374 and 406 from MAO A by site directed mutagenesis, and Cys 156, 365 and 397 from MAO B resulted in complete loss of catalytic activity. The same results were seen affecting only MAO B when its C terminal sequence was exchanged with that of MAO A. Interchange of the N terminal sequences had no effect on either of the two enzymes. Finally, exchange of amino acids 161-375 from MAO A with amino acids 152-366 from MAO B converted the selective catalytic activity of MAO A into that of MAO B. The reciprocal experiment putting a piece of MAO A into B rendered an inactive molecule. These experiments obviously suggest that, beyond their environment, MAO selectivity depends on
specific regions of the two molecules. More recently Geha et al. (2000, 2001) reported that an exchange of MAO A Phe 208 for MAO B Ile 199 and *vice versa* did not change the substrate or inhibitor specificities of the enzymes. However, the same experiment using site directed mutagenesis caused mutant MAO A I335Y to behave like MAO B, while mutant MAO B Y326I behaved in a reciprocal fashion.

**Activity and its regulation**

The most widely used method for the quantification of MAO activity is an assay using radiolabeled substrates for platelet activity in whole blood samples. Fluorometric assay for brain samples, rapid bioluminiscence, monoclonal antibody radioimmunoassay and high pressure liquid chromatography, amongst others have also been used to measure MAO activity. Activity levels of MAO tend to vary not only amongst species but also intra-species (Weyler et al., 1990).

The existence of two different genes encoding MAO may explain the functional differences that are apparent even in primitive organisms, such as the toad, suggesting the early duplication in evolution of a primitive MAO gene. These two genes have been shown to have different mechanisms of regulation during transcription. Variations in the 5' and 3' ends of the MAO genes translate into different sites of transcription initiation, splicing and polyadenylation sites (Weyler et al., 1990). Breakefield and Edelstein on their part (1980) warned about the influence of other genes upon different mechanisms involved in MAO synthesis and catalytic activity. They also suggested considering a multigenetic model in understanding the complex pathways involved in the determination of activity levels in the different species and individuals. Despite these points to be considered, activity of MAO is strongly influenced by MAO genes, as
revealed by twin studies that have found heritability values as high as 0.77 (Pedersen et al., 1993).

Kinetically speaking, neurotransmitters could "induce" their own catabolism if their levels increase in the proximity of MAO molecules due to enzymatic up-regulation. Because oxygen is a required component for the reaction, the reduced substrate-enzyme complex shows up to 200 fold higher affinity for oxygen in comparison to the reduced enzyme on its own. This allows alternative pathways to occur depending on the quantity of substrate present in the cell environment. This situation varies with the affinity for the different substrates, which in turn influences the rates of product dissociation, new substrate binding and availability of the enzyme to be oxidized (Ramsay, 1998).

Activity regulation is also related to the environmental conditions that surround the enzyme. The lipid environment, for example, may exercise an influence on MAO's activity as demonstrated with in vitro experiments. In vivo, dietary lipid restriction diminished MAO B and A activity in rat liver by 50% and 70%, respectively. The normal level of activity was restored with the administration of fat supplemented diets. Other experiments have shown that the liver and heart suffered between 20 and 30% reduction in MAO activity when a low fat diet was fed to the experimental animal. Riboflavin and iron deficiency tend to produce the same effects. Surprisingly, the brain and lungs do not experience the same level of disruption in either of these two circumstances (Weyler, 1990).

Aging increases MAO B activity up to 50% between the ages of 50 to 60 years (Saura et al., 1997). This increase affects brain, platelet and plasma activity measurements (Robinson et al., 1972). MAO A, on the other hand, does not seem to experience the same increase and may actually decrease in relationship to earlier levels (Saura et al.,
The reason for these changes is not yet understood, as the amount of biogenic amines decreases simultaneously. They probably occur in response to changes in mitochondrial morphology or lipid environment or maybe it is the effect of transcriptional factors that are active during the aging processes (Weyler et al., 1990).

As detailed later, early in development MAO A appears in most tissues and reaches near-adult levels of activity at birth. Its activity increases 1.5-2 fold through life. In rat central nervous system, the increase over age follows a caudal-rostral pattern in parallel with neuronal differentiation. Comparatively, in most tissues MAO B activity appears after birth and increases sharply during the neonatal period. In the brain, this increase is more evident than in other tissues and it follows a dramatic neonatal astrocyte proliferation (Weyler et al., 1990).

Hormones play an important role in MAO activity. Hypophysectomy in experimental animals is followed by variation of activity that depends on the tissue in question. Ovariectomized females show no changes in activity until they are given testosterone, hydrocortisone or progesterone, which increase MAO activity in the uterus. In human fallopian tubes, levels of MAO activity change with the menstrual phases, reaching a peak during the secretory phase, probably to protect the fertilized egg from biogenic amines (Weyler et al., 1990). Chakravorty and Halbreich (1997) reviewed the literature and found a consistent report of MAO inhibition during the estrogenic phases of the menstrual cycle (Baron et al., 1980). Castration produces an increase in MAO A activity and a decrease in B activity in liver. The inverse is observed after the administration of estradiol. Progesterone and adrenalectomy augment activity for both isozymes. Dexamethasone also produces an important stimulatory effect on
MAO A activity (up to 14 times), but only a discrete one (up to 3 times) on MAO B (Weyler et al., 1990).

The objective of this brief introduction is to highlight the roles of MAO isoforms in evolution and their physiological involvement in a great variety of metabolic activities in different species, and in different tissues in the human body. Taking this into account, more important for the purposes of the present thesis is the fact that both forms of MAO have pivotal roles in brain metabolism. Equally important is to realize how versatile their actions are not only in the different organisms, and in the different tissues as said before, but also through ontogenetic evolution. In the following chapters, we will analyze in depth how MAO can modify behavioural traits through its catabolic actions during critical periods of brain development.
**HYPOTHESIS**

Monoamine oxidases modulate the development of aggressive-impulsive behaviour, through the metabolic disregulation that results from the absence of MAO activity secondary to genetic distortions or pharmacological induction.

**OBJECTIVES**

To investigate the possible association between genetic predisposition, aggressive behaviour, and MAO.

- To investigate the existence of associations between mutations and aggressive behaviour.

- To investigate the existence of associations between genetic polymorphic markers, behavioural traits and enzymatic activity

- To analyze the effects of pharmacologically induced MAO inhibition in pregnant mice on behaviour and monoamine metabolism.
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CHAPTER II

GENETICS OF MONOAMINE OXIDASES
Gene localization

Early studies aimed at localizing the MAO genes were carried out by hybridizing human chromosomes in a rodent background. Pintar et al. (1981a) used human fibroblast chromosomes in mouse neuroblastoma cells. As these cells lack MAO activity, identification of the hybridized cells was achieved by mapping the partially digested proteins labeled with $^3$H-pargyline (an irreversible MAO inhibitor). Karyotypic analysis directed the search towards the X chromosome. Later studies with monoclonal antibodies against MAO B also pointed to the X chromosome as the potential locus for this gene (Kochersperger et al., 1986; Shih, 1991). Pintar et al. (1981b) also produced hybrids of rat chromosomes on a MAO-deficient murine background; the expression of both forms of MAO was only achieved when the rat's X chromosome was integrated to the mouse genome, which confirmed the former findings (Hsu et al., 1989; Weyler et al., 1990).

An analysis of genomic DNA of subjects having Xp deletions, variations in the number of X chromosomes, and hybrids that retained different portions of the X chromosome, allowed the assignment of a locus for MAO A to the short arm of the X chromosome, in position p21-11 (Ozelius et al., 1988; Shih, 1991). Inside that region, the MAO A gene was localized to p11.23-p11.4 by in situ hybridization (Lan et al., 1988; Levy et al., 1989; Shih, 1991). Linkage analysis in large pedigrees with the use of restriction fragment length polymorphisms has placed MAO A approximately 10 centimorgans (cM) away from OTC and 14 cM from OATL1 markers (Ozelius et al., 1988; Harris et al., 1993). MAO B has been located close to the assigned MAO A position (Lan et al., 1988; Levy et al., 1989; Hsu et al., 1989; Shih et al., 1999a; Shih and Thompson, 1999).
Norrie disease is a disorder caused by a microdeletion in the X chromosome involving the Norrie disease gene. The most extensive deletions also include both MAO genes, resulting in the absolute lack of MAO enzymatic activity, which suggests that the three genes are located in close proximity to each other (Hsu et al., 1989; Shih, 1991; Berger et al., 1992; Collins et al., 1992; Chen et al., 1995). The distance between the MAO genes has been calculated by linkage analysis to be 2.7 cM in an order telomere - MAO A - MAO B - Norrie disease gene - centromere (Harris et al., 1993). Physical maps have revealed that in a telomere > centromere direction MAO genes are 140 base pairs (bp) apart from DXS7; the distance sequence continues with MAO A (95 kb) - 50 kb - MAO B (95 kb) - 50 kb - Norrie disease gene (Chen et al., 1995; Shih et al., 1999a).

Structure

In 1988, Bach et al. succeeded in producing clones containing the cDNA for each one of the MAO enzymes; this work unequivocally demonstrated that MAO A and MAO B are encoded by distinct genes. However, in their experiments Bach et al. also demonstrated that the two proteins have up to 70% similarity in their amino acid sequences. As expected, such similarities are present in the exonic regions of both genes, while the non-coding 5' and 3' regions in both genes show little resemblance. This may also suggest the presence of differential regulatory mechanisms of expression for these enzymes (Bach et al., 1988).

Both genes are constituted by 15 exons and 14 introns. In the MAO A gene the first exon is separated from exon two by a stretch of 30 kb, while the last five exons are clustered together. The MAO B gene shows a similar exonic distribution. Twenty out of the 28 exonic
5' or 3' border sequences are identical between the two genes. MAO A has exons that range from 54 to 440 bp, while MAO B has exons that range from 54 to 1000 bp. Intron sizes are poorly conserved between the two genes. All intron donor and acceptor sites are consistent with classical GT/AG splice junction rules. The number of amino acids encoded by each exon is identical between genes except for exons 1 and 15. Compared with exon 1 of MAO B, exon 1 of MAO A encodes 9 additional amino acids. Exon 15 of MAO A encodes two amino acids less than its counterpart in the MAO B gene. There are 858 nucleotides in exon 15 that are not translated as they are located after the TAA termination codon (Bach et al., 1988; Grimsby et al., 1991).

In both genes, amino acids distributed between exons 1 and 2 form an adenin monophosphate binding site that may be important for catalytic activity as it is involved in binding flavin adenin dinucleotide non-covalently. In addition, flavin adenin dinucleotide binds in a covalent fashion to the pentapeptide Ser-Gly-GlyCys-Tyr encoded by exon 12 in both genes (Grimsby et al., 1991). Furthermore, the percentage of similitude between MAO A and MAO B amino acid sequences is as follows: exon 1 = 60%; 2 = 72%; 3 = 74%; 4 = 77%; 5 = 70%; 6 = 75%; 7 = 74%; 8 = 68%; 9 = 66%; 10 = 67%; 11 = 80%; 12 = 94%; 13 = 89%; 14 = 48%; and 15 = 60%. The highest agreement is found in sites related to the flavin adenin dinucleotide binding area (exon 12). Exons 14 and 15 show the lowest levels of correspondence, contributing perhaps to different affinities for particular substrates observed between the two forms of the enzyme. Individually, the structure that each exon maintains shows a high degree of conservation across different species. Other examples of duplicated proteins tightly linked over evolution and with identical function have proved that one of the two proteins tends to appear in different tissues and/or at different developmental phases in various
organisms, as if they had specialized in a developmental role or location over phylogeny, after being duplicated (Chen et al., 1991 and 1992a; Shih et al., 1999a; Shih and Thompson, 1999). This also may be the case for MAOs.

The chromosomal region in which both genes reside (Xp21-11) spans approximately 240 kb. As stated above, inside that region, MAO A occupies the position closest to the telomere, while MAO B is proximal to the centromere. Both 3' ends lay adjacent to each other separated by 50 kb, while the 5' ends lay in the opposite extremes. The approximate size of each gene is 95 kb. Regions rich in GC content are located in areas that include exon 1 in both genes, which is a common feature of "housekeeping" genes (Chen et al., 1992a).

The organization of the MAO genes in this region (telomere - MAO A - MAO B - centromere) is very similar to that observed in mice, except that the telomeric extreme in humans corresponds to the centromeric end in mice (Chen et al., 1995). Also interesting is that teleosts and some amphibia possess only a single form of MAO (most similar to MAO A), while toads have both forms of the enzyme (Chen et al., 1992a). This may be the result of an endoreduplication event that occurred just after formation and unequal crossing over. It is thought that this happened some 500 million years ago during amphibian evolution (Chen et al., 1991). Such genetic arrangement forces transcription to occur in different strands, eliminating the possibility of different forms of the enzyme resulting from different splicing events. The fact that diverse levels of activity and amounts of enzyme are present in the different tissues at different periods in life further strengthens the idea that the mechanisms involved in expression of MAO genes have to be independent from one another (Chen et al., 1992a; Chen et al., 1995).
Genetic regulation of expression

MAO activity varies from tissue to tissue in a single organism. This also applies to the presence of each of the two different forms of the enzyme in a given tissue. As enunciated before, these facts intrinsically indicate that the regulation of gene expression is independent between genes and tissues. One of the most important structures in a gene related to such differences is the promoter. The isolation of the 5' promoter-containing regions of both genes (Grimsby et al., 1991; Chen et al., 1991) has shown that 5' regions in MAO A and B genes are GC rich and are surprisingly homologous (60% sequence identity) (Shih et al., 1995). Maximal transcription activation was demonstrated in segments 140 bp long for MAO A and 150 bp long for MAO B (Shih et al., 1999a).

MAO A promoter activity is found in a *PvuII/DraII*, 240 bp long fragment, 62 bp in a 5' direction from the ATG initiation codon. The region upstream from the promoter (819 bp long) has a down-regulating effect (Shih et al., 1995) and it is constituted by 7 repeats including those two described as part of the core promoter (I and II) (fig 1). An eight repeat downstream from repeat I (called -I), is also part of this extended promoter structure. Repeat I spans from nucleotide -89 to -178 while repeat II goes from -179 to -268. Each 90 bp repeat is constituted by a 42 bp fragment, and a 48 bp fragment with an Sp1 binding site. Homology between repeats I and II is 83% while that of the four smallest fragments is just 74% (Zhu and Shih, 1997).

Repeats III and IV have an third 42 bp fragment attached to their sequences, while repeat V has only half of that extra sequence. Hence, repeats I, II, VI, VII and -I are 90 bp long. Repeat III is 132, repeat IV is 131, and repeat V is 108 bp long. Homology between all the basic 90 bp repeats equals 50% excluding repeats I and VII (Zhu and Shih,
Upstream, extending from repeat II to base pair -1400, there are some structures that could have promoter-like activity such as a TAATAA sequence, two CCAAT boxes, four CACCC elements, a TGACCTCA adenin monophosphate response element, four direct identical repeats 30 bp long, and 3 potential Sp1 binding sites. Despite such great potential for promoter activity, in vitro experiments have shown that only repeats I and II play a substantial role in promoter activity (Zhu et al., 1992 and 1994).

Initially, Zhu et al. (1992) proposed that MAO A transcription was initiated at several points inside the -250 to -130 region that includes repeats I and II and four Sp1 protein binding sites. To elucidate if other transcription factors interact with Sp1 or different binding sites in promoting expression of the MAO A gene, a DNase I footprint experiment was implemented. The results showed that only the Sp1 sites were protected from digestion, confirming that promoter activity happened only in Sp1 binding sites bound by Sp1 proteins (Zhu et al., 1994; Shih et al., 1995). Denney et al. (1994), using RNAse protection and rapid amplification of cDNA ends (5'-RACE) assays in several different tissues, suggested that transcription starts at a GC rich point 40 bp upstream from the ATG initiation codon. This point matches a weak consensus sequence for an Inr initiator element capable of specifying accurate transcriptional initiation in a Sp1-stimulated manner. In those experiments, smaller transcripts were initiated in the -95 bp and -129 to-140 bp GC rich regions that contain the 90 bp long repeats mentioned above. The rapid amplification of cDNA ends assay also yielded a product in which transcription had started between -80 to -75 bp (Denney et al. 1994; Denney, 1995). In the case of MAO A though, the Inr sequence seems to have no effect upon the promoter and, as it sits in an upstream
region that down-regulates the promoter's activity, the net effect is one of down-regulation (Zhu and Shih, 1997).

Zhu et al. (1994) on his part suggested that the actual point of transcription initiation is located at -73 in all human tissues. In the same study it was also demonstrated that transcription occurs in a bidirectional fashion. The TAATAA structure upstream of repeats I and II when mutated, did not have any forward effect but increased significantly the reverse promoter activity, implying that it has a reverse promoter-inhibitor structure. Sp1 sites 4 and 1 down-regulate the forward activity while increasing the reverse activity. Sp1 sites 2 and 3 promote transcription in both directions, while the GC rich region 3' of repeat I (containing the -73 point) had the inverse effect, and promotes forward activity while inhibiting reverse activity. The reverse or forward promoter activities are directly proportional to the concentration of Sp1 proteins. This suggests that a different gene may be transcribed on the reverse sense strand, depending perhaps on the cellular conditions prevailing at the moment of transcription initiation (Zhu et al., 1994).

The presence of other transcription factors capable of joining the GC rich sequences in the promoter may explain to some extent the differences in MAO A activity amongst tissues (Zhu, 1994; Shih et al., 1995). Sp1 proteins are found in all cells of higher organisms and it is clear that the activity of promoter Sp1 elements is directly proportional to the concentration of Sp1 proteins. The said concentration varies up to 100-fold between tissues and between developmental periods. Keeping in mind such characteristics it is possible that Sp1 factors play a regulatory role in the expression of MAO A gene (Shih et al., 1995).

The promoter sequence of MAO A contains a repetitive sequence 30 bp long that shows polymorphic variation. The core sequence is
made out of six nucleotides \([\text{ACC(A/G/C)G(C/T)}]\) repeated six times per fragment. Alleles found to date consist of 3, 3.5, 4 and 5 repeats of the above cassette. The location of this polymorphic region is -1142 to -1262 bp upstream of the initiation start site. The relevance of this promoter region polymorphism resides in the possible connection between genotype and rate of transcription of the MAO A gene. Alleles having 3.5 and 4 repeats of the said sequence are associated with a higher rate of transcription, such that they may be characterized as transcription activators (Sabol et al., 1998).

The **MAO B promoter** is less well defined. It is located in a 150 kb \(\text{PstI/NaeI}\) fragment, 99 bp upstream from ATG codon. The 5' sequence that follows the promoter, down-regulates the expression of MAO B, as it is also the case with the MAO A promoter (Shih et al., 1995). The MAO B gene has several consensus sequences that display promoter activity in transient expression assays; however, that should not imply that transcription is naturally initiated in this way (Chen et al., 1995). Zhu et al. (1992) demonstrated that the MAO B promoter is constituted by two clusters of overlapping Sp1 sites (located - 176 and -226 respectively), separated by a CACCC element (-210). At position -146 a potential TATA box (TAATATA) was found; there is another Sp1 binding site 3' to the TATA box (-82). Three more potential Sp1 binding sites, three CACCC sequences and two CCAAT boxes, lie 5' of the mentioned structures. At position -709, there is a CACCC element in the form of GGGTG, a "adenin monophosphate-response-element" like sequence (TGATGTCA, -807), and an AP-1 binding site (TGACTCTA at position -1340). Two identical repeats 29 bp long were found from -750 to -778 and from -779 to -807. Two novel factors F and M may also have a role in transcription regulation. None of these elements, however, were superior in promoter activity to the basic Sp1 sites (Zhu et al., 1992 and 1994).
More recently Wong et al. (2001) reported that the MAO B promoter is a 2kb region flanking the 5' end of the MAO B gene. Inside this region, section -246 to -99 was sufficient to perform the basic promoter activities in several cultivated cell lines. In agreement with Zhu's findings, this promoter was found to contain two clusters of consensual Sp1 elements, a repressor CACCC structure and a functional TATA box (-146 bp). Several members of the Sp protein family, as well as other regulatory proteins, are capable of joining the promoter structures. However, only Sp1 and Sp4 can trans-activate the promoter. Counteracting this action, overexpression of Sp3 and BTEB2 can repress the Sp1 and Sp4 activation. Sp4 is mainly expressed in brain while BTEB2 is expressed in placenta and testis.

This may explain why most of the catabolic activity in brain is due to MAO B, while MAO B activity is negligible in placenta. Consequently, different regulatory mechanisms may play an important role in tissue expression of MAO B activity. Adjacent Sp1 binding sites must have at least 10 bp between them to allow simultaneous binding of two protein molecules. As the Sp1 binding elements in the MAO B promoter overlap, only one Sp1 binding site can be used during each transcriptional cycle (Wong et al., 2001).

Sp1 binding proteins are also subjected to ontogenetic regulation which causes their activity and cellular content to change throughout development, cell proliferation and apoptosis (Shih et al., 1995; Wong et al., 2001). Sp3 sites, on the other hand, act as repressors or activators depending on the promoter and the cellular environment. In consequence, regulation of MAO B genes depends on the integrity of Sp1 sites and availability of different Sp proteins that compete to bind to Sp1 sites. Finally, mutations produced in the CACCC element by site-directed mutagenesis increased the promoter activity 2 to 13 fold.
and enhanced Sp1 mediated activation; this suggests that the action of the CACCC element is one of suppression (Wong et al., 2001).

**Mutations and deletions found in man**

MAO enzymatic activity is under strong genetic regulation (Murphy et al., 1976; Rice et al., 1984; Hotamisligil and Breakefield, 1991; Lenders et al., 1998). Activity measured in platelets obtained from monozygotic twins shows strong correlation \( r = 0.76-0.88 \), in comparison to dizygotic twins \( r = 0.39-0.45 \), with a heritability of 0.8 and higher variance among families (1.06) than within families (0.36) (Weyler et al., 1990). Individual cell lines have highly consistent enzymatic activity from one subculture to the next (Rice et al., 1984; Hotamisligil and Breakefield, 1991). In a segregation analysis Rice et al. (1984) considered as acceptable models of transmission a codominant model without polygenic background or a recessive locus with polygenic background, strengthening the assumption of a major single locus as reasonable explanation for the genetic basis of MAO activity (Rice et al., 1984). However, this model did not account for the X-linkage of the structural gene, which somewhat reduces the validity of the conclusions. These results stress the influence of inherited factors on the enzymatic activity of MAO.

**Point mutations that affect enzymatic activity**

A remarkable exception to the genetic conservation observed in MAO genes is the mutation causing Brunner's Syndrome. Other exceptional genetic events are the microdeletions causing Norrie disease, described later in this chapter.

In 1993, Brunner et al. (Brunner et al., 1993a) published the discovery of a large Dutch family in which 14 males were affected by
mental retardation with violent behaviours. The study revealed that only males were affected, that they did not have any congenital abnormality or dysmorphological traits, and that the average IQ for an affected person was 85. Patients displayed stereotyped movements such as plucking, fiddling or wringing their hands. Behavioural disturbances were characterized by episodes of aggressive and/or violent responses triggered by insignificant stimuli. Those episodes appeared in clusters of one to three days coexisting with insomnia and night terrors. In one case, one male raped his sister and was committed to a forensic institution at age 23. There, he frequently incurred fights with other inmates, the reason why he was constantly transferred to other areas in the same institution. Twelve years later he stabbed a warden in the chest with a pitchfork after being told to get on with his work. Another man attempted to run over his boss with a car at the sheltered workshop where he was working, because he was told that his job was not up to par. A third affected subject forced his sisters to undress in front of him by threatening them with a knife. Two other members of the family were known arsonists whose criminal acts were associated with stressful periods. Some other disturbing behaviours seen amongst the affected males were voyeurism, exhibitionism and a tendency to grasp or inappropriately hold onto their female relatives.

Karyotypic analysis showed no abnormalities. Multipoint linkage analysis (Brunner et al., 1993a) pointed towards the region of the X chromosome (Xp11) to which MAO maps, with a maximum lod score of 3.69. Biochemically, MAO A substrates were significantly elevated, while MAO A metabolites were severely reduced, suggesting a primary MAO A deficiency. Platelet MAO B activity was not affected in any family members. Unaffected males of this family were behaviourally and biochemically normal. All females were also normal,
notwithstanding that the mothers of the affected males were considered obligate carriers.

In a subsequent study, Brunner et al. (1993b) cultured fibroblasts from three affected males, two carrier females and a non-carrier female to investigate the production and activity of MAO A and its response to dexamethasone challenge (a treatment known to increase activity up to 14 times). As fibroblast MAO is mainly A in type, it was no surprise to find that the affected males showed negligible amounts of activity and no response to the dexamethasone challenge in their fibroblast cultures. Carriers and non-carrier females had low to moderate activity, a normal pattern of excretion of metabolites and substrates, and responded adequately to the dexamethasone challenge and selective inhibition with clorgyline. DNA sequence analysis demonstrated four single base substitutions. Three of these are neutral substitutions (941 G→T; 1077 T→A; 1460 T→C). The fourth, at position 936, changed a CAG codon encoding glutamine to a TAG codon containing a termination message. The mutated gene produces a truncated protein incapable of carrying on the metabolic activities of normal MAO A. The metabolic consequence is high urinary excretion of substrates and low urinary levels of metabolites. Abeling et al. (1998) further analyzed this excretory pattern in an attempt to develop a diagnostic tool. They found the ratios for normetanephrine/vanillylmandelic acid, and normetanephrine/3-methoxy-4-hydroxyphenylethylenglycol to be the most discriminative measures of deficient metabolism between affected and non-affected populations in 48 hour urine.

One might also want to know how frequent these MAO deficiency states are and how often they are due to genetic mishaps. In the case of MAO A, Murphy, et al (1998) studied a population constituted by healthy subjects, neuropsychiatric patients treated with MAO
inhibitors, violent alcoholics and non-alcoholics, paraphilics, and attention deficit and hyperactivity disordered adults. Their approach included a metabolite-screening test (3-methoxy-4-hydroxyphenylethylenglycol plasma concentration and normetanephrine/3-methoxy-4-hydroxyphenylethylenglycol ratio), followed by a search for single strand conformational polymorphisms from those individuals with the lowest 3-methoxy-4-hydroxyphenylethylenglycol levels. Out of 224 individuals included in the study only two displayed biochemical levels suggestive of MAO enzymatic activity deficiency, and for that reason no single strand conformational polymorphisms analysis was conducted.

**Point mutations with no effect on enzymatic activity.**

In a similar study to the one by Murphy et al. (1998), Schuback et al. (1999) found no subjects with gross deficiency of MAO A in a population of more than 600 subjects including a control group and others affected by paraphilias, Parkinson's or Lesch-Nyhan syndromes, or mental retardation (all disorders in which low activity of MAO has been suggested in one or more reports). In those subjects with the lowest plasma levels of 3-methoxy-4-hydroxyphenylethylenglycol, no mutation could be found in the coding and splice junction regions of the MAO A gene by single strand conformational polymorphisms analysis. In another study, Tivol et al. (1996) looked for mutations in ten fibroblast cultures from 7 controls and 3 Lesch-Nyhan patients. Enzymatic activity of MAO A showed a wide (>100-fold) range of variation. DNA sequencing and single strand conformational polymorphisms analysis demonstrated the presence of a few polymorphic variants that did not have any
influence on the protein assembly or enzymatic activity. These variants are: a) 435 A→C (arg→arg); b) 941 T→G (arg→arg) in a Fnu4Hl site; c) 1076 A→T (pro→pro); d) 1460 C→T (asp→asp) in an EcoRV site; and e) 1609 A→G (lys→arg) (Hsu et al., 1995). Given that none of these alterations changes protein function, any potential effect upon enzymatic activity might well be related to changes in the non-coding (intronic and/or regulatory) regions of the gene. This was suggested earlier by the striking similarity observed in the amino acid sequences of the two forms of the enzyme amongst MAO in different tissues intra-species and inter-species (Chen et al., 1995). Should that be the case, the changes in activity may result from changes in transcription regulation, splicing efficiency and other steps involved in the synthesis of MAO enzymes (Chen et al., 1995; Hsu et al., 1995; Tivol et al., 1996).

MAO B has shown substantially fewer mutations than MAO A. Chen et al.(1993) compared the sequences of MAO B in brain, platelets and liver to determine the relationship between platelet MAO B measurements and MAO B activity in the brain. Compared to the liver sequence, brain genes showed a 794 C→T substitution changing a GAC codon to GAT, both encoding Asn. Position 440 has a A→T substitution, changing codon ACA into ACT, both encoding Thr. Finally, in position 825 a C→T substitution changes a CTA codon into TTA, both codons for Leu. No other changes in the nucleotide sequences were noted, rendering the protein sequences identical for the three enzymes, and confirming (within the restrictions imposed by tissue-specific regulation) that platelet MAO B activity should be highly correlated with brain MAOB activity (Chen et al., 1993; Chen et al., 1995).

**Deletions** (Norrie Disease)
Norrie disease was first described by Norrie in 1927 and later by Warburg (1966). It is an X linked recessive disorder characterized by blindness secondary to degenerative and proliferative changes in the neuroretina. Those changes involved pseudotumors, retinal-, ciliar- and iris- pigment epithelium hyperplasia, necrosis of the inner layer of retina, cataracts, eye atrophy, white cornea and obliterated anterior chamber. Neurologically there were mental disorders with psychotic features in at least half of the patients; 30% of patients also developed sensorioneural deafness. Severe cases showed delayed sexual development, growth failure, profound mental retardation, myoclonus, seizures and proneness to infections (de la Chapelle et al., 1985; Sims et al., 1989; Zhu et al., 1989; Berger et al., 1992). However, manifestations could be mild to the point of allowing some patients to suffer no deafness and achieve university education as reported in two cases whose pathological features were linked to point mutations in the Norrie disease gene instead of the classic microdeletions which also may involve MAO genes (Fuentes et al., 1993). The biochemical phenotype for each patient is strongly influenced by the extent of the deletion such that variable sized deletions give rise to a continuous syndrome in which the different deleted genes are responsible for various manifestations. Murphy et al. (1990) analyzed the enzymatic and metabolite profile of two subjects with Norrie disease described by Sims et al. (1989). Murphy's characterization was later confirmed in a family where the proband had a complete deletion of Norrie disease gene and both MAO genes, with the mother and grandmother being obligate carriers. Cerebrospinal fluid levels of serotonin and norepinephrine were much higher in these Norrie disease patients than in normal subjects, while metabolites of serotonin and norepinephrine were decreased. Dopamine and its metabolite homovanillic acid were not affected. As expected, MAO B activity was practically negligible and 3-methoxy-4-hydroxyphenylethylenglycol
was absent in plasma or greatly reduced in urine. This patient also demonstrated a severe tyramine reaction, twice as intense as that elicited in MAO inhibited subjects. No signs of delayed sexual maturation were detected. Interestingly, the heterozygous females in this family showed abnormalities similar to those noted in the proband but in a less severe form (Zhu et al., 1989; Collins et al., 1992). The patient's mother, though, had signs and symptoms of chronic hypomania, and even though no behavioural manifestations were evident in the grandmother, she had a low intelligence quotient (Collins et al., 1992).

In 1992 Berger et al. sequenced fragments of DNA from patients affected by this disease. The most relevant finding was that of a putative gene that could account for the presence of Norrie disease in the vicinity of MAO B and MAO A genes as was previously suspected (Levy et al., 1989; Berger et al., 1992). These studies also demonstrated that the Norrie disease gene is expressed in retina and choroid in the eye, and fetal brain. Berger discovered a wide range of microdeletions in patients with Norrie disease, but also noticed that the severity of the phenotype does not correlate linearly with the size of these microdeletions. The sequence analysis (Chen et al., 1992b) predicted a protein of 133 amino acids and a molecular weight of 15,043, with homology to mucins and the von Willebrand factor (Fuentes et al., 1993; Meitinger et al., 1993). The protein in question is similar in its tertiary structure to transforming growth factor β. Histological analysis suggested that Norrie disease gene protein may be involved in the growth or angiogenesis of the eye and brain. When at fault, the retinosensorial neurons and the highly vascular retrolental membrane become disorganized, giving rise to the manifestations already explained (Meitinger et al., 1993).
Transgenic MAO murine models

As demonstrated in a number of ways summarized above, the role of each form of MAO is much more complex than that of being simply complementary to each other. One way of understanding such a riddle is by analyzing the effects of naturally occurring genetic disorders that cause a total deprivation of function in affected persons. It is for that reason that Brunner's syndrome and Norrie disease were introduced before. However, they also represent models that are not completely "clean" from the standpoint of environmental influences, and neither do they allow a study of what happens during development. The development of transgenic mice with the specific genes for each enzyme experimentally deleted illustrates the effects of selective lack of one or the other form of MAO.

MAO A

In 1995 Cases et al. reported the development of transgenic mice that lack MAO A activity after an interferon $\beta$ transgene was inserted into MAO A gene. MAO RNA obtained from the MAO A transgenic mouse (Tg8) demonstrated 4 species of RNA. One species included the interferon $\beta$ in substitution of exons 2 and 3; the remaining three had deletions that eliminated exons 2+3, 2+3+5, or 2+3+4+5. Because exon 2 is vital to flavin adenin dinucleotide binding, transcription of any of these transgenic RNAs produced MAO proteins devoid of enzymatic activity. This was experimentally demonstrated in brain and liver. MAO B activity was shown to be intact in the said animals.

Behaviourally, the mice showed changes that tended to disappear along with the aging process. Newborns showed intense head nodding. At 5-10 days of age, trembling was present upon locomotion and tail suspension together with prolonged righting, moving backwards when on a new surface (as opposed to pivoting), and
strong and prolonged reactions to pinching. Between ages 11-16 they ran frantically, constantly jumped and fell over, hid by digging into the bedding in the event of moderate sounds and movements, shook and jumped while sleeping, kept a hunched posture, and when suspended by the tail adopted a bat and ball position. Aggressive behavior was manifested as a high propensity to bite the experimenter. As adults, Tg8 mice continued to be offensive-aggressive as deducted from the numerous bite wounds and scars on genitals and rumps of their cage mates. Resident-intruder tests exhibited a shorter latency to the first attack and a hunched, static, fluffed-fur posture in response to the exploratory moves of control intruder animals (Cases et al., 1995). The use of ketanserin (non-selective serotonin\textsubscript{2A} and vesicular monoamine transporter 2 antagonist), MDL 100907 (selective serotonin\textsubscript{2A} antagonist), or tetrabenazine (selective vesicular monoamine transporter 2 antagonist) was effective in suppressing aggressive behaviours when administered to Tg8 mice, which made the authors suspect that the aggressive responses in transgenic mice may be mediated by serotonin\textsubscript{2A} and vesicular monoamine transporter 2 receptors. The non-selective antagonism of ketanserin proved to be more effective in reducing aggressivity than the selective effects of either MDL 100907 or tetrabenazine (Shih and Thompson, 1999; Shih et al., 1999b).

Sexual behaviour was also abnormal in Tg8 mice, who tended to grasp non-receptive females much more frequently than their wild counterparts. This was concluded from the frequent and intense squeaking of control females in the presence of virgin Tg8 males. In Porsolt's swimming test, normal mice spent 150 (male) to 200 (females) seconds immobile (out of a 4 minute trial); Tg8 mice comparatively, spent only 40 to 85 seconds immobile (average for males and females respectively), while the remainder of the time was
spent trying to escape from the water. Open field tests showed that Tg8 mice venture significantly more time in the center of the field and hesitated quite often about the direction to take, while normal mice tended to stay in the corners of the field where they would be less exposed to predators. Control mice may display similar behaviours to those seen in Tg8 animals but at a much lower intensity. Non-specific inhibition of MAO elicited transiently those behaviours that disappeared over time from the behavioural repertoire of adult Tg8 mice (Cases et al., 1995). Transgenic mice have also shown increased fear conditioning and avoidance learning, with no changes in maternal behavior and eye-blink conditioning (Kim et al., 1997; Kim and Thompson, 1998).

Exploratory behaviour and startle reflex were reported to be decreased in MAO A transgenic mice. An alcohol preference test did not show any significant difference between MAO A knock-outs and control mice, except that Tg8 mice had prolonged latency- and shorter duration of sleep. These effects were also observed, on a smaller scale, in control mice after administration of clorgyline at 5-10 mg/kg doses; this may suggest that such changes result more from the lack of MAO A enzymatic activity than from developmental changes secondary to the genetic MAO A deficiency in Tg8 mice (Popova et al., 2000).

**Metabolically**, Tg8 mice had higher levels of brain amines serotonin, dopamine and norepinephrine and their metabolites were significantly reduced (Cases et al., 1995; Lajard et al., 1999). These abnormalities were corrected as the animals aged. During adulthood, a single dose of MAO inhibitors was enough to bring back the young Tg8's metabolic profiles (Cases et al., 1995).

**Histologically**, Tg8 mice suffered disarray of the barrel shape organization present in normal mice in granular layer IV of the somatosensory cortex. A flat layer of cells substituted the barrel-like
The formation of barrels is influenced by peripheral input via trigeminal and thalamic nuclei. The development of such relays also follows a peripheral-cortical pathway (Cases et al., 1996). Thus, beyond the relevance of having a morphological effect secondary to MAO inhibition, this finding suggests that the primary target of this metabolic disturbance may be located at the origin of the thalamo cortical afferents. Peripheral receptors and brain stem relays showed normal patterned distributions; it is only when the axons leave their central nuclei to travel to the cortex that the barrel like configuration is lost, even when the crude topography appears preserved. This experiment also helped to demonstrate that:

- MAO A inhibition was responsible for the morphological changes as demonstrated in normal mice by administering a MAO A inhibitor. MAO A inhibited mice developed the same lack of barrel formation in the somatosensory cortex as Tg8 mice did (Cases et al., 1996).

- Pharmacological inhibition of MAO A with clorgyline during the first postnatal week was capable of inducing disruption in the formation of the barrels in the somatosensory cortex in normal mice, similar to that seen in Tg8 mice. This suggests that the critical period for these effects to occur may be restricted to the first days of neonatal life. Earlier or later inhibition of MAO A did not induce the same changes. (Cases et al., 1996)

- The neural disarray is exclusively the consequence of excessive levels of serotonin but not of catecholamines. This was deduced from data showing that the administration of para-chlorophenylalanine, a serotonin synthesis inhibitor, given to Tg8 mice during development prevented the histological changes seen in untreated Tg8 mice. On the other hand, the same effect was not achieved with the administration of the catecholamine synthesis
inhibitor α-methylparatyrosine (Cases et al., 1996). Functionally, cells in layers I - III of the barrel cortex in Tg8 mice respond faster than equivalent cells in wild mice when the corresponding D2 principal whiskers are stimulated. This may be due to a more profuse innervation of those layers by the thalamo-cortical afferents that bring the sensory input to those layers, or to a more profuse innervation originating in the first relay cortical cells coming from layer IV in the barrel cortex (Yang et al., 2001).

Other morphological abnormalities demonstrated in phrenic motoneurons of Tg8 mice, consisted of multipolar dendritic trees with high density of dendritic spines and varicosities, contrary to the usual bipolar trees seen in normal neurons. As in previous examples, para-chlorophenylalanine was capable of correcting such changes in Tg8 mice. Interestingly, a serotonin2A agonist was also able to replicate the multipolar dendritic trees seen in wild-type animals which suggests that the effect of excessive serotonin during development in phrenic motoneurons is mediated by serotonin2A receptors, perhaps through excessive stimulation by the neurotransmitter (Bou-Flores et al., 2000).

In the visual tract, retinal ganglion cells showed a pervasive and aberrant pattern of segregation at the level of the dorsolateral geniculate nucleus in which ipsilateral and contralateral projections appeared mixed instead of being positioned in their normal regions. This effect disappeared in transgenic mice that were given injections of para-chlorophenylalanine (serotonin synthesis inhibitor) during pregnancy. Optical nerves and tracts had a normal pattern of distribution. Segregation of inputs from retina is known to be activity-dependent. The results in this experiment point to abnormalities caused by the blockade of activity-dependent segregation mechanisms, perhaps by acting upon serotonin1B presynaptic
receptors (which results in inhibition) and/or by saturating postsynaptic serotonin$_{2A}$ and serotonin$_{7}$ receptors. Also important was the discovery of transient expression of the serotonin transporter and the vesicular monoamine transporter. The presence of these receptors would permit the presynaptic vesicular storage of serotonin which in time would decrease the amount of serotonin available to serotonin$_{1B}$ receptor decreasing its inhibitory effect. Neurons expressing serotonin transporter and vesicular monoamine transporter 2 would be consequently more active at enhancing the activity-dependent mechanisms of guidance. Excessive concentrations of serotonin would result in disruption of such mechanisms by over-stimulation of those receptors involved (Upton et al., 1999).

**Immunolabeling** studies showed that serotonin tends to accumulate transiently in non-serotonergic neurons in Tg8 mice, which naturally was not seen in control mice. Seven-day-old Tg8 mice had increased serotonin immunoreactivity in serotonergic fibers over several brain regions. The affected places are the catecholaminergic cells of substantia nigra, ventral tegmental area, hypothalamus, and locus coeruleus. Other affected areas were: - telencephalon: areas CA1 and CA3 fields of the hippocampus, central amygdala, indusium griseum and deep layers of the anterior cingulate and retrosplenial cortices; - diencephalon: primary sensory nuclei, mediodorsal-, centrolateral-, oval paracentral-, submedial posterior-, and lateral posterior thalamic nuclei, and -brainstem: neurons of the superior olivary nucleus and anteroventral cochlear nucleus.

The presence of serotonin in those cells was concluded to be the result of reuptake as the distribution of L-aromatic amino acid decarboxylase, necessary for the synthesis of serotonin, was normal. Furthermore, serotonin immunoreactivity was positive even in non-serotonergic neurons lacking aromatic amino acid decarboxylase.
Reuptake in these cells suggests that serotonin transporter may be expressed in the said neurons, as happens in developing thalamic neurons. This suggestion was strengthened when the blockade of the transporter with paroxetine or fluoxetine resulted in serotoninergic immunoreactivity being abolished. Alternatively, non-serotoninergic neurons lacking the serotonin transporter may reuptake serotonin using dopamine and norepinephrine transporters. This phenomenon was implied when selective blockade of dopamine and norepinephrine transporters resulted in abolition of immunolabeling of non-serotoninergic neurons previously positive to the staining technique (Cases et al., 1998).

Tryptophan hydroxylase immunoreactivity in Tg8 mice is normal in quantity and localization, and is normally suppressed by the tryptophan hydroxylase inhibitor para-chlorophenylalanine (Cases et al., 1995). This suggests that increased synthesis cannot account for elevated levels of serotonin in Tg8 mice. Given that MAO B activity was normal in Tg8 mice, catabolic compensation neither seem to play a role in decreasing excessive levels of serotonin during early life (Cases et al., 1995).

The storage in the presynaptic axon is normally done in vesicles, however, electric activity would be expected to stimulate the release of serotonin from its vesicles, as happens in normal mice. However, the excess of serotonin present in Tg8 mice may act upon the serotonin_{1B} receptors preventing electrical stimulation from taking place and making the immunolabeling the more intense due to a greater concentration of serotonin (Cases et al., 1998).

At ten weeks of age serotoninergic immunoreactivity was back to normal, perhaps because of the emergent compensatory activity of MAO B. Confirmatory findings with regard to the above mentioned MAO B catabolic activities in Tg8 mice were reported by Ikemoto et al.
In that experiment, MAO B positive regions detected by histochemistry were no different between controls and transgenic MAO A knockouts. This is consistent with enzymatic evidence showing that MAO B activity remains intact despite the complete absence of MAO A. However, the lack of significant differences in the levels of 5-hydroxyindoleacetic acid (a serotonin metabolite) between controls and Tg8 adult mice can only be explained if MAO B has taken charge of the catabolism of serotonin.

Amongst other anomalies, an interesting difference in regional cerebral blood flow and related to the described histological changes was found by Holschneider et al. (2000). The somatosensory and barrelfield neocortex showed higher blood flow in Tg8 mice in comparison to wild mice, while blood flow was lower in entorhinal and midline motor cortex, also in comparison to control. The administration of acute doses of fenfluramine increased cortical blood flow in both populations and in all regions of the brain. However, changes were more pronounced in MAO A knockouts in comparison to wild type mice.

At the molecular level serotonin1A, serotonin2A and serotonin2C receptors exist in decreased numbers in the brains of mutant Tg8 mice (Shih and Thompson, 1999; Shih et al., 1999b). Also interesting is the observation that the activity of the various receptors is differentially influenced by the excess of serotonin resulting from the inactivation of MAO A. Bou-Flores and Hilaire, studying medullar-spinal cord preparations demonstrated that serotonin2A responses were preserved in transgenic MAO A knockout mice, while responses were lost in serotonin1B receptors. This is in sharp contrast to the facilitation that normally occurs in wild mice by a stimulus to the serotonin2A receptors and an inhibition due to serotonin1B receptors. Perhaps the lack of serotonin1B response may leave the neurons
uninhibited as suggested above, inducing potentially serious developmental consequences through activity-dependent mechanisms (Bou-Flores and Hilaire, 2000).

Further confirmation of the findings described above comes from the report of Salichon et al. (2001) where single, double and triple knockouts of MAO A, serotonin transporter, and serotonin1B receptor genes were used to "dissect" the role of each of these molecules in causing the abnormalities seen in MAO A knockout mice. Serotonin1B overactivation prevents the normal segregation of retino-geniculate, somatosensory projections to the thalamus, and the thalamo-barrel field cortex projections in a gene-dosage fashion. However, control mice do not need serotonin1B to develop normally. Serotonin transporter knockout mice had defects similar to, but of a lesser degree than, those seen in MAO A knockouts. The elimination of serotonin1B in the double transgenic serotonin transporter-serotonin1B showed a wild type of segregation of retinal fibers as seen in the dorsal-lateral geniculate nucleus, suggesting some contribution from the said receptor in the absence of the serotonin transporter. The pattern appears distorted in the MAO A knockout and is corrected in the MAOA-serotonin1B double knockout probably because of the absence of serotonin1B receptors. Perhaps the lack of reuptake results in an increased amount of serotonin in the extracellular space that overestimates the serotonin1B causing inhibition of activity and deficient activity-dependent guidance mechanisms.

**MAO B**

Studies in transgenic MAO B mice are much less abundant in comparison to the growing MAO A knockout literature. One of the
earliest studies described the creation of a mouse that over-expressed MAO B and was used in the study of the effects of MPTP on Parkinson's disease (Andersen et al., 1992). For long time it has been known that MAO B metabolizes 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine to 1-methyl-4-phenyl pyridium; when 1-methyl-4-phenyl pyridium is taken up into dopaminergic neurons it inhibits component 1 of the mitochondrial electron transport chain, decreasing adenin monophosphate levels and resulting in cell degeneration and death (Andersen et al., 1994). Notwithstanding this fact, Andersen et al. demonstrated that the mere presence of MAO B is not sufficient to induce cell damage, as most of the conversion of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine into 1-methyl-4-phenyl pyridium happens in non-dopaminergic glial and serotoninergic cells that express MAO B. Perhaps the dopamine transporter plays an active role by introducing 1-methyl-4-phenyl pyridium into the dopaminergic cells where it causes its deleterious effects after remaining sequestered for some time (Andersen et al., 1994).

A knockout MAO B mouse generated by inserting a neomycin resistant gene in exon 6 of the MAO B gene was described by Grimsby et al. in 1997. Behaviourally, these mice did not show any difference from controls with respect to exploratory activity (open field test) or anxiety (elevated plus maze). However, when subjected to inescapable stress (forced swim test), MAO B knockout mice showed an increase in activity that corresponds to an increased vigilance in response to stress. This response was still present with the same intensity four weeks after the first assessment, while wild mice showed adaptive responses by decreasing their physical activity. Such phenomena could not be explained by deficits in memory, as the execution of the Y maze was no different between strains. From the biochemical point of view, MAO B transgenic mice had elevated levels of
phenylethylamine, but normal levels of norepinephrine, dopamine and serotonin and their metabolites (Grimsby et al., 1997).

More recently, the same transgenic model was used to study dopamine metabolism (Fornai et al., 1999). This mouse has no MAO B activity and no compensatory increase in the activity of MAO A. Phenylethylamine was eight times higher when compared to wild mice (Fornai et al., 1999). Under basal conditions no difference in the amount of dopamine or its metabolites was noted between the MAO B knockout and wild mice. This suggested that MAO A is capable of maintaining Dopaminergic catabolism in the presence of any challenge (Chen et al., 1999; Fornai et al., 1999). Administration of clorgyline resulted in an increase in dopamine concentration and a decrease in metabolite production in both groups, supporting the previous conclusion (Fornai et al., 1999). When dopamine was increased by systemic administration of 10 mg/kg of L-dopa both groups experienced similar increases in dopamine and its metabolites (homovanillic acid and dihydroxyphenylacetic acid). Administration of 50 mg/kg of L-dopa produced similar increments in dopamine concentration but higher augmentation in dihydroxyphenylacetic acid and homovanillic acid in wild type mice. Finally, after 100 mg/kg of L-dopa, knockout had higher levels of dopamine and lower levels of its metabolites in comparison to wild type animals, suggesting that MAO A is capable of inactivating dopamine when it is present in excessive amounts to a certain limit. This was confirmed by the simultaneous administration of l-deprenyl and L-dopa to both populations, which produced an elimination of the differences that appeared between the two groups when L-dopa was administered alone as described above (Fornai et al., 1999). Using microdialysis, Chen et al. (1999) evaluated the effect of blocking the dopamine transporter by acute administration of cocaine to MAO B knockout and control mice. After
acute administration of 15 mg/kg of cocaine, the amount of dopamine in the microdialysates of both groups experienced a twofold increase, while decreasing dihydroxyphenylacetic acid and homovanillic acid, but increasing 3-methoxytyramine which is produced extracellularly by the catabolic effect of catechol-O-methyltransferase on dopamine. This suggests that recapture is necessary for the metabolism of dopamine and underscores the presence of alternative pathways of dopaminergic metabolism. (Chen et al., 1999). Additionally, neither an immunoreactivity analysis of tyrosine hydroxylase or binding assay for the dopamine transporter demonstrated differences between the two groups of mice related to synthesis or transport of dopamine (Chen et al., 1999). Dopamine receptor binding analysis showed no differences for D1 between wild and transgenic mice, however, D2, D3, and D4 were increased by 30% in comparison to wild mice, suggesting the presence of an independent up-regulation given that dopamine metabolism seems untouched in knockout mice. A possible mechanism for this to happen may involve phenylethylamine. Given that it is degraded by MAO B, knockout mice have an excess of phenylethylamine that makes it a likely candidate to be the agent of receptor modification in MAO B knockout mice (Chen et al., 1999).

An additional effect of the lack of MAO B activity is the redistribution of cerebral blood flow in transgenic mice. Scremin et al. (1999) demonstrated that MAO B knockout mice have a more abundant cortical blood flow in the central sensory and motor regions of the brain compared to wild type animals. The continuous administration (30 min) of phenylethylamine increased the blood flow in lateral frontal and piriform cortex of both groups. Globally, phenylethylamine decreased cortical blood flow in MAO B knockout by 31-41% in comparison to controls. These differences may be secondary to differential neuronal activation resulting from chronic
and acute increases of phenylethylamine levels in wild and transgenic mice. Also, these effects may be influenced by the anxiogenic effects that high levels of phenylethylamine have *in vivo* (Scremin et al. 1999).

**Functional differences of the monoamine oxidases: Lessons from transgenic mice and human MAO deficiency states**

To understand the function of these two very similar molecules let us review what differences have emerged from the naturally occurring disorders affecting one, the other or both. The absolute and combined absence of MAO activity occurring in subjects affected by Norrie disease produces blindness, hearing loss and mental retardation, with autistic like behaviour. Also present are altered autonomic function and metabolic disarray secondary to the catabolic disturbance (Sims et al., 1989; Murphy et al., 1990; Collins et al., 1992; Lenders et al., 1996). Selective MAO A deficiency as described by Brunner et al. (1993 a and b) is associated with borderline intelligence and impulsive aggressive behaviours with some sexually perverse traits such as exhibitionism and voyeurism. Selective MAO B human deficiency has never been reported however, Norrie disease compromising only MAO B and not MAO A, has shown no behavioural component despite the presence of blindness and hearing loss (Lenders et al., 1996 and 1998). From the metabolic point of view, a comparative study of two subjects with selective MAO A deficiency, two with selective MAO B deficiency and Norrie disease, and five with combined deficiency of A and B with Norrie disease, showed that plasma levels of norepinephrine and adrenaline were normal for all subjects. However, the metabolites of norepinephrine and adrenaline showed reliable patterns of variability. Normetanephrine, metanephrine, 3,4-dihydroxyphenylglycol, 3-methoxy-4-hydroxyphenylethylenglycol and
vanillylmandelic acid were normal in MAO B deficient subjects, but high in the MAO A and MAO A+B deficient groups. Dopamine was high only in those with combined MAO deficiency. Its metabolites dihydroxyphenylacetic acid and homovanillic acid also had the same pattern of variation as did the metabolites of and norepinephrine (Lenders et al., 1996 and 1998; Shih and Thompson, 1999).

Urinary tests showed that MAO B deficient subjects had high concentration of phenylethylamine, but all other amine and metabolite levels were comparable to those in control subjects. MAO A deficient subjects had higher urinary excretion of normetanephrine, serotonin and 3-methoxytyramine, but lower excretion of 3-methoxy-4-hydroxyphenylethylenglycol, vanillylmandelic acid, and homovanillic acid. The combined deficit of MAO produced a urinary test pattern resembling that seen in MAO A deficient subjects, but with broader variation (Lenders et al., 1996 and 1998).

Maximal elevations of phenylethylamine are seen in MAO B deficient subjects, but the fact that combined deficiency of MAO produces higher levels of phenylethylamine excretion than those found in individuals deficient only in MAO B activity suggests that MAO A does contribute in part to phenylethylamine metabolism (Lenders et al. 1996 and 1998). Comparatively, MAO A knockout mice had higher levels of serotonin, and norepinephrine in frontal cortex, cerebellum and hippocampus in relation to controls; MAO B knockout mice had only higher levels of phenylethylamine. Behaviourally, both types of knockout mice demonstrated a higher reactivity to induced stress in the forced swim test; this may be due to the role that norepinephrine and phenylethylamine have in inducing fear and stress responses in each model. Finally, MAO A knockouts spent a higher proportion of time exhibiting aggressive and defensive behaviours in comparison with controls. MAO B knockout mice did
not share these behavioural abnormalities. (Shih & Thompson, 1999; Shih & Chen, 1999).

Hence, it could be concluded that these enzymes are not able to fully compensate for one another in the metabolism of neurotransmitters despite the abundance of MAO B in the brain (Berry et al., 1994). This is particularly the case when MAO A is absent during development; in this case, there is global deficiency of MAO until after the late fetal period, at which time MAO B begins to be expressed. Thereafter, MAO B plays an auxiliary role in the metabolism of catecholamines even when MAO A is permanently absent or inhibited (Lenders et al., 1996; Shih and Thompson, 1999).

Due to its relevance, Brunner's syndrome mutation is a genetic abnormality that bears an important weight in the way we conceive behavioural disorders in general, and aggressive responses in particular. However, its rare occurrence warns scientists not to draw the conclusion that an aggression gene has been found (Morell, 1993). The first duty then is to assess how generalized is the existence of such a mutation amongst people that display aggressive phenotypes in comparison to controls. In the next chapter, we introduce a population study carried on in search of the said mutation, in a population of male subjects who had been followed since early infancy. Some of these individuals, now teenagers, had displayed aggressive behaviour in an intermittent or pervasive fashion. Others behaved normally and were considered as controls. Our results demonstrated that C936T mutation is not present in any of our subjects; hence it has no influence in the causation of aggressive behaviours observed in a small percentage of our subjects. That allows us to conclude that aggressive behaviour may be influenced by MAO activity, but it is not necessary for it to be completely blocked to cause aggressive phenotypes as in Brunner's syndrome, as illustrated
by the wealth of literature correlating low levels of monoamine oxidase activity with aggressive behaviors that are part of various psychopathological entities.
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Figure 1: MAO A promoter. Repeats appear numbered from I to VII.
CHAPTER III

AGGRESSIVE BEHAVIOUR AND BRUNNER'S SYNDROME: NO EVIDENCE FOR THE C936T MUTATION IN A POPULATION SAMPLE

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Aggressive behaviour and Brunner's syndrome: No evidence for the C936T mutation in a population sample

Mejia, JM1,2 Ervin, FR2 and Palmour, RM1,2,3

Departments of Biology1, Psychiatry2 and Human Genetics3

McGill University.

1033 Pine Av. West.


Canada.

T: (514) 398-7303

F: (514) 398-4370

Running head title: MAO A gene mutation and aggressive behaviour
Abstract

Brain catabolism is highly dependent on the effects of monoamine oxidases (MAO) because they terminate the action of recaptured neurotransmitters. In 1993, Brunner and colleagues reported the discovery of a family with a deficiency of MAO A activity due to a point mutation in the 8th exon of this X linked gene. The phenotype of the affected members included impulsive and/or aggressive behaviours and borderline mental retardation. This finding suggested the hypothesis that MAO A activity might be related to the development of aggressive behaviours, at least in these atypical cases. To evaluate the generalizability of that hypothesis, we studied a population of 100 adolescents drawn from an age cohort which has been followed longitudinally for more than 15 years.

We amplified the region that contains the mutated sequence and identified the mutation by means of restriction enzyme cleavage (Bfa 1). None of the subjects in our sample carried the C936T mutation, notwithstanding the aggressive phenotype. In agreement with others, we conclude that total absence of MAO A is not a common event in population sample. The phenotype of the kindred described by Brunner, as well as that of MAO A knockout mice, demonstrate that complete deficiency of MAO is sufficient, but not necessary, for the development of pathological impulsive aggressive behaviours.

Keywords: Monoamine oxidases; neurodevelopment; MAO A deficiency; impulsive-aggressivity.
Monoamine oxidases (MAO) are important enzymes for the catabolism of the amine neurotransmitters dopamine, serotonin, norepinephrine, and adrenaline. Levels of MAO activity have been associated since 1965 with several psychiatric disorders, some of which are characterized by aggressive behaviour. Other traits also related to aggressive behaviour, such as impaired impulse control, have been correlated with low MAO activity in numerous reports (reviewed by Oreland and Hallman, 1995).

In 1993 a robust demonstration of this relationship was reported by Brunner and colleagues, who studied a family which included 14 males affected by a syndrome with prominent features of impulsive aggressive behaviours and other pathological conducts, such as arson, voyeurism, and exhibitionism. Because this disorder only affects males, a search for candidate genes was focused on the X chromosome (Brunner, 1993a and b).

In a subsequent analysis it was found that all affected males, as well as unaffected female carriers, have a point mutation in the eight exon of the MAO A gene, changing a glutamine codon to a stop codon. Affected subjects, who also display borderline mental retardation, had no measurable activity of fibroblast MAO A. Female carriers and other non-carriers in this family were completely asymptomatic (Brunner, 1993a). Despite exhaustive efforts to look for this mutation in different samples of aggressive subjects (Murphy, et al., 1998; Schuback, et al., 1999), there is as yet no positive report.

We searched for this mutation in a group of 100 young male subjects of French Canadian origin, followed for more than ten years as part of a longitudinal cohort that aims to understand the relationship of predisposing factors to the development of aggressive behaviour in childhood and adolescence (Tremblay et al., 1991). The sample has a normal range of IQ scores, with approximately 7%
showing borderline mental retardation. For the present study, subjects were classified according to their SBQ scores with respect to aggressive behaviour. This classification system, which yields a balanced proportion of stable non-aggressive, unstable aggressive and stable aggressive subjects, considers the evolution of aggressive behaviour over time, rather than utilizing single point evaluations, which are intrinsically unstable in young individuals (Nagin & Tremblay, 1999). A number of reports (e.g., Le Marquand et al., 1998; Seguin et al., 1999) described increased levels of impulsive responding in this population sample.

For the purposes of genotyping, DNA was extracted from venous blood with the phenol chloroform technique. Primers 5'ctt ctt ctt cca gaa ggc c 3’ and 5’ gac ctt gac tgc caa gat 3’ were used to amplify a region that included exon 8 of MAO A gene. Conditions for PCR were 35 cycles of 1' @ 94 °C; 30 sec @ 52 °C; 1' @ 72 °C followed by 5' @ 72°C. The amplified product was digested with Bfa1. After separation by PAGE, C936T mutation would be detected by a pair of bands, while uncut products would appear as a single band. None of the 100 subjects showed double bands which was interpreted as an absence of the C936T mutation. Genomic DNA was used as a positive control to ensure that the Bfa1 restriction enzyme had normal activity.

Thus, in a population sample enriched for aggressive behaviour, but not for borderline mental retardation, we (like many others) failed to find any evidence for the C936T mutation originally described by Brunner et al. (1993a). According to a recent review (Murphy et al., 1998) the persistent failure to identify another subject or set of subjects affected by Brunner's syndrome indicates that this is an extremely rare syndrome in which loss of MAO A activity appears to be sufficient to account for the phenotype. In the affected subjects, the pleiotropic effects of the loss of MAO activity illustrates the
multiple ways in which elevated levels of neurotransmitters could disturb critical phases of brain development.

It is well known that neurotransmission induces neuronal development in serotoninergic cells and their targets. It is possible that a narrow “window” in the concentration of neurotransmitters is required for the fine tuning of the brain to occur. Thus, extreme changes in the activity of MAO (either high or low) could certainly preclude normal development (Whitaker-Azmitia, 1996).

More often however, subtle biochemical changes may give rise to a broad variety of states that would be manifested as differential levels of quasi-pathological aggressive behaviours that are still considered to be within the “normal” range of human behaviours. These changes do not occur in isolation, but are typically the result of multiple genes interacting and creating a very complex environment at critical periods of neurological development. In consequence, these subtle alterations will become evident only under challenging circumstances.

Future research should focus on the different effects that neurotransmitter changes during neural development have on the specific systems involved in higher neurological functions.
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CHAPTER IV

ENZYMATIC ACTIVITY, COMMON POLYMORPHISMS AND BEHAVIOURAL TRAITS
There is a long history of studies of platelet MAO activity in both normal and psychiatric populations. Much of this literature predates the current era of molecular genetics and it is based solely on studies of enzymatic activity. Although a complete review of this work is beyond the scope of this thesis, an appreciation of some of the key studies is necessary to establish a conceptual framework for current reports in this field.

One of the earliest studies in this area is that of Murphy et al. (1976), who documented MAO activity in 680 subjects using a normal population constituted by a minimum of 40 individuals representing each decade of life and each gender. The objective was to establish the influence that gender and age might have on enzymatic activity in a normal population. Both plasma and platelet MAO activity were measured. There was no correlation between the two types of activities (r = 0.08) (Murphy et al., 1976; Schooler et al., 1978). Plasma activity ranged from 5 to 39 nMoles/ml/hr, the most prevalent activities being those between 10 and 30 nMoles/ml/hr. Comparatively, platelet activity spanned from 2 to 40 nMoles/10^4 platelets/hr, with the most prevalent values falling between 5 and 25 nMoles/10^4 platelets/hr. Contrary to previous reports, Murphy and colleagues did not find any age difference. Gender differences appeared only in the platelet measurements, which were 20% higher in females than in males (Murphy et al., 1976).

This degree of variability in MAO activity has been repeatedly reported in both normal and clinical populations (Hotamisligil & Breakefield, 1991). Over a wide range of studies the distribution is almost always skewed to the left such that low enzymatic activity predominates over high levels of activity (Murphy et al., 1976). Interestingly, an abundant body of literature has associated extreme levels of MAO activity with behavioural traits and psychopathological
Relationship of MAO activity with neuropsychological and behavioural traits in normal populations

Shaughnessy et al. (1980) studied 65 normal females, ages 18 to 50, with no personal or family history of psychosis. MAO activity was measured using tyramine as substrate. Personality traits were assessed using the clinical analysis questionnaire (Delhees & Cattell, 1975). A multivariate analysis showed no significant differences between the high and low activity groups with regard to personality traits. However, those women with the lowest levels of MAO activity were found to have a) lower superego strength, b) high rates in anxiety/depression scales, c) high tension and irritability, d) emotional liability, and e) low levels of self-confidence. In their review, Shaughnessy et al. (1980) stated that Murphy et al. (1977) found a positive correlation between MAO activity and the hypochondriasis and psychastenia scales of the Minnesota Multiphasic Personality Inventory (Hathaway & McKinley, 1943). Negative correlations between low MAO activity and the paranoid scale (Murphy et al., 1977) or the hysteria scale of the Minnesota multiphasic personality inventory (Castrogiovanni et al., 1990) have also been reported in other studies. Schooler et al. (1978) found negative correlations between MAO activity and the general sensation seeking and thrill seeking scales of the Zuckerman's Sensation Seeking Scale (Zuckerman, 1974). Also, out of 93 subjects studied, those having low MAO activity demonstrated high ego strength as measured with the Barron Strength Scale (Barron, 1956) and positive affect measured with the Nowlis Mood Scale (Nowlis, 1965). Finally, Donelly et al. (1979) demonstrated the discriminating value of grouping high vs. low
MAO activity subjects to perform a factor analysis, by taking some of the items of the Minnesota multiphasic personality inventory obtained from a group of women that participated in their study. Women with low MAO activity showed higher social contact seeking, strong positive affect and self-confidence, and high sensation seeking.

Using the high-risk paradigm, Buchsbaum et al. (1976) and Coursey et al. (1979 and 1980), studied a sample of 68 normal subjects drawn from an original group of 375 male college students assessed for MAO activity. The highest and lowest 10% of the population were selected to study vulnerability to psychiatric disorders by analyzing their family history, psychological test results and average evoked potentials. Low MAO subjects were more sociable as described also by Donelly et al. (1979). They had twice as many personal and familial antecedents of psychiatric illness than controls (Buchsbaum et al., 1976; Coursey et al., 1979). Low MAO activity was also correlated with more criminal convictions, higher and more diverse drug consumption, and higher scores on the Minnesota multiphasic personality inventory antisocial scale. Similar to the study of Murphy et al. (1977), Coursey et al. (1979) confirmed that low MAO activity is linked to higher scores in the hysteria and hypochondriasis scales of the Minnesota multiphasic personality inventory (reviewed in Oreland et al., 1998).

Shekim et al. (1989) studied 58 healthy adult males and confirmed the reported associations of low MAO activity with hypochondriasis in the Minnesota multiphasic personality inventory, and higher sensation seeking in Zukerman's sensation seeking scale (Murphy et al., 1977; Schooler et al., 1978; Coursey et al., 1979). A negative correlation with the dependence and impulsivity scales translates into paranoid and impulsive tendencies, while a positive correlation with the order scale of the Personality Research Form-E
(Murray, 1938), indicated neatness and organization at an increasing level when MAO activity is high.

Average evoked potentials permitted distinguishing two groups of low MAO activity individuals according to their response to increasingly intense stimuli. They were called “augmenters” if the amplitude of the evoked potentials increased with the growing intensity of the stimuli, or “reducers” if the amplitude decreased as the stimuli increased. Augmenters with low MAO activity had a significantly higher number of antecedents of suicide attempts in their personal and family histories (Buchsbaum et al., 1976; Coursey et al., 1979). Additional psychological testing and factor analysis of the same population showed differences established by gender, identifying low MAO males and high MAO females as those with the highest psychopathological tendencies (Coursey et al., 1980).

Finally, Klinteberg et al. (1990 and 1992) studied 37 male subjects with a neuropsychological battery. It included a finger tapping and alternation test, a reaction time test, a perceptual maze test, a perspective fluctuation test, a lexical decision task, and a reaction time task that also assessed motor disinhibition. Low levels of MAO activity were related to shorter times of response and higher scores in the perspective fluctuation test, suggesting differences in the perception of deceiving images. A strong negative correlation appeared between levels of MAO activity and failed inhibitions in the motor disinhibition task. These results link MAO activity to higher right hemisphere activation, and to frontal inhibitory activity which may underlie the failures in motor disinhibition (Klinteberg et al., 1990 and 1992; Oreland & Hallman, 1995; Oreland et al., 1998).
MAO activity in subjects with psychopathological conditions

**Alcoholism**

Associations between low levels of platelet monoamine oxidase and alcohol abuse were reported as early as 1975 by Gottfries et al. who found that MAO activity was decreased in the brains of alcoholics that committed suicide. Additional reports by Takahashi et al. (1976), Wieberg et al. (1977), Major and Murphy (1978), and Sullivan et al. (1978) ratified this discovery (reviewed in Gorkin, 1983). Other studies such as that of Anthenelli et al. (1998), who studied 1473 individuals including 140 probands, 876 relatives and 457 non-related subjects, did not concur. He found that alcoholism does not contribute a substantial variability to the low activity of MAO once controls for gender and tobacco smoking are introduced in the analysis. Farren et al. (1998) recruited 46 alcoholics and 22 non-alcoholic subjects, ethnically homogeneous. They found absolutely no correlation between MAO activity and alcoholism despite numerous attempts of grouping subjects by gender, smoking habits, length of abstinence, severity of alcoholism, length of alcohol use, type of alcoholism (I or II according to Cloninger's classification) (Cloninger, 1981), family history of alcoholism, presence/absence of sociopathic traits or history of sociopathic traits.

In an earlier study by Tabakoff et al. (1988), platelet MAO activity was no different in 95 abstinent alcoholics (abstinent for 5-90 days) in comparison to 33 normal subjects. MAO activity was measured in relationship to protein content in platelets while most other studies report results in relationship to platelet count. Tabakoff et al. (1988) also confirmed the inhibitory effects of alcohol upon MAO activity in *vitro*. Alcoholics have a stronger inhibitory effect of alcohol upon
platelet MAO compared to that observed in non-alcoholics. This difference is obvious when the concentration of substrate is ten times higher than the Km. However, it tends to disappear as the substrate concentration decreases towards Km (Tabakoff et al., 1985 and 1988). Sherif et al. attempted unsuccessfully to replicate these findings in a study of 25 male chronic alcoholics. Despite using various different concentrations of substrate, no correlation between MAO inhibition and alcohol consumption was ever seen. As in some other studies discussed below, in this experiment type II alcoholics demonstrated lower MAO activity in comparison to type I alcoholics and control subjects (Sherif et al., 1992).

The issue of alcohol being a cause or an effect of low levels of MAO activity has been explored in several studies. Major and Murphy et al. (1978) reported that there was no influence of the length of alcohol consumption upon levels of MAO activity. Sullivan et al. (1978) stated that levels of MAO did not changed from the pre-abstinence levels after a year of abstinence. Finally, Major et al. (1981) studied 12 subjects admitted to an alcoholism rehabilitation center to better understand how MAO activity is affected by abstinence. They monitored MAO activity 72 hours after the last drink and noticed a mean increase of activity of 66% compared to pre-abstinence levels on each individual. A week later, levels of MAO activity were still 37% higher; two weeks later they were 19% higher and four weeks after the last drink, activity levels were still 14.9% above their pre-abstinence values. These results suggest that acute alcohol abstinence increases MAO activity, with a subsequent and gradual decrease back to pre-abstinence levels.

As seen above, some studies in alcoholics (von Knorring & Orelan, 1985; Pandey et al., 1988; Sullivan et al., 1990; von Knorring & Orelan, 1996) have differentiated those designated type I
from the ones characterized as type II according to Cloninger's classification (Cloninger, 1981). Beyond the clinical characteristics that define this typology (early consumption of alcohol, early social problems, resistant to rehabilitation, comorbid psychopathic traits, familial antecedents of substance abuse and other psychopathologies, etc), these studies demonstrated other differences amongst type II and type I alcoholics and normal subjects. Type II alcoholics obtained high scores in: a) the boredom susceptibility and in the thrill/adventure seeking scales of Zuckerman's sensation seeking scale (Zuckerman, 1974); b) the extraversion scale from Eysenck's personality inventory (Eysenck & Eysenck, 1975; Eysenck, 1976); and c) the novelty seeking dimension of the Tri-dimensional Personality Questionnaire (Cloninger, 1987). The presence of these traits had suggested that rather than being only associated with alcoholism, low MAO activity coexists with those traits of type B personalities and substance abuse of which alcoholism is also part (von Knorring & Oreland, 1996).

Family history of alcoholism did not seem to play an important role in predicting the level of MAO activity measured in a "high risk" population of subjects who had parents who suffered alcoholism. In this study, Schuckit et al. (1982) paired 15 men by age, height/weight ratio, and drinking history, and measured MAO platelet activity before and after drinking 0.59 g/kg of alcohol. No statistically significant differences were perceived amongst the two groups at risk (high and low). However, a tendency towards lower levels of MAO activity was perceived amongst the "high risk" subjects. Comparatively, using a family study approach, Devor et al. (1993) studied 13 two- and three-generation families (108 subjects, 49 of them alcoholics). In addition to the findings of lower MAO platelet activity in those affected by alcoholism, they demonstrated that alcoholism and low MAO activity co-segregate in the families studied. Unfortunately, this finding is
confounded by the fact that 16 of 49 alcoholic subjects also met criteria for antisocial personality disorder. A segregation analysis of MAO activity at two substrate concentrations supported a major Mendelian locus mode of transmission with a moderate multifactorial background effect. Despite their encouraging results, the authors fell short of demonstrating that MAO is a marker for the genetic susceptibility of alcoholism according to Reich's criteria (Reich, 1988). This was due to the fact that at risk members are still unaffected despite the substantial heritability \((H = 0.2)\) of the marker, and to the presence of an excess of affected relatives amongst the putative carriers (54.3\%) (Devor et al, 1993).

**Tobacco Smoking**

In a recent review of the literature, Oreland et al. (1999) pointed out that the level of MAO activity in smokers is lower than that of non-smokers and ex-smokers regardless of gender. However, nicotine does not inhibit MAO, implying that other components in cigarettes may be responsible for the enzymatic inhibition caused by smoking (Fowler et al., 1998 and 2000). Some studies have indicated that MAO inhibition is irreversible and probably caused by adducts of 1,2,3,4-tetrahydroisoquinoline (Mendez- Alvarez; et al., 1997).

Other studies have also shown an inhibitory effect on MAO A secondary to tobacco smoking, which implies that some antidepressant effects may be part of its physiological actions. This is still in question as cigarette smoking only inhibits between 24 and 32\% of the enzymatic activity while the required MAO A inhibition to achieve clinical effects has been reported to be between 20 to 80\% (Fowler et al., 1996 b). In a recent publication, Hauptmann and Shih (2001) report that 2-naphthylamine inhibits MAO activity (up to 60\%
MAO B and 40% MAO A at 200 µM), irreversibly through a mixed mechanism (competitively and non-competitively), and in proportion to the dose obtained from cigarette smoking.

The influence of cigarette smoking in studies associating low platelet MAO activity and the personality trait of sensation seeking was specifically analyzed by Ward et al. (1987) in a study of 87 smoking and non-smoking student volunteers. Zukerman's sensation seeking scale; (Zukerman, 1974) was used to assess sensation seeking behaviours. Smokers of both sexes had lower levels of platelet MAO activity. Scores from the disinhibition scale of the sensation seeking scale had a negative correlation with MAO activity. Male smokers scored higher in disinhibition in comparison with same gender non-smokers, while female smokers had higher total scores in comparison with their non-smoking counterparts. The authors concluded that low MAO activity is not necessarily due to cigarette smoking, as differences between smokers and non-smokers were not statistically significant (Ward et al., 1987). von Knorring and Oreland (1985) applied a similar study design to a population of 1000 recruits, and found that platelet MAO activity was low only in smokers compared to non-smokers and ex-smokers. Correlations similar to those established by Ward et al. (1987) were found in this study with the thrill and adventure seeking-, and the modified experience seeking scales of Zukermann's sensation seeking scale (Zukerman, 1974).

Fowler et al. (1996a and b) studied the effect of tobacco smoking upon MAO activity with positron emission tomography. By analyzing the influx constant which varies with MAO B concentration, or by using radiolabeled clorgyline which selectively binds to MAO A, they were able to quantify activity for each form of the enzyme. Eight smokers, eight non-smokers and four former smokers were recruited for the MAO B study (Fowler et al., 1996a). Concomitant
measurements such as glucose metabolism and blood flow did not differ between the three experimental groups, demonstrating that the effects of smoking were selective on inhibition of enzymatic activity and not the result of other effects. Levels of MAO B were reduced 40% in the brains of smokers compared to non-smokers and former smokers, while MAO B levels between the last two groups were comparable. MAO B platelet activity returned to normal values after a few weeks of smoking cessation. As reviewed by this team (Fowler et al., 1996 a), cigarette smoke, extracts of cigarette smoke and smoker's saliva have an inhibitory effect on MAO activity which proves that low levels of MAO activity in smokers may be an added effect to the existent MAO activity in each subject.

Fowler et al. (1996 b) recruited 15 non-smokers and 16 smokers of both genders to study MAO A activity in different regions of the brain in relationship to smoking. The activity in smokers was reduced 24-32% on average for all cortical and sub-cortical structures. However, some regions (e.g. occipital) were reduced up to 38%.

In a large twin study of 690 men and 861 women, Whitfield et al. (2000) explored the relationship between tobacco smoking, alcoholism and other psychiatric disorders. All subjects were followed up longitudinally and were assessed with respect to MAO activity, personality characteristics assessed with tri-dimensional personality questionnaire (Cloninger, 1987) and Eysenk's personality inventory (Eysenck & Eysenck, 1975), and psychiatric diagnosis made with a telephone-adapted form of the Semi-Structured Assessment for Genetics of Alcoholism (Bucholz et al., 1994). The results showed that the diagnosis of alcohol dependence (33% men, 11% women), conduct disorder and depression were significantly more frequent amongst smokers in comparison to ex- and non- smokers, regardless of gender. Smokers had lower levels of MAO activity that were inversely
proportional to the amount of cigarettes consumed. Smoking less than 10 cigarettes a day had almost no effect on MAO activity, but the group that consumed 11-19, or >20 had significantly lower levels of MAO activity in comparison to non-smokers. Twin status did not have any relevant influence on the predisposition to smoking habits. Yet, non-drinkers with a monozygotic twin dependent on alcohol had lower levels of MAO activity in comparison to non-drinkers with concordant twins. A personal history of alcohol abuse, particularly in the year previous to the study, was significantly associated with lower levels of MAO activity. In conclusion, the authors suggested that MAO is a state marker for tobacco smoking and it is associated with other disorders insofar as they are comorbid with the former. Notwithstanding their findings, it is possible that due to the recruitment techniques that failed to attract severe and violent alcoholics, MAO may also be a trait marker for this particular type of alcoholism (Whitfield et al., 2000).

**Personality traits and disorders**

As mentioned above, several personality traits have been associated with low MAO activity. Those traits aggregate together in the criteria for personality disorders in the cluster B type (narcissistic, histrionic, borderline and antisocial) (Oreland & Hallman, 1995; Hallman et al., 1996; Oreland et al., 1998). To illustrate this point Yehuda et al. (1989) studied 15 male individuals diagnosed with borderline personality disorder. Those diagnosed with borderline personality disorder had lower MAO activity when compared to control subjects. Furthermore, a subgroup of subjects with borderline personality disorder and antisocial personality disorder as a comorbid entity displayed the lowest levels of MAO activity. Fowler et al. (1980) reviewed the literature and came to the conclusion that MAO activity
relates to those behavioural traits much better than it does to particular psychiatric diagnoses. Perhaps what this reveals is a constitutional vulnerability that predisposes individuals to such disorders in the presence of environmental factors. Under this hypothesis Fowler et al. (1980) studied 59 individuals, 18 of whom belonged to a mountaineering club (interpreted as a sign of sensation seeking behaviour); 9 had some interest in mountaineering but did not practice it, and the rest were not interested in mountaineering at all. The 3 groups were assessed with Zuckerman's sensation seeking scale (Zukerman, 1974), the extraversion/impulsivity and lie scales from Eysenck's Personality Inventory (Eysenck & Eysenck, 1975; Eysenck, 1976), and the impulsivity and monotony avoidance scale from the Karolinska Scales of Personality (Shalling & Edman, 1993). Active and non-active mountaineers had significantly lower platelet MAO activity in comparison with the control group and scored higher in the thrill and adventure seeking scale, general sensation seeking, extraversion-impulsivity, and impulsivity and monotony avoidance (Fowler et al., 1980). A very similar model was used by Schalling et al. (1987) to study 40 men with regard to their personalities. The instruments used were the Karolinska scales of personality, sensation seeking scale and Eysenck's personality inventory. These results suggested that individuals with high MAO activity display temperament characteristics compatible with dysthymia, while subjects with low MAO show patterns associated with extraverted and uninhibited behaviours.

In a study of persistent criminal behaviour, 68 juvenile delinquents were contacted as adults to assess their MAO activity and its relationship to psychopathy, as measured by the psychopathy check list (Hare, 1991) and persistent criminal behaviour. There was no relationship between MAO activity levels and psychopathy check
list scores (Alm et al., 1996; Stalenheim et al., 1997). Nonetheless, people with low MAO activity and psychopathy check list scores higher than 0 had a significantly higher rate of recidivism with regard to commission of criminal acts in the 25 year follow-up period of the study (Alm et al., 1996). Similarly, Belfrage et al. (1992) studied 59 males undergoing forensic psychiatric examinations. They were classified as violent or non-violent according to the nature of their current crime, and aggressive or non-aggressive according to their history of aggressive behaviour. MAO levels proved to be low in those violent or aggressive in comparison with those who were neither violent nor aggressive. When each subgroup was subdivided into psychotic and non-psychotic, the differences in MAO activity disappeared amongst the psychotic groups, but prevailed amongst the non-psychotic.

In a third forensic population, Stalenheim et al. (1997) used MAO activity levels and psychopathy check list scores to analyze the relationship between these three factors. They recruited 58 individuals admitted for a forensic psychiatric assessment after one month of detention. They used the Karolinska scales of personality, psychopathy check list and MAO activity measurements and compared their results with those obtained from 400 members of a comparison group. Significant relationships between levels of MAO activity and the following Karolinska scales of personality scales were found: impulsiveness, monotony avoidance, psychastenia, socialization, verbal aggression, irritability, psychopathy factor and aggression factor. No association emerged with psychopathy check list scores, though. Type II alcoholism and borderline personality disorder were diagnosed significantly more often amongst those with low MAO activity.
Attention Deficit and Hyperactivity Disorder

One of the earliest studies in this field is the one from Shekim et al. (1982), who found that levels of MAO activity were reduced in 8 attention deficit and hyperactivity disorder children in comparison to control children. Interestingly, the differences in MAO activity disappeared after two weeks of treatment with d-amphetamine. In a more sophisticated study, Stoff et al. (1989) found that high MAO activity is associated with laboratory measurements of impulsivity such as a continuous performance task, a delay task similar to differential reinforcement of low rate responding, and a matching familiar figures test (Kagan et al., 1964). Children with high levels of MAO activity, from the original population of 32 showing signs of behavioural disorder, made more commission errors, more delay errors, and had a faster matching familiar figures test tempo of responding. A canonical correlation of these three factors rendered an $r = 0.53$ in discriminating low vs. high MAO activity 73% of the time. Shekim et al. (1986) studied 22 boys diagnosed with attention deficit and hyperactivity disorder in relationship to MAO activity and their performance in a neuropsychological battery similar to that detailed above. MAO activity was low in attention deficit and hyperactivity disorder children and was associated with impaired performance in the matching familiar figures test and the continuous performance task in comparison with non-attention deficit and hyperactivity disorder children.

Bipolar Affective Disorder

Murphy and Weiss (1972) studied MAO level of activity in a group of 23 bipolar patients, 34 monopolar depressed patients and 52 normal subjects. Those subjects suffering bipolar affective disorder
showed 45% lower MAO activity in comparison to the other two groups similar in age and gender (Murphy & Weiss, 1972; Murphy et al., 1974). Other studies have found no difference between monopolar and bipolar MAO activity in platelets (reviewed in Oreland, 1979).

**Schizophrenia**

Murphy et al. (1974) studied a group of 33 individuals to test if MAO activity was any different in schizophrenics from that in control and bipolar patients. They found that, indeed, levels of enzymatic activity were markedly reduced in chronic schizophrenics as well as in patients with bipolar depression or acute psychosis in comparison to unaffected individuals or those with unipolar depression. Knowing that twin studies have proven that MAO activity is under genetic control they explored whether that influence would prevail in the presence of schizophrenia, or if this disorder would have any influence on enzymatic activity. They looked at MAO activity in discordant schizophrenic twins and found that low levels of MAO were present not only in the affected twins, but also in the unaffected co-twins, confirming the presence of genetic control upon MAO activity, and that schizophrenia has no influence in MAO activity. Finally, Murphy et al. postulated that MAO activity acts as a genetic predisposition or vulnerability factor towards some psychiatric disorders.

**Suicide**

As reviewed by Buschbaum et al. (1977), low levels of MAO activity were reported by Gottfries et al. (1974 and 1975), Sourkes (1976) and Buschbaum et al. (1976) in individuals who committed suicide. However, Grote et al. (1974) found a negative association in a similar
analysis. In other study, Buschbaum et al. (1976) analyzed whether average evoked potentials and MAO activity would be better predictors of suicide when used together. As previously explained, average evoked potentials response depends on the electroencephalographic responses of subjects presented with a stimulus. The amplitude of the response could increase (augment) or decrease (reduce) as the stimuli increases in intensity (Buschbaum et al., 1976). Out of the 79 psychiatric patients, "augmenters" with low MAO had an increased incidence of suicidal attempts in comparison with those with high MAO activity or those with low MAO activity/non-augmenters. Amongst normal subjects (n = 68) low MAO activity and family history of suicide had a high correlation with augmenting in the average evoked potentials of the electroencephalogram (Buschbaum et al., 1977).

**Polymorphic variants of MAO**

In contrast to the very rare mutations described in Chapter II, there is a large number of different genetic variations that occur much more frequently in both normal and clinical populations. Although there are other possibilities, in general these changes are of three main types: a) those in which a nucleotide is changed for another nucleotide [single nucleotide polymorphisms or point mutations]; b) those in which a small number of nucleotides (2, 3, etc.) are deleted or duplicated; c) those in which there is a variable number of tandem repeats. Some single nucleotide polymorphisms may result in a change in a restriction enzyme cutting site and thus will also be known as restriction fragment length polymorphisms. Often these polymorphisms occur in non-coding regions of the gene or fail to change protein sequence. Still, some of these polymorphisms do cause a change in protein structure or function and even more of them have
an effect on the transcriptional or translational efficiency of the gene and on the quantity of functional protein available.

An increasing number of such polymorphisms are now known in the MAO A and B genes. In an early study, using an "exon-scanning" technique, Hsu et al. (1995) reported the presence of 5 different point mutations in MAO A exons, in a population of 11 cell lines of fibroblasts. Four of them were silent while the one that changed a Lys for an Arg did not have any repercussions on MAO A activity. Tivol et al. (1996) identified the same point mutations with the use of Reverse Transcriptase Polymerase Chain Reaction, single strand conformational polymorphism and DNA sequencing in 40 normal subjects. Measurements of MAO activity failed to be correlated to the presence of the different polymorphic variants found in this population (Tivol et al., 1996).

Polymorphic variants and MAO activity

Although the study of Tivol et al. (1996) failed to identify an association between 5 polymorphic MAO A markers and MAO activity measured in skin fibroblasts, other studies have been able to demonstrate an association between the different alleles of a polymorphism and enzymatic activity. One such example involves polymorphisms generated by three restriction fragment length polymorphism sites (Fnu4HI, EcoRV and MspI), each of which corresponds to a specific point mutation in the MAO A gene. Out of the eight possible haplotypes only three were found in the population under study. These three haplotypes showed a statistically significant association with the levels of enzymatic activity (MAO A) measured in cultured fibroblasts (Hotamisligil & Breakefield, 1991; Ozelius et al., 1989; Hsu et al., 1995). More recently Denney et al. (1999) studying a
group of normal subjects found that MAO A activity from fibroblasts is directly correlated to the length of the U-variable number of tandem repeats located in the promoter region of the MAO A gene described by Sabol et al. (1998). This functional polymorphism is located in position -1142 to -1262 and comprises a fragment 30 bp long with a core sequence ACC(A/G/C)G(C/T) that repeats itself 6 times inside this fragment. The fragment may appear two to 5 times, the most frequent alleles being those with 3 and 4 repeats. When constructs containing promoters with the polymorphic marker were fused with a luciferase coding sequence and were transfected into human neuroblastoma cells, those cell lines containing alleles with 3.5 or 4 repeats showed the highest expression of the reporter gene. Comparatively, alleles with 2, 3 and 5 repeats had the lowest activity. The ratio of transcription ranged from 9.6 for alleles 3.5/4, to 2.4 for alleles 3.5/3. Ratios for the most popular alleles 4/3 were equal to 4.8. This polymorphism was also found to be in strong linkage disequilibrium with the MAO A CAn polymorphism described by Black et al. (Black et al., 1991). Also related to this marker's size is the proportion of cells expressing MAO A activity in each tissue as studied in cultures of fibroblasts taken from 15 males ages < 1 to 90 years old. The mean specific activity was calculated by dividing the culture enzymatic activity by the number of immunopositive cells. The smallest mean specific activities were registered in the 3 repeat allele cells (7.2±3.1 nMol/h/mg prot.) while those with 3.5 had the highest mean specific activity (23.9±9.5 nMol/h/mg protein) (Denney et al., 1999).

Using a different approach Jonsson et al. (2000) reported no correlation between the U-variable number of tandem repeats promoter polymorphism (Sabol et al., 1998) and the concentration of monoamine metabolites in cerebrospinal fluid of 37 healthy males. In
contrast, 51 healthy women had higher levels of homovanillic acid and 5-hydroxyindoleacetic acid if at least one of the "high transcription" alleles of this marker (3.5 and 4 repeats) was present. When women were genotypically grouped in high (3.5/4 and 4/4), low (2/3 and 3/3) and combined genotypes (3/3.5 and 3/4) with regards their transcriptional capacity, the same differences persisted achieving again statistical significance (Jonsson et al., 2000).

Another polymorphic marker was described by Hinds et al. based in a variable number of tandem repeats that occurs in the first intron of the MAO A gene (Hinds et al., 1992). The authors reported no attempt to associate this marker with activity or any specific pathological entity, but nonetheless considered it to be a highly informative marker for genetic studies, particularly in comparison to the restriction fragment length polymorphism markers from the study by Hotamilsigil et al. (1991). This is due to the fact that the variable number of tandem repeats is much more polymorphic (15 alleles) than the restriction fragment length polymorphism, commonly reporting 3 alleles, which are in strong linkage disequilibrium with one another, limiting even more the potential for variation in populations.

In the case of MAO B the first polymorphic markers were described in 1992 (Grimsby et al., 1992; Konradi et al., 1992). They are located in the second intron of the gene and are constituted by two dinucleotide repeats. A GT repeat produced fragments that span from 168 to 198 bp in length and has 12 alleles, while a TG repeat produced fragments that go from 195 to 213 bp in length with 9 alleles. Girmen et al. (1992) failed to correlate the presence of different alleles of the two markers with platelet MAO activity. A linkage analysis was used by Saccone et al. (1999) in trying to find a connection between MAO B activity and gene loci in a genome-wide
screening. Two hundred and ninety one markers were used in an analysis of 148 nuclear families with a total of 1008 non-independent sibpairs. Two point- and multipoint linkage analysis rendered modest positive lod scores for two markers in chromosome 2 and one in chromosome 6. Surprisingly, lod-scores were non-significant in the X chromosome (Saccone et al., 1999).

Mejia et al. (submitted) in a follow-up study of 100 teenagers (introduced in the following chapter), found no association between markers MAO B 1.1/1.2 (Grimsby et al., 1992), MAO B gtct/gtgt (Konradi et al., 1992) and two other MAO A markers, with platelet MAO activity using tryptamine as substrate.

**Association of specific polymorphisms with pathological conditions.**

**Panic and anxiety disorders**

Due to the functional relevance of the polymorphic marker described by Sabol et al. (1998), Deckert et al. (1999) studied the association of the different alleles with the occurrence of panic disorder in two independent populations. Alleles with 3 and 4 repeats were the most prevalent in the whole population. However, the female panic disordered population showed a higher proportion of long alleles (3.5, 4 and 5 repeats), compared to the frequency of short ones (2 and 3 repeats). This difference was not observed amongst males of the same sample. Hamilton et al. (2000) followed up on this model and applied a family based design to look for linkage or association between panic disorder and the same polymorphism (Sabol et al., 1998). They studied 620 subjects in 70 multiplex families and 81 triads. No association was found between allele frequencies and the
population of study; linkage was not demonstrated either (Hamilton et al., 2000).

Finally, Jorm et al. (2000) failed to demonstrate any association between the MAO A V-variable number of tandem repeats (Sabol et al., 1998) polymorphism with depression and anxiety, or with personality traits (neuroticism, behavioural inhibition, negative affect and psychoticism) that they considered might predispose an individual to the said disorders.

**Affective disorders**

In the field of affective disorders, Ho et al. (2000) studied several markers in different candidate genes: (serotonin transporter (Ogilvie et al., 1995), dopamine receptor D2 (Furlong et al., 1998), tyrosine hydroxylase (Turecki et al. 1997), tryptophan hydroxylase (Nielsen et al., 1994) and MAO A CAn (Black et al., 1991), Fnu4HI (Hotamisligil & Breakefield, 1991) and U-variable number of tandem repeats (Sabol et al., 1998)). The populations studied suffered monopolar depression (n=131) or bipolar affective disorder (n=139). The MAO A marker U-variable number of tandem repeats showed a weak association with suicidal behaviour in the bipolar population. This association became stronger when the bipolar group included only females. The same subgroup (bipolar females) showed also a weak correlation with the MAO A Fnu4HI marker. In both cases alleles related to lower MAO A activity were more prevalent amongst the subjects with higher incidence of suicidal behaviour.

Schulze et al. (2000) reported that women with recurrent depressive episodes have longer alleles in the MAO A promoter polymorphism (3.5, 4 and 5 repeats) in comparison to control men and women and to populations with a single episode of depression.
Given that such alleles are associated with increased levels of transcription, the authors suggested that high MAO A enzymatic activity might predispose to depression in women.

In a population of 106 bipolar and 125 unipolar individuals, Furlong et al., (1999) explored the associations between affective disorder and the *Fnu4HI* restriction site (Hotamilsigil & Breakefield, 1991), the MAOA-CAn polymorphism (Black et al., 1991), and MAOA-pro-variable number of tandem repeats (similar to that described by Sabol et al., 1998). A meta-analysis was also performed to look for associations that may have been missed in previous studies due to the small size of the populations. Individual marker analyses rendered no significant associations between the alleles of each marker and the presence/absence of affective disorder. The meta-analysis showed a significant association between alleles 116 vs. 122 and 114 vs. 122 of the marker described by Black et al. (1991) in both ethnic and gender combined subgroups. *Fnu4HI* marker displayed significant associations only in the female group. In this study, the MAOA-pro-variable number of tandem repeats was found to extend from 72 to 162 bp in length with imperfect repeats 12 or 30 bp in size, formed by a basic ACC(G/A/C)G(C/T) pattern that was repeated several times in each fragment. Alleles 132 and 102 accounted for 98% of the variability amongst the control group and no associations were found with affective disorders. Interestingly the haplotype 132-114-1 for markers MAOA-pro-variable number of tandem repeats, MAOA-CA and *Fnu4HI*, was found in 54.9% of male controls. In conclusion it is possible that these markers contribute to the expression of the MAO A gene. An alternative explanation is that they are in linkage disequilibrium with other genes more relevant to MAO function. Whatever the case, their effect on enzymatic activity remains to be defined.
Rubinsztein et al. (1996) compared a group of unrelated males (n=67) and females (n=113) affected with bipolar disorder to a control group matched by gender. The markers used were again the \textit{Fnu4HI} restriction site (Hotamilsigil & Breakefield, 1991) and the MAOA CAn (Black et al., 1991). This time, significant associations occurred between MAOA CAn and the population under study but not with the \textit{Fnu4HI} polymorphism. When the data were combined with that available from two previous studies in the United Kingdom, significant associations were found for both markers.

Probably the most recent report using the U-variable number of tandem repeats described by Sabol is that of Syagailo et al. (2001) which examined associations between the different marker alleles (2, 3, 3.5, 4 and 5 repeats) and the occurrence of schizophrenia and affective disorders. No association was found in any of the analyses for the complete or for stratified populations.

Another study involving three markers, two for MAO B and one for MAO A (Konradi et al., 1992; Grimsby et al., 1992; Black et al., 1991), found no association when bipolar or unipolar populations were compared as a whole to controls. Nevertheless, when stratified by gender, female bipolar and unipolar patients showed strong associations with a few alleles of each marker. Relative risk calculations confirmed the presence of one allele per marker significantly associated with the presence of bipolar or unipolar affective disorder in female patients (Lin, 2000).

Craddock et al. (1995) and Muramatsu et al. (1997) found no association between bipolar disorder and the 23 bp variable number of tandem repeats in intron 1, the GTn repeat in intron 2 and the \textit{Fnu4HI}-restriction fragment length polymorphism in exon 8. Despite this, Lim et al. (1995) and Parsian and Todd (1997) found an association with the GTn marker.
<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Phenotype</th>
<th>Associations</th>
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</tr>
</thead>
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<td>and drug abuse</td>
<td></td>
<td>Craddock et al., 1995; Lim et al., 1995; Muramatsu et al., 1997</td>
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<td></td>
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<td>(GT)n repeat intron 2</td>
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<td></td>
<td></td>
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<td>Alcoholism</td>
<td>Yes</td>
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<td>Aggression</td>
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<td></td>
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<td>EcoRV restriction fragment length</td>
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<td></td>
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Table 1 continued

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<tbody>
<tr>
<td>Promoter variable number of tandem repeats Sabol et al., 1998</td>
<td>Panic disorder</td>
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<td></td>
<td>Panic disorder</td>
<td>No</td>
<td>Hamilton et al., 2000</td>
</tr>
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<td></td>
<td>Antisocial personality disorder Healthy volunteers monoamine metabolites Symptoms depression or anxiety, personality disorder traits Impulsive aggression Antisocial alcoholism Major depressive disorder</td>
<td>No</td>
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</tr>
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<td></td>
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<td></td>
<td></td>
<td>Yes</td>
<td>Manuck et al., 2000</td>
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<td></td>
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<td>Yes</td>
<td>Samochowiec et al., 1999</td>
</tr>
<tr>
<td></td>
<td></td>
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Table 2. Polymorphic variants in the MAO B gene

<table>
<thead>
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<th>Associations</th>
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<td>(TG)n repeat intron 2 Grimsby et al. 1992</td>
<td>Bipolar disorder</td>
<td>No</td>
<td>Muramatsu et al., 1997</td>
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<td></td>
<td></td>
<td>Yes</td>
<td>Lin et al., 2000</td>
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<tr>
<td>(GT)n repeat intron 2 Konradi et al. 1992</td>
<td>Bipolar disorder</td>
<td>Yes</td>
<td>Lin et al., 2000</td>
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<td></td>
<td></td>
<td>No</td>
<td>Parsian and Todd, 1997</td>
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<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>Garpenstrand et al., 2000</td>
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</table>
Alcoholism, with or without antisocial behaviour

Following up on previous information about low levels of MAO activity in association with type II alcoholism, Parsian (1999) studied mutations in exons 8 and 14 previously described by Hotamisligil and Breakefield in relationship to MAO A activity (Hotamisligil & Breakefield, 1991). These mutations create restriction sites for Fnu4HI and EcoRV respectively. The mutated allele in exon 14 is significantly more frequent in type II alcoholics, but the one in exon 8 is not. Both mutations exist in strong linkage disequilibrium with the polymorphic marker MAO A CAn described by Black et al. (1991). When the three markers are combined, two of the resulting haplotypes (F1E1C6 and F1C6) are significantly more frequent amongst Type II alcoholics in comparison to controls or Type I alcoholics. Taking into account this association and its relationship to MAO A activity, Parsian suggested that such haplotypes could be considered as predictors of low MAO A activity in Type II alcoholics. Previously, Vanyukov et al. (1995a and b) published two reports about the relationship between the MAO CAn marker (Black et al., 1991), early onset alcoholism, and measurements of aggressive behaviour. Long alleles (> 115 bp) were found to be associated with early onset alcoholism in males but not in females (Vanyukov et al., 1995a). Nonetheless no significant association was found with respect to aggressive behaviour (Vanyukov et al., 1995b).

Samochowiec et al. (1999) used the U-variable number of tandem repeats promoter polymorphism (Sabol et al., 1998) to study a population of antisocial alcoholics, non-antisocial alcoholics and controls. The group of antisocial alcoholics showed the 3 repeat allele, which translates into low transcriptional activity, significantly more often than controls and non-antisocial alcoholics, suggesting again that low MAO activity may be associated with antisocial/violent
behaviour. Mannuck et al. (2000) studied a community sample of 110 men in search of associations between the said polymorphic markers and impulsive/aggressive behaviour (assessed with the Life History of Aggression interview, the Barrat Impulsiveness Scale and the Buss-Durke Hostility Inventory) and a fenfluramine challenge. Subjects with 3 or 5 variable number of tandem repeats in the promoter polymorphism (lower transcriptional activity) scored slightly lower in dispositional aggressiveness and impulsivity, and higher in log-peak fenfluramine induced prolactin secretion, compared with those showing 3.5 and 4 repeat variable number of tandem repeats (higher transcriptional activity).

In contradiction to these positive findings, three groups have reported no association between MAO A polymorphisms and antisocial personality disorder or alcoholism. These studies included the U-variable number of tandem repeats promoter polymorphism (Sabol, 1998) and the EcoRV restriction fragment length polymorphism (Hotamisligil et al., 1991; Hsu et al., 1996; Lu et al., 1999).

**MAO B polymorphic markers and neurological disorders**

A fewer number of studies include or focus on MAO B markers. A linkage analysis was conducted on 40 CEPH (Centre D'Etude du Polymorphisme Humain) reference families using the EcoRV polymorphism from Hotamisligil's study (Hotamisligil & Breakefield, 1991) and the MAO B polymorphic marker described by Kurth et al. in order to deduce the approximate distance between MAO genes (Kurth et al., 1993). The result suggested that both genes are tightly linked as the linkage analysis showed that the region between the two of them measures only 2.7 centimorgans (Harris et al., 1993).
An association analysis approach for the correlation of specific alleles of multiple markers in both genes with the incidence of narcolepsy rendered a weak association between a dinucleotide repeat in each gene and the presence of the sleep disorder (Koch et al., 1999).

A polymorphic variant was identified by single strand conformational polymorphism analysis of a digested product that included intron 13 of the MAO B after DNA from patients affected by Parkinson's disease was digested with *Hae* III. Allele 1 was more frequent in Parkinson's disease patients than in controls (62% and 45% respectively) (Kurth et al., 1993; Shih et al., 1999). In a subsequent publication, Costa et al. (1997) specified that the polymorphic difference was related to a single base substitution (A/G), the A allele being the one most frequently found amongst the Parkinson's patients in previous studies. They, however, found that the G allele was more frequently found in the Parkinson group, counterbalancing the previously suggested association. Three other studies, one confirming and the others denying these findings, later added information to this matter (Hotamisligil et al., 1994; Nanko et al., 1996; Shih et al., 1999).

Unfortunately, most studies focus on a limited aspect of this complicated series of mechanisms. In our view, more integrative approaches should be taken if the understanding of MAO influences in brain function are ever to be understood. For this reason we have evaluated the enzymatic activity of MAO, in conjunction with a series of genetic markers at this locus. Our results demonstrated that two polymorphic markers in MAO B are associated with platelet enzymatic activity, and that one of these markers is also associated to pervasive aggressive behaviour. However, no correlation could be established
between activity and behaviour, hampering the possibilities of establishing a model that depicts how genes cause behaviour.

As seen in this chapter, it is clear that there is overwhelming evidence in favour of an association between low levels of MAO activity and a great variety of psychiatric disorders. Some of these diseases involve behavioural traits such as aggression and impulsivity that may be the elements in the disorder that are directly influenced by MAO enzymatic activity. As explored in detail in the preceding sections, findings with polymorphic markers in the MAO genes have also established a solid correlation with a great variety of mental ailments, which in the end corroborates the hypothesis about MAO activity being in the etiological framework of mental disorders. Despite all the available evidence, not enough attempts have been made to bridge the biochemical, genetic and behavioural factors that may be intimately involved in the etiopathogenic mechanisms of various neuropsychiatric disturbances. In the following chapter, we introduce a study where the objective was to explore the connections between aggressive behaviour, gene markers and enzymatic activity. This was done under the hypothesis that the physiopathogenic mechanisms manifested as impulsive-aggressive behaviour must involve variations in gene expression that influence the biochemical processes and determine or regulate the various functions of neurotransmitters.
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CHAPTER V

NO ASSOCIATION BETWEEN PLATELET MONOAMINE OXIDASE ACTIVITY AND MAO B ALLELES IN A POPULATION OF FRENCH-CANADIAN MALES
Contribution by authors:

**Mejia JM:** Co-designed study, executed biochemical experiments, analyzed results and prepared report.

**Tremblay RE:** Supervised design and collaborated in the preparation of the report.

**Nagin D:** Collaborated in the analysis and preparation of the report.

**Palmour RM:** Co-designed study, supervised biochemical experiments, analyzed results and prepared the report.
No association between platelet monoamine oxidase activity and MAO B alleles in a population of French-Canadian males

Jose M Mejia¹, Richard E Tremblay², Daniel Nagin³, Roberta M Palmour¹

¹Departments of Biology, Psychiatry and Human Genetics, McGill University, Montréal (Québec) CANADA, ²Departments of Psychology and Psychiatry, Université de Montréal, Montréal (Québec) CANADA; ³Department of Biostatistics, Carnegie Mellon University, Pittsburgh PA

Running title: Platelet MAO activity and MAO B alleles

Address for correspondence: Roberta M. Palmour, Ph.D. Department of Psychiatry McGill University 1033 Pine Avenue West Montréal (Québec) H3A 1A1 CANADA

Tel: 514-398-7303 FAX: 514-398-4370 e-mail: mc23@musica.mcgill.ca
ABSTRACT

Monoamine oxidase, one of the two principal degradative enzymes for catecholamine and indoleamine neurotransmitters, has long been implicated as a candidate gene in neuropsychiatric disorders. Despite ample evidence of genetic contributions to levels of platelet MAO activity, it has been difficult to identify specific markers that might subserve this variation. We report here the lack of a positive association between the maximal velocity (Vmax) or Km of platelet MAO enzymatic activity and two intronic microsatellite polymorphisms (MAO B1.1. and MAO Bgtgt) of the MAO B gene. We also found no association between any measure of enzymatic activity and exonic microsatellite polymorphisms of the MAO A gene.

Similarly there was no association between enzymatic activity and aggressive behaviour or between genotypes and behaviour, nor were we able to identify an interaction between genotype, enzymatic activity and behaviour. Given the large number of polymorphic variants at each locus, in future studies it will be important to apply a similar model to a larger population of subjects.

KEYWORDS

Monoamine oxidase, enzymatic activity, polymorphic markers, aggressive behaviour
INTRODUCTION

MAO, the principal degradative enzyme of amine neurotransmitters, has long been a target of investigation in neuropsychiatry. In both clinical samples and in normal populations (Murphy et al. 1974; Buchsbaum et al. 1977; Buchsbaum et al. 1984; Shekim et al. 1985; Ward et al. 1987; Yehuda et al. 1989; Weyler et al. 1990; Devor et al. 1993; Oreland and Hallman, 1995; Chen and Shih, 1998; Farren et al. 1998; Murphy et al. 1998; Samochowiec et al. 1999; Shih et al. 1999b), low activity of platelet MAO has been associated with increased vulnerability to suicide, drug abuse, legal difficulties and impulsive aggression. The most dramatic example of this relationship was reported in 1993 by Brunner and colleagues (Brunner et al. 1993a,b). In a highly atypical Dutch family, a pattern of pathological impulsive aggressive behaviour was linked to the region of the X-chromosome bearing the structural genes for MAO A and MAO B. Affected males were subsequently found to carry a point mutation in exon 8 of the MAO A gene and to be severely deficient in fibroblast MAO A activity.

MAO A and MAO B are highly homologous but show distinct patterns of tissue expression and some specificity with respect to substrate affinities (Weyler et al. 1990). Both forms of the enzyme reside in the external mitochondrial matrix in the presynaptic terminals and cell bodies (Abell & Kwan, 2001). Platelet MAO (mainly type B) displays a broad range of enzymatic activity in normal populations (Murphy et al. 1976; Hotamisligil and Breakefield, 1991). It is known that many environmental factors, such as alcohol consumption (Parsian et al. 1995), tobacco smoking (Fowler et al. 1996 a,b; Oreland et al. 1999), menstrual cycles (Baron et al. 1980) hormonal changes and age (Youdim and Holzbauer, 1976; Weyler et al. 1990) influence enzymatic activity. However, there is also robust
data indicating that MAO activity is highly heritable (Nies et al. 1973; Breakefield and Edelstein, 1980; Pandey et al. 1981; Friedl et al. 1979; Oxenstierna et al. 1986). The latest finding have allowed researchers to assume that platelet MAO activity is a good indicator of brain MAO activity. In spite of this, Young et al., (1986) reported no correlation between brain and platelet MAO in a study of 14 epileptic subjects. MAO activity was measured in temporal and frontal cortical brain samples obtained during neurosurgical procedures. Platelet and brain samples were stored frozen for 131 and 180 days, respectively on average. In contrast (Bench et al., 1991) a study of the dose range of Ro 19-6327 for the induction of MAO B inhibition using positron emission tomography, demonstrated a strong correlation (r=0.949) of positron emission tomography measuring gradual inhibition of brain MAO activity with that of platelet MAO in eight normal subjects. Finally, Da Prada et al., (1988) reviewed the literature about this issue and concluded that the pharmacological similarities between platelets and serotonin containing neurons indicate that platelets are a reliable and accessible indicator of MAO B activity in the central nervous system.

Unfortunately, the search for polymorphic genetic markers that might subserve genetic contributions to enzymatic activity has not been particularly productive. Only a few population-based studies relate enzymatic activity to polymorphic markers in genes encoding MAO -A or -B (Hotamilsigil and Breakefield 1991; Girmen et al. 1992; Hsu et al. 1995; Sabol et al. 1998; Schuback et al. 1999), and the reported associations are inadequate to explain the genetic component of variance (Shuback et al. 1999). In contrast, robust associations with behaviour have been found in relation to genetic or chromosomal abnormalities that completely obliterate enzymatic activity (Shih, 1991; Collins et al. 1992; Brunner et al. 1993a,b; Hsu
et al. 1995; Lenders et al. 1998; Shih et al. 1999a,b,c). Studies in transgenic animal models also underscore the behavioural implications of obliterating MAO activity (Cases et al. 1995; Grimsby et al. 1997; Lenders et al. 1998; Shih et al. 1999c).

The present study addresses two questions:

Might there be an association between platelet MAO activity and MAO A or B alleles?

Might there be an association between these genotypes and/or platelet MAO activity with aggressive behaviour in a population sample characterized longitudinally?

To answer these questions we studied platelet MAO (B) enzymatic activity, measured in a biochemically rigorous fashion, and four polymorphic markers in the untranslated portion of the MAO genes. The possibility that there might be an interaction between genetic markers, biochemical status and behavioural characteristics was also studied.

SUBJECTS

Subjects in this study were drawn from an ongoing longitudinal cohort of Caucasian males recruited in 1984 at the age of 6 (Tremblay et al., 1991). All boys attending kindergarten that year in a working-class Montréal catchment area of 53 Catholic schools, were invited to participate. The original sample included 1161 boys of whom 1037 met inclusion criteria.

To be included in the study it was only required to be French-speaking and to have parents born in Canada whose mother tongue was also French. Each boy was followed from age 6 to 16 with evaluations of personality, school performance, cognitive function,
and psychosocial adaptation. Parent and teacher ratings were collected. A number of reports detail demographic, psychosocial and other characteristics of this epidemiological cohort (reviewed in Nagin and Tremblay, 1999).

**Construction of a sample for laboratory studies:** In 1991 a sample of 200 subjects was selected for laboratory investigation. The average age of the participants was 13 years. On the basis of the Social Behaviour Questionnaire (SBQ), fighting subscale scores at ages 6, 10, 11 and 12, boys were grouped as follows: a) those whose physical aggression score was always below the 70th percentile (19%); b) those who scored above the 70th percentile once or twice (46%), and c) those who scored above the 70th percentile at least 3 times (35%). The laboratory sample was weighted to include equal representation from each aggression group, but individual subjects from each group were selected at random (Tremblay et al., 1991).

A wide range of laboratory evaluations, including psychosocial, psychological and other diagnostic measures (neurophysiological and neuropsychological tests), and more recently endocrine and neurochemical challenges have been reported on this cohort. At age 16, 189 boys were invited to contribute blood for studies of enzymatic activity and genetic polymorphisms. Platelets and DNA were available from 103 of the 109 who provided informed consent for this study.

**Statistical analysis of behavioural phenotypes:** Of particular importance to the present paper is the teacher's SBQ (Table I), which was used to assess physical aggression, opposition and hyperactivity on an annual basis from ages 10 till 15. In a recent paper Nagin and Tremblay (1999), have analyzed the longitudinal trajectories of these three factors [which correspond well to externalizing behaviours (Achenbach and Edelbrock, 1991)], and derived models that best fit the group data. In the present report, we utilize their physical
aggression trajectory to explore possible hypothesized relationships between MAO activity or genotypes and physical expressions of aggressive behaviour.

Four specific trajectories (Nagin and Tremblay, 1999) were identified in a sub-sample of 893 boys for whom SBQ data were available at age 6, and each year between the ages of 10 and 15:

a) Non-aggressive: between 15 and 25% of the sample never showed significant physical aggression.

b) Low-level desisters: Nearly half of the sample, exhibited physical aggression at age 6, but desisted by age 10 or 12.

c) High-level near-desisters: A third group (25-30% of the population) included subjects with very high levels of physical aggression at age 6 but substantially reduced levels of fighting by age 15.

d) The fourth group (approximately 5% of the population) showed high levels of physical aggression with no decrease over time.

Under Nagin's classification scheme, 15% of the subjects who provided platelets and DNA for the present study never showed physical aggression, 44% were low-level desisters, 36% were classified high-level near-desisters and 5% showed chronic physical aggressive behaviour. This distribution resembles very closely that of the original sample, such that this sample is representative of the total population with respect to aggressive behaviour.

METHODS

Genotyping. DNA was extracted from whole blood by the phenol-chloroform technique. For each marker, appropriate regions of
the genome were amplified by PCR with the use of specific sets of primers and annealing temperatures as shown in Table II. All reactions were performed with 200 mM of dCTP, dTTP, dGTP and 100 mM of dATP plus 100 mM of $^{35}$S dATP; 500 ng of each primer, 1.5 U taq polymerase; and 1x taq polymerase buffer [50mM KCl, 10 mM tris-HCl (pH 9), 1mM MgCl$_2$, 1% Triton X-100]. Each reaction comprised 30 cycles of 30 sec at 96 °C, 30 sec at the annealing temperature defined in Table II, and 30 sec at 72 °C. The final cycle was followed by a 5 min extension period at 72 °C. Genotypes were determined by autoradiography after separation of alleles by gel electrophoresis in 5% denaturing polyacrylamide. Allele size was determined through reference to a sequencing ladder of known size.

**Measurement of enzymatic activity:** Activity of platelet MAO was quantified using a modification of the original technique of Axelrod and Wurtman (Wurtman and Axelrod, 1963). Platelets were prepared from each blood sample by differential centrifugation, and stored in 0.3 M sucrose at -70 °C until assay. Platelet concentrates (50 µl aliquots) were diluted 1:5 in 20 mM sodium phosphate buffer, pH 7.4. The mean protein concentration of these preparations was 14.9 ± 3.8 mg/ml. From this diluted solution, 10 µl aliquots were incubated for 15 minutes with each of five graded concentrations (125, 62.5, 20, 9 and 6µM) of $^3$H-tryptamine (New England Nuclear, 28.5 - 33 Ci/mMol), in triplicate. Reactions were stopped by adding 5 µl of 1N formic acid and by placing the samples on ice.

The product of the reaction [indoleacetic acid (IAA)] was separated from the unreacted substrate by thin layer chromatography (TLC) in a solution of n-amyl alcohol, glacial acetic acid and water (2.4 : 0.6 : 1), at room temperature. Cellulose TLC plates with fluorescent indicator
(Kodak, Rochester NY) were spotted with 5 μls of sample. IAA and tryptamine carriers (5 mg/ml. each) were included to visualize the areas of interest for ulterior analysis. After chromatography, the product and remaining substrate were observed under UV light. The regions in the plates containing product and substrate were excised, and each piece was counted by liquid scintillation (ICN Betamax scintillation fluid) in an LKB Wallac Rackbeta Counter, Model 1211.

The maximum velocity of reaction (Vmax) and substrate affinity (Km) were calculated by non-linear regression analysis using a single site (i.e. hyperbolic) model (Prism version 3; GraphPad Software Inc. San Diego, Cal.) in which substrate concentration is plotted against the metabolic product (indoleacetic acid, expressed as pMol/mg protein/minute).

**Statistical analysis:** Relationships between genotype, kinetic variables, and behavioural measures were examined by ANOVA.
**RESULTS**

**MAO genotypes:** The distribution of the five microsatellite markers in this population (Figure 1) was not significantly different from that observed in other Caucasian populations, or in a reference sample of the French Canadian population (data not shown). All MAO A markers had pronounced bimodal distributions but this was not the case for MAO B markers.

**Platelet MAO activity and genotype:** Platelet MAO activity was found to have a broad spectrum of values (Figure 2), ranging from 1.5 to slightly over 470 pMoles IAA/mg protein/min. Km varied from 0.3 to 974 μM. There was no significant association between platelet MAO activity and alleles of the MAO A or MAO B gene markers (Table III).

**MAO genotypes and behaviour.** There was no significant relationship between a longitudinal measure of physical aggression (Nagin and Tremblay, 1999) and MAO A 1.1/1.2, MAO A 3-1/3-1 or MAO B 1.1/1.2, B 1.1/1.2 markers, and the high vs. low strata of Nagin’s physical aggression trajectories (Table IV).

**MAO activity and behaviour.** Neither Km nor Vmax were related to the trajectories of physical aggression (Vmax: $F_{3,89} = 0.38$, $p=0.77$; Km: $F_{3,89} = 0.28$, $p = 0.84$).
The objective of the present study was: (1) to evaluate the relationship between MAO genotype and biological activity of the platelet form of this enzyme, and (2) to explore the relationships between MAO genotype and/or platelet MAO activity and physical aggression in a community-based sample of boys studied longitudinally from age 6-16. Insofar as we know, the results reported here provide no evidence of a significant association between measures of enzymatic activity (Vmax and Km) and two microsatellite markers within each MAO gene. We also report the lack of significant association between a longitudinal measurement of physical aggression and alleles at MAO microsatellite markers.

As mentioned previously, MAO exists in two forms, A and B, which have different profiles of substrate specificity, different patterns of tissue expression and are encoded by different genes (Breakefield and Edelstein, 1980; Weyler et al., 1990; Shih, 1991). In platelets, the tissue most readily available from human subjects, MAO B is the predominant form (Weyler et al., 1990). It would thus be logical to expect a relationship between platelet MAO activity and MAO B alleles. Similarly, there is at least one report of association between microsatellite polymorphisms of the MAO A gene and enzymatic activity in cultured fibroblasts, a cell type that expresses MAO A almost exclusively (Hotamisligil and Breakefield, 1991).

Even though expected, these types of relationships have not been uniformly demonstrated. For example, Girmen et al. (1992) found no evidence of an association between platelet MAO activity and MAO B genotypes, contrasting with the positive reports of correlation between MAO A genotypes and MAO A activity (reviewed in Shuback et al., 1999). One important exception to these challenging observations is the exon 8 point mutation in the Dutch kindred reported by Brunner.
(Brunner et al. 1993a, b), and associated with a complete loss of MAO A activity.

Despite having a larger sample than other studies presented to date (nearly 100 subjects) and a rigorous multi-point assessment of enzymatic activity, the intuitive relationship between MAO activity and separate untranslated MAO markers escapes demonstration as previously reported by some other investigators (Sullivan et al., 1990; Anthenelli et al., 1998). In this study there was also a non-significant relationship between a longitudinal measure of aggressive behaviour and the genotyped MAO markers.

Some explanations to the absence of correlation between the three variables here studied point to an insufficient sample size to protect against Type II error. Also, assessment of aggressive behaviour in young persons is difficult, as such behaviours are both intrinsically subject to measurement error and even when measured accurately, they tend to be unstable. In the present study though, the use of repeated measures, a longitudinal design, and a trajectory approach to analysis may have provided some degree of protection against this measurement error (reviewed in Lyons et al., 1995; Nagin and Tremblay, 1999).

Some other important limitations must be considered in evaluating the present data. Despite the relative large size of the sample in relation to previous evaluations of MAO activity-genotype relationships, the present sample would still be considered small in the context of current genetic association studies. While this has little impact on the relationship between genotype and enzyme activity, it may adversely affect the robustness of any possible genotype-behaviour relationships. Indeed, a sample of 2000 or more individuals would be required in order to have sufficient power to evaluate allelic
contributions to the chronic aggressive phenotype that accounts for only about 5% of the total population.

Variation in ethnic background of the subjects within a sample tends to confound and sometimes preclude the establishment of relevant relationships amongst variables. In the present study, the collection of data from a specific ethnic population provided some protection against false positive associations resulting from population stratification. However, the 1991 decision (Tremblay et al., 1991) to assemble a laboratory sample in which externalizing disorders were oversampled might potentially lead to other types of bias. A positive aspect of this decision is that the final composition of the laboratory sample, with respect to proportions of each aggression trajectory, did not differ significantly from that observed in the epidemiological sample.

A final concern that has repeatedly surfaced in studies of MAO activity is the confounding influence of environmental factors such as smoking (Fowler et al., 1996a,b; Oreland et al., 1999) or alcohol consumption (Anthenelli et al., 1998; Palmour et al., in press), which epigenetically reduce the activity of platelet MAO. As the present cohort comprised only males of a specific age, there was no age- or gender-associated variability to be considered.

In summary, we present here additional evidence against an association between platelet MAO enzymatic activity and genetic polymorphic variants in the MAO genes. There was no association between enzymatic activity and aggressive behaviour, genotype and aggressive behaviour, nor were we able to identify an interaction among genotype, enzymatic activity, and behaviour.
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Table I: The Social Behaviour Questionnaire: Teacher Version. Bold items comprise the Fighting subscale; italic items, the Anxiety subscale.

<table>
<thead>
<tr>
<th>1. Fights with others</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Bullies and intimidates others</td>
</tr>
<tr>
<td>3. Kicks, bites and hits others</td>
</tr>
<tr>
<td>4. Is not liked by others</td>
</tr>
<tr>
<td>5. Irritable; gets carried away easily</td>
</tr>
<tr>
<td>6. Disobedient</td>
</tr>
<tr>
<td>7. Tells lies</td>
</tr>
<tr>
<td>8. Destroys the property or things of others</td>
</tr>
<tr>
<td>9. Will not share</td>
</tr>
<tr>
<td>10. Blames others</td>
</tr>
<tr>
<td>11. Has no regard or consideration for others</td>
</tr>
<tr>
<td>12. Attempts to stop quarrelling or fighting among others</td>
</tr>
<tr>
<td>13. Invites another who is alone to join his group and play</td>
</tr>
<tr>
<td>14. Tries to help another who is hurt</td>
</tr>
<tr>
<td>15. Spontaneously offers to look for something another has lost</td>
</tr>
<tr>
<td>16. Takes time to praise the work of others less able</td>
</tr>
<tr>
<td>17. Offers sympathy to another who has made a mistake</td>
</tr>
<tr>
<td>18. Offers to help another who has difficulty with a task</td>
</tr>
<tr>
<td>19. Helps another who is sick</td>
</tr>
<tr>
<td>20. Consoles another who is crying or upset</td>
</tr>
<tr>
<td>21. Helps to clean up a mess made by another</td>
</tr>
<tr>
<td>22. Worries; is worried about many things</td>
</tr>
<tr>
<td>23. Plays by himself in the corner; always alone</td>
</tr>
<tr>
<td>24. Always appears sad, unhappy, near to tears</td>
</tr>
<tr>
<td>25. Is frightened of new situations or things</td>
</tr>
<tr>
<td>26. Cries easily</td>
</tr>
<tr>
<td>27. Has little capacity for concentration; cannot attend to one thing for any length of time</td>
</tr>
<tr>
<td>28. Easily distracted</td>
</tr>
<tr>
<td>29. Quits easily</td>
</tr>
<tr>
<td>30. Is moony or daydreamy</td>
</tr>
<tr>
<td>31. Very active, always moving and jumping. Cannot stay in one place</td>
</tr>
<tr>
<td>32. Moves continually; squirms, cannot stay still</td>
</tr>
</tbody>
</table>
### TABLE II. Conditions for amplification of microsatellite markers at MAO A and MAO B loci

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primers</th>
<th>Sequence</th>
<th>Annealing temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAO A 1.1/1.2 (Black, 1991)</td>
<td>MAO A PCR1.1/1.2</td>
<td>5' aga gac tag aca agt tgc cac 3' 5' cac tat ctt gtt cga tca ct 3'</td>
<td>58°C</td>
</tr>
<tr>
<td>MAO A 3-1/3-2 (Hinds, 1992)</td>
<td>MAOA 3-1/3-3</td>
<td>5' ggt agg ctc ctt taa gaa aag gtt aaa a 3' 5' caa taa atg tcc tac acc tt 3'</td>
<td>57°C</td>
</tr>
<tr>
<td>MAO B 1.1/1.2 (Grimsby, 1992)</td>
<td>MAO B PCR1.1/1.2</td>
<td>5' ctt cac agc ctc tct ccc ag 3' 5' ctt cct ctt ctt ctc tct gtc 3'</td>
<td>58°C</td>
</tr>
<tr>
<td>MAO B GT-CT/GT-GT (Konradi, 1992)</td>
<td>MAOB GT-CT/GT-GT</td>
<td>5' gaa gca tcc aag tta gga gt 3' 5' att tgg cct cat aca gtt ag 3'</td>
<td>59°C</td>
</tr>
</tbody>
</table>
TABLE III: Relationships between polymorphic MAO markers and platelet MAO activity

<table>
<thead>
<tr>
<th>Marker</th>
<th>Vmax (pMol IAA/mg protein/min)</th>
<th>Km µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
</tr>
<tr>
<td>A 1.1/1.2</td>
<td>(7,85) 0.52</td>
<td>0.82</td>
</tr>
<tr>
<td>A 3-1/3-2</td>
<td>(7,85) 1.17</td>
<td>0.32</td>
</tr>
<tr>
<td>B 1.1/1.2</td>
<td>(8,84) 0.50</td>
<td>0.85</td>
</tr>
<tr>
<td>B gtgt/gtgc</td>
<td>(7,85) 0.67</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Platelet MAO activity was measured radiochemically with 5 concentrations of \(^3\)H-tryptamine as substrate, as described in the text. Kinetic constants were determined by a non-linear regression analysis.
TABLE IV. Relationship between MAO genotypes and physical aggression

<table>
<thead>
<tr>
<th>Markers</th>
<th>SBQ Teachers rated physical aggression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\chi^2$</td>
</tr>
<tr>
<td>A 1.1/1.2</td>
<td>20</td>
</tr>
<tr>
<td>A 3-1/3-2</td>
<td>12.29</td>
</tr>
<tr>
<td>B 1.1/1.2</td>
<td>26.28</td>
</tr>
<tr>
<td>B ggtg/gtcg</td>
<td>15.58</td>
</tr>
</tbody>
</table>

Aggression categories were determined by trajectory analysis of SBQ aggression scale scores, measured at ages 10, 13, 14, 15 and 16, as described by Nagin and Tremblay (1999).
FIGURE LEGENDS

Figure 1: Frequency distribution plots for MAO A (Panels A and B) and MAO B (Panels C and D) microsatellite markers in 103 healthy young males from a French Canadian epidemiological sample.

Figure 2: Frequency distribution plots of platelet MAO Vmax (Panel A) and Km (Panel B). Enzymatic activity was determined radiochemically as described in the text. Vmax is expressed as pMols/mg of protein/min, while Km is expressed as nM. Both Vmax and Km were normalized by natural logarithmic transformation prior to parametric analyses.
Figure 2
CHAPTER VI

MONOAMINE OXIDASE AND DEVELOPMENT
As introduced in chapter one, MAO has various mechanisms of regulation. Thus, during the life of all species that have MAO activity different levels of activity are expressed depending on the ontogenetic stage. Interestingly, this type of regulation seems to start even before birth, during brain development, and may exercise an important role in central nervous system development and function (Weyler et al., 1990). In their review of the biochemistry and genetics of MAO, Weyler et al. (1990) noted that MAO A appears in the embryos of human, rat and mice before MAO B does. After birth, MAO A increases its activity 1.5 to 2-fold following the caudal-rostral pattern of neuronal differentiation from brainstem to cortex (Tsang et al., 1986). Comparatively, MAO B appears shortly before birth and increases its activity 2 to 5-fold, reaching adult levels of enzymatic activity by the day of birth. Such an increment is thought to follow the proliferation of MAO B-producing astrocytes (Tsang et al., 1986). A somewhat different study in rat brain claims that activity for both forms of the enzyme continues to rise until 36 months of age. However, the proportion of growth is much more pronounced for MAO B than for MAO A, such that A:B ratios on the order of 1:4 or 1:3 are maintained (Rao et al., 1995).

These differences were noted as early as 1969 in a report by Shih and Eiduson. Through electrophoresis of MAOs on a 16 day old embryo, a 1 day old chick and an adult New Hampshire red hen, they revealed that the embryo's pattern of bands resembled that of the newborn. In the adult, a slow moving band had disappeared, but at least 5 remaining bands showing MAO reactivity were common to all stages. This suggested the presence of different forms of MAO even at those early stages of life (Shih & Eiduson, 1969 and 1971).

Strolin-Benedetti et al. (1992) reviewed studies of the development of MAOs in different tissues and found that the relative
concentrations of the two forms of the enzyme vary from one tissue to another. Studies focusing on brain tissue from rats and mice showed that MAO A reaches maximal activity by 2 to 4 weeks after birth; however, it is detectable by the first postnatal week (Horita, 1968). In brainstem and forebrain, MAO A activity was higher from 0-15 days of age than later in life. Contrary to this, MAO B increases its activity to reach adult levels by day 20 after birth (Bourgoin et al., 1977). After irreversible inhibition with pargyline, the rate of recovery is faster in the first week of life compared to the period of 8 to 16 weeks of age, at which time 4 weeks of recovery are required (Horita, 1968). Selective inhibition studies demonstrated that MAO B activity measured 6 days before birth was only 2.5 to 3% of the total brain MAO activity, but by day 25 it reached 32% of the total activity (Mantle et al., 1976). Studies in rat fetuses (Liu et al., 1987; Fiszman et al., 1991) indicated that MAO activity could be detected in embryos by day 13 and that such activity increases considerably by day 19 of development.

Postnatally, MAO A and B follow different trends. MAO A activity peaks at 15 days of age and is reduced in adult rats. Meanwhile MAO B, which represents only 30% of the activity on day 15, continues to increase and peaks by the 45th postnatal day (Jourdikian et al., 1975). Mice display a similar ontogenetic evolution of MAO. By 2 months of age, MAO A activity is stable and continues to be so for other 26 months, when it starts to decline slightly. MAO B reaches its peak in some regions not earlier than 16 months of age (2 months for most structures) and remains stable for the rest of the mouse's life (Koide & Kobayashi, 1984; Strolin Benedetti et al., 1991; Irwin et al., 1992). Binding studies demonstrated slightly different times for the same phases, though the trajectories over time are essentially the same. According to these techniques, the average decrease of 31% in MAO A binding starts between 4 to 9 weeks of age becoming stable
from 9 weeks to 19 months and finally increasing by 20-27% between 19 and 25 months. MAO B showed the same age related increase described before. The increase in binding is twice as big (54% on average), compared to that of MAO A and occurs between 4 weeks and 25 months of age (Saura et al., 1994).

In human brain, MAO A activity is very high at birth and decreases sharply during the first 2 years of life (Kornhuber et al., 1989). This parallels the decline of serotonin and catecholamine metabolites (Anderson et al., 1988). MAO B is low at birth and remains low during early childhood increasing steadily until an advanced age. It is possible that such changes follow the decline of neuronal density and the increase of synaptic density as MAO A is predominantly intracellular, while MAO B is mainly found in glia (O'Carroll et al., 1987; Westlund et al., 1988; Kornhuber et al., 1989).

While focusing on the ontogenetic changes of MAO one has to wonder about other elements of the embryo's environment that may influence the final developmental result. One element that comes to mind is the **blood-brain barrier** and its control of access to potentially damaging substances. Brain capillary endothelial cells constitute a barrier which chemically blocks the entrance of biologically active compounds, at least in part, by virtue of the cellular content of degradative enzymes. MAOs participate actively in this barrier by oxidating monoamine neurotransmitters coming from outside the central nervous system (Kalaria & Harik, 1987). An early experiment by Sessa and Perez (1975) showed that MAO activity in capillary endothelial cells decreases sharply in the first three weeks of postnatal life but peaks back to its original level ten days later. In a later study exploring the activity of MAO during ontogenetic development in the rat, Kalarial and Harik (1987) used various techniques to measure MAO activity and to distinguish the different
forms of the enzyme. With $^3$H-pargyline binding in the endothelial cells they found that the concentration of MAO increased six times during the first post-natal week and peaked to adult levels by the third week of age. Experiments using clorgyline and deprenyl demonstrated that the increase is predominantly of MAO B, with a MAO B/MAO A ratio that changed from 2 to 7 in the same period. Cortical increases during the same time are not as dramatic compared to those in microvessels, with ratios changing from 0.6 to 1.6 respectively. Enzymatic activity measured with benzylamine or 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine as substrate revealed a 20-fold increase in cerebral microvessels compared to a fivefold increase in cortical cells. This demonstrates that MAO activity, and particularly MAO B activity is extremely important to the functions of the blood-brain barrier.

Another element of this complex interaction is the mammalian placenta. It has been reported in several studies that placental tissues possess predominantly MAO A activity and almost no MAO B activity (Grimsby, et al., 1990; Abell & Kwan, 2001). Physiologically, it has been assumed that MAO protects the fetus by decreasing the amount of biogenic or bioactive amines that cross the placenta through catabolism (Gorkin, 1983; Abell & Kwan, 2001). It has also been suggested that MAO may have a role in maintaining adequate blood flow to the placenta by metabolizing biogenic amines as these components tend to decrease blood flow if they overload the placental circulatory system (Barnea, et al., 1986). Placental MAO has threefold higher affinity for serotonin in comparison to catecholamines (Barnea, et al., 1986). Serotonin was thought to be transported from the maternal circulation as injections of serotonin increase transiently its concentration in the fetus. That seems to be confirmed by studies that have not been able to demonstrate synthetic capacity for
serotonin in placental tissues. Hence the only viable explanation for high immunoreactivity in the giant cells of the ectoplacental cone and those of the syncytiotrophoblast is that serotonin is recaptured from the maternal circulation by the said cells (Yavarone, et al., 1993). However, Huang et al. (1998) reported that they detected serotonin synthesis in trophoblastic cells. These cells also have serotonin receptors predominantly in syncytiotrophoblast cells. The serotonin immunoreactivity observed in the experimental cells had a tendency to decrease with gestational age, reaching its minimum concentration just before birth. The concomitant accumulation of serotonin was thought to facilitate delivery (Gorkin, 1983; Huang, et al., 1998). All of this suggests that placental structures may play an active role in regulating the flow of biogenic amines into the fetal environment.

As presented in former sections, several studies have demonstrated the importance of MAO in brain development. Naturally occurring disorders such as Norrie Disease or Brunner's syndrome (Lenders et al., 1998) have set up the basis for an experimental analysis of MAO deficiencies during development. In themselves, these disorders are limited as sources of information because they are rare, and because they do not allow investigation of integrated pathogenic aspects necessary to understand the effects of MAO deprivation during development. Consequently, biological models have been developed in search of knowledge of how MAO activity influences brain development and behaviour. In 1994, Whitaker-Azmitia et al. administered combined MAO inhibitors (deprenyl and clorgyline) to pregnant rats in two periods, from gestation until birth, or from birth until sacrifice. Neurological development showed no impact, as developmental milestones were no different between experimental animals and controls. MAO-inhibited mice had seizures, stereotypic behaviours (grooming the head between the ears to the point of
rubbing off the fur), and they were very aggressive in comparison to controls. They also exhibited deficits in tests of passive avoidance and were more active in an open field. Paroxetine binding demonstrated a significant increase of the density of serotonin terminals in MAO-inhibited animals in several regions of the brain at early postnatal ages, with the tendency to become corrected in the subsequent weeks. This study suggested that all described anomalies are directly related to the decrease in MAO inhibition, and secondarily to the increased serotoninergic concentrations in which these brains are developing. Furthermore, it also suggested that prenatal and postnatal periods are equally sensitive times with respect to MAO inhibition (Whitaker-Azmitia et al., 1994).

In 1995, Cases et al. developed a transgenic mouse that lacks MAO A as the relevant gene has been interrupted by the insertion of a β interferon fragment. As detailed in chapter II, the behaviour of such knockout mice was characterized by tremors, difficulty in righting, fearfulness and aggression. Interestingly, extreme concentrations of serotonin and norepinephrine were detected in the pups of the knockout mice. All behavioural characteristics were reversed by the aging process until animals reached adulthood and by the use of a serotonin synthesis inhibitor para-chlorophenylalanine. Adult transgenic mice relapsed to early behavioural disturbances when given a single dose of MAO inhibitor. Histological analysis demonstrated cytoarchitectural changes in the somatosensory cortex characterized by the loss of the barrels in the IV layer of such cortex (Cases et al., 1995). Further investigations using the same model had confirmed that the histological changes are directly related to the excess of serotonin (Cases et al., 1996). It has also been demonstrated that in such animals, serotonin tends to be concentrated in neurons
that normally do not have serotonin pre- and even postnatally (Cases, et al., 1998).

Notwithstanding all these data, an excess of serotonin cannot be considered in isolation. It is well known for example, that MAO inhibitors have been part of the psychotropic armamentarium against depression without such dramatic effects. Hence it appears necessary to consider when MAO inhibition is taking place what lies beneath the cytoarchitectural and behavioural changes seen in knockout mice, and potentially in Norrie disease and Brunner's syndrome. Pharmacological inhibition of MAO during brain development has been a useful model for studying such phenomena. In a study complementary to that introduced in the present chapter by the author, Vitalis et al. (1998) administered clorgyline to several groups of pregnant mice. A set of mice was used to find out which dose or regime of administration (daily or three times a day) was the most effective in producing the histological changes observed in the knockout mice. A 10 mg/kg dose, given every 8 hrs was chosen to be optimal. Next, in order to know precisely when in brain development the MAO inhibitory effects occur, five groups were given clorgyline at different times: E 15 to P7; E 15 to P0; P0 to P7; P0 to P4 or P4 to P7, where E = prenatal and P = postnatal day. Only when clorgyline was administered during E 15 to P7 and P0 to P7 did the barrel formations disappear. The P4 to P7 group had some disturbances in the histological pattern, but they were not severe enough to be considered significant. This study confirms that there is a very specific period of time during which changes in morphology reflect the effects of severe increases of serotonin in the developing brain secondary to MAO inhibition.

Chapter VII presents an original study carried out by the candidate (Mejia et al., submitted for publication) which also used
pharmacological inhibition in the study of the effects of MAO deprivation during development. In this study, we contrasted the effects of inhibiting either MAO A or MAO B selectively, or inhibiting both enzymes simultaneously to prenatal development in the absence of inhibition. Continuous inhibition was maintained during the complete period of pregnancy and nursing by chronic microinfusion of drug to the dam. As compared to the neurohistological measures described by Vitalis et al (1998), the primary goal of our study was to conduct a detailed study of the behavioural effects of these manipulations.
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CHAPTER VII

MONOAMINE OXIDASE INHIBITION DURING BRAIN DEVELOPMENT INDUCES PATHOLOGICAL AGGRESSIVE BEHAVIOUR IN MICE

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**Glen Baker**: Measurement and analysis of monoamine oxidase activity and supervision of the manuscript.
Monoamine oxidase inhibition during brain development induces pathological aggressive behavior in mice

Jose Maria Mejia¹,², Frank R. Ervin², Roberta M. Palmour¹,²,³ and Glen B. Baker

Departments of ¹Biology, ²Psychiatry, and ³Human Genetics
McGill University, 1033 Pine Avenue West, Montréal, Québec H3A 1A1 Canada, and Neurochemical Research Unit, Department of Psychiatry, University of Alberta 1E7.44 Walter C. Mackenzie, Health Sciences Centre, Edmonton, Alberta T6G 2R7 Canada

Address for correspondence: Roberta M. Palmour Ph.D.
1033 Pine Avenue West
Montreal, Quebec, H3A 1A1
CANADA

Tel: 514-398-7303
FAX: 514-398-4370
e-mail: mc23@musica.mcgill.ca

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ABSTRACT

Monoamine oxidase (MAO) is historically a focus of concern in research on impulsive and aggressive behavior. Recent studies in a single kindred with a point mutation in the MAO A gene, together with phenotypic evaluations of MAO A knockout mice have sharpened this interest. The goal of the present study was to investigate the behavioral consequences of MAO inhibition during brain development and to determine the extent to which specific effects could be attributed to MAO A vs. MAO B.

MAO A and B inhibitors were administered, separately or in combination, during gestation and lactation. Behavioral evaluations included neurological testing, delay of rewarded response and the resident-intruder aggression paradigm, conducted before and after an acute pharmacological challenge. Total prenatal MAO inhibition produced a severe pattern of disturbed behavior, while MAO B-inhibited mice demonstrated a similar pattern but of a lower intensity. Aggression was elevated in MAO A-inhibited mice only after acute pharmacological challenges, suggesting prenatal sensitization.

Thus, developmental inhibition of MAO activity engenders behavioral effects which parallel those observed in animals with genetic ablation of MAO function. These data underscore the importance of neurochemical changes during development and provide a possible model for uninhibited aggression, common in clinical populations.
INTRODUCTION

In 1993, Brunner et al. (1993a) described a kindred in which several males exhibited borderline intelligence and pathological impulsive aggression. The pattern of inheritance suggested an X linked disorder. Subsequent analysis revealed a point mutation in exon 8 of the monoamine oxidase A (MAO A) gene (Brunner et al., 1993b), which resulted in complete deficiency of MAO A activity but normal MAO B activity (Brunner et al., 1993a). The notion that MAO A deficiency might be related to aggressive behaviour was strengthened by studies in experimental animals such as that of Cases et al. (1995) in which transgenic MAO A knock-out mice exhibit aggressive behaviours including a shorter latency to attack in a resident-intruder paradigm, compared to wild-type mice, and that by Whitaker-Azmitia et al. (1994) in which rat pups were exposed to MAO inhibition during brain development. The MAO-inhibited rats were impaired in acquiring passive avoidance and spent more time in exposed regions of an open field. This was interpreted as reflecting higher impulsivity, increased sensation seeking behaviours and lower anxiety in threatening situations.

Clinically, MAO inhibitors are effective and widely used antidepressants (Kaplan and Sadock, 1998), but aggression is not a reported side effect of these drugs. What is the basis for this paradox? Perhaps it is related to the developmental timing of MAO inhibition pervasive in the Brunner patients, prenatal in the experimental animal studies, and postnatal in patients treated with MAO inhibitors. This conceptualization would be consistent with the developmental expression of MAO activity in which MAO A appears early, while MAO B is not expressed until after birth and rises sharply during astrocyte proliferation (Weyler et al., 1990). In the absence of MAO activity,
substrates and alternative metabolites would increase in concentration and compensatory mechanisms may appear. The present study was designed to investigate the behavioural consequences of inhibition of MAO during embryogeny and early postnatal life, and to determine the extent to which specific effects could be attributed to selective or combined inhibition of MAO A and/or B.
METHODS:

Overview: This experiment comprised two phases. In phase one, equal numbers of male and female offspring were studied. In the second phase, a replication using only male offspring confirmed the initial results. A pilot study was implemented to analyze the behavioural effects of paroxetine, a specific and widely-used SSRI.

Subjects and drug treatment: Retired CD1 dams were purchased from Charles River Laboratory and received a day after impregnation. After one day of adaptation, weight was recorded and continuous administration of medication started. Microosmotic pumps (ALZA 2002) containing either: a) 100 μl clorgyline, 4.9 μg/μl; b) 100 μl deprenyl, 1.225 μg/μl; c) 100 μl 4.9 μg/μl clorgyline + 1.225 μg/μl deprenyl; or d) 100 μl 0.9% sterile saline, were implanted. In each dam the daily dose of deprenyl was 0.25 mg/kg and of clorgyline was 1mg/kg, alone or in combination. The vehicle for all drugs was sterile physiological saline. Independently, four dams were treated with paroxetine (100 μl, 49 μg/μl), diluted in a 50% solution of ethanol/sterile physiological saline, at a daily dose of 10 mg/kg. (Doses of medication were adopted under suggestion of Dr. Pierre Blier, verbal communication based in his ample experience working with the inhibitors in mice).

Four dams were allocated to each experimental group in each phase. Surgical implantation of the pumps was performed under ketamine/xylazine anesthesia (200:10 mg/kg respectively). Each pump was inserted subcutaneously through a 1cm. long incision in the scruff of the neck. Two weeks later the pumps were replaced by pumps (ALZA 2004, 240 μl.), that remained in place for four weeks to complete 6 weeks of MAO inhibition.
After weaning, equal numbers of male and female pups were randomly chosen from each dam to integrate groups of 10 subjects per treatment for the first phase (50 total; 25 females and 25 males, including paroxetine group). The second phase had 5 males per group randomly selected from the available litters (20 total, no paroxetine replication group).

Mice were housed in transparent cages, in single sex groups of two or three. Light and dark cycles were from 7am to 7pm. Grain based food pellets and water were accessible ad libitum.

**Neurological assessment:** During the first three months of age, neurological evaluations (McLearn et al., 1970) were performed to identify early signs of impairment. The evaluation included several maneuvers to elicit reflexes, equivalent to those used to identify neurological deficits in human babies. A descriptive statement of each item with operational criteria for scoring is presented in appendix 1.

**Open Field Tests:** This test was used to estimate motor activity. Each subject was placed into the center of an open black plexiglas box (50 x 50 x 20 cm). The floor of the box was divided in 10 cm² squares. Motor activity was measured by counting the number of squares entered by the mouse (1 point = all 4 extremities inside the square) during a three-minute period. All tests started at approximately 10 AM and were videotaped for additional evaluation. This test was conducted at 1, 2, 3 and 6 months of age.

**Differential Reinforcement of Low rate responding (DRL):** DRL tests evaluated the capability of each mouse to withhold a response when presented with a time delay. After being deprived of food for 24 hr., three-month-old mice were individually placed inside an operant-conditioning chamber (MED Associates Model # ENV-007) for 15 minutes. In the first set of trials mice learned to use the nose poke
device to obtain food. Once 30 pellets were obtained in a trial, a fixed delay was introduced after which the subject could poke in again to get a new pellet. If the delay was not respected the mouse was punished by restarting the latency time. The delay started at 5 seconds and was increased to 15 and 30 seconds in subsequent phases. Once a mouse reached the threshold of 30 successful responses it was taken to the next longer delay trial. Each mouse was tested for a maximum of 5 trials per phase. Animals that could not learn how to get pellets, and those that could not tolerate fasting, were excluded from the test (2 from group A, 1 from group B, 1 from AB and 1 from paroxetine).

**Resident-Intruder test:** At five months of age, each mouse was tested in a resident intruder paradigm for offensive aggression three times at weekly intervals (Mos et al., 1984; de Boer et al., 1999). Sex- and weight-matched untreated mice were used as intruders. As this has previously been shown to enhance aggression, all mice were isolated for 4 weeks before testing (Mos et al., 1984; de Boer et al., 1999). Each trial started by placing an intruder into the home cage of the experimental mouse. Trials lasted for 3 minutes and were videotaped for subsequent scoring. Scored behaviours included: a) the time elapsed between the placement of the intruder into the resident's cage and the first attack (latency to the first attack) and b) number of attacks per trial. An attack was defined as any attempt by a mouse to bite the opponent regardless of whether it was or was not successful. Females were unresponsive in this paradigm.

**Post-natal pharmacological challenges.** The effect of post-natal MAO inhibition on aggressive behaviour was tested in all groups of animals. Three weeks after the third trial of pre-natal MAO inhibition behavioural testing, a single dose of combined MAO inhibitors (clorgyline 1mg/kg + deprenyl 0.25 mg/kg) was administered.
intramuscularly to all males. Fifteen minutes later a resident intruder test was performed. A week later this procedure was repeated with a higher dose of combined MAO inhibitors (clorgyline 3mg/kg + deprenyl 0.75 mg/kg).

**Sacrifice and monoamine oxidase inhibition tests.** At the end of the behavioural experiments, animals were sacrificed by decapitation after hypoxic stunning. Brains were dissected and preserved in isopentane at -80 °C. Monoamine oxidase activity was measured in the brains of animals that were sacrificed at 21 days of age at the end of the MAO inhibitor administration. These animals were not subject to any experimental maneuvers. Activity was measured by the radiochemical procedure of Lyles and Callingham (1982) with 14C-labelled serotonin and 14C-2-phenylethylamine as substrates of MAO A and MAO B respectively. Results are presented in table 3 as the percentage of inhibition that experimental animals exhibited after subtracting the enzymatic activity of each one of the prenatally treated subjects, from the mean activity of control mice. In time this value was subtracted from a 100% of hypothetical inhibition to give the percentage of inhibition per subject and their mean as a group. Control subjects are referred to as C subjects and experimental animals correspond to A = clorgyline, B = deprenyl, and AB = clorgyline + deprenyl inhibition groups.

**Statistical analysis.**

(1) **DRL.** Differences between groups were analyzed using ANCOVA. The independent variable was treatment group while the dependent was the ratio of successful attempts/total number of attempts (a measure thought to better illustrate the ability to withhold a response than the absolute number of successful trials).
(2) **Resident-Intruder.** Differences between groups with respect to latency to first attack were analyzed by survival analysis (Kaplan Mayer and *post-hoc* Mantel Cox), using 200 seconds as a censoring variable and the time in seconds to the first attack as the dependent variable. This test was applied given the nature of the data in which the discriminating variable is the time in which the mouse attacks for the first time, which may reflect how "tolerant" it is to the presence of an intruder. This is similar to a survival curve in that once the first attack has occurred the subject is eliminated from the analysis as it happens when subjects in clinical trials of survival, die. Differences between groups with regard to the number of attacks were analyzed with repeated measures ANOVA using the number of attacks as the dependent variable and treatment group as the independent variable.

(3) **Post-natal pharmacological challenges.** Two-way analysis of variance, with dose and treatment as grouping factors, was used to analyze the differences amongst groups in attacks and latency to first attack.

(4) **Paroxetine as an enhancer of serotonin levels.** The hypothesis that behavioural effects seen in these studies could parsimoniously be explained by prenatal elevation of serotonin levels was tested indirectly by examining the effects of paroxetine as compared to placebo on DRL and on resident-intruder responding. A statistical plan identical to that described above was used.
RESULTS

The number of mice born to each dam ranged from 7 to 12. All groups but paroxetine had a total of 10 males and 5 females. Experimental groups did not differ (p> .05) in body weight (Table 1). There were no morphological abnormalities in the newborn mice. Neurological evaluations at 1, 2 and 3 months of age showed the expected developmental changes, which did not differ between groups. No clinically relevant neurological aberration occurred in any member of the study population. Motor activity, as reflected by the number of squares entered in the open field test at different ages, also showed no difference attributable to experimental manipulations [F (3,34)= 1.5; p>0.2] (Table 2).

Preliminary analysis entering the phase of testing (phase 1 or phase 2) as a factor, failed to reveal any significant contribution of test phase (# attacks: t=.11, p=.93; latency to the first attack: t=.59, p=.61; DRL: t=.90, p=.40). In consequence, behavioural data were merged across phases.

Inhibition of monoamine oxidase.

Table 3 shows the percentage of inhibition achieved in each one of the prenatally treated groups. Judging by the means there was equal proportions of inhibition for MAO A and B activity in those animals inhibited with clorgyline during development, while those inhibited with deprenyl showed twice the proportion of inhibition for MAO B activity in comparison to MAO A activity. Those animals undergoing inhibition of both enzymes prenatally show no difference in proportion of inhibition for one enzyme or the other. The last pair of groups shows also the highest inhibition of them all.
**Ability to withhold a response as measured by DRL.**

Differential Reinforcement of Low rate responding (DRL) is a test in which mice must delay responding in order to achieve a maximal number of food rewards. The inability to acquire this skill is thought to relate to impulsivity as impulsive mice are expected to be impatient, hence they poke before the delay is finished and are punished by the restarting the delay time. Prenatal exposure to MAO inhibitors decreased \([F(3,186)=3.58; p= .015]\) the number of successful attempts to obtain a food reward in the operant conditioning chamber (Figure 1).

A potentially more informative DRL measure is the ratio of successful attempts to the total number of attempts (Figure 2). Prenatal treatment with MAO inhibitors also decreased this ratio \([F(3,186)=3.51, p<0.02]\). Pair-wise comparison (Fisher's PLSD) revealed significant differences between A vs. AB (p <0.01), A vs. B (p < 0.05) and AB vs. C (p<0.02).

**Offensive-aggressive behaviour as measured by Resident Intruder test**

In this experiment, analysis of variance revealed a significant effect \([F(3,110) = 5.88, p < .001]\) of prenatal MAO inhibition on the number of attacks, a measure of aggressive behaviour (Figure 3). *Post-hoc* analysis showed that significant pair-wise differences occurred between groups A vs. AB and C vs. AB (p<.001). There was a trend towards differences between B vs. AB (p<.05).

A survival analysis was used to compare the latency to the first attack (treated as a repeated measure) across treatment groups (Figure 4). Observations in which no attack had occurred by 200
seconds were censored. Given the characteristics of our population and variables, statistical significance was assessed by the Mantel-Cox test. Latency to first attack was significantly affected by prenatal drug exposure ($\chi^2 = 19.43, p<.001$). Group AB had the shortest latency while group A showed the longest latency.

**Pharmacological Challenges and Fighting Behaviour**

The extent to which acute postnatal exposure to MAO inhibitors might sensitize postnatal behaviour was tested in adults with acute treatment with MAO inhibitors. In order to achieve complete enzymatic inhibition, MAO A and MAO B inhibitors were combined and administered at two different doses. The dependent variables in these analyses were computed for each mouse by subtracting the baseline values (number of attacks, latency to the first attack) from the values obtained in the two challenged states (low dose and high dose). Two-factor ANOVAs (prenatal treatment group, challenge dose) were used to evaluate the data.

As shown in Figure 5, the number of attacks increased in animals treated prenatally with MAO A inhibitors and to a lesser extent in those treated with MAO B inhibitors or saline [$F(3,65) = 3.56; p=0.019$]; there was no effect of challenge dose [$F(1,65)=0.017; p=0.89$] and no interaction between group and dose [$F(3,65)= 0.19; p=0.90$]. There was also no effect on attacks in animals treated prenatally with combined MAO A and -B inhibitors, which probably reflects a ceiling effect. Post-hoc analysis confirmed significant pair-wise differences between groups A vs. AB ($p<.002$).

Similarly, prenatal treatment influenced the latency to first attack after acute administration of a combined MAO A/MAO B inhibitor
challenge \( F(3,65) = 6.58; p=0.0006 \). There was no significant effect of challenge dose alone \( F(1,65)=0.12; p=0.73 \) and no significant interaction between dose and prenatal treatment group \( F(3,65)=0.28; p=0.84 \). Post-hoc analysis demonstrated that the main differences were related with those increases occurring on groups A and B compared with AB and control. [A vs. AB, \( p=0.0003 \); A vs. C, \( p=0.0009 \); B vs. AB, \( p=0.02 \) and B vs. C, \( p=0.04 \)].

As shown in Figure 6, the latency to first attack was reduced in animals treated prenatally with either MAO A or MAO B inhibitors. There was no change in animals treated prenatally with the combined regimen or with saline.

**Effects of paroxetine on behaviour**

Paroxetine is a selective serotonin re-uptake inhibitor that increases availability of serotonin in the synaptic cleft by obstructing the uptake of neurotransmitter molecules by the pre-synaptic serotonin transporter. Under normal conditions, the transporter terminates post-synaptic receptor stimulation by recapturing serotonin and provides an important mechanism for the regulation of neurotransmission. Once in the pre-synaptic axon, serotonin is catabolized by MAO (Barker and Blakely, 1995). In the absence of MAO activity, cells could become exposed to elevated levels of serotonin. Despite their different mechanisms of action paroxetine treatment would in a way mimic this phenomenon by increasing the amount of serotonin available in the synaptic cleft. Accordingly, we studied a group of mice treated prenatally with paroxetine. Pups from the paroxetine treatment group, like those in other treatment groups, showed no neurological differences after birth. The paroxetine group
also did not vary by weight [Males: C (10) = 25.4 ± 2.1 g vs. P (10) = 25.4 ± 1.98; Females: C (5) = 20.2 ± 1.48 g vs. P (5) = 19.3 ± 0.67 g. The t-test for open field activity showed a mean number of squares entered of 162.16 compared to 83.56 of the control group, yielding an \( F(1,12) = 6.714, p = .024 \). In DRL tests, the paroxetine group do not differ significantly from controls (number of successful attempts: \( F(1,88) = 0.14, p = 0.72 \); ratio of successful vs. total attempts: \( F(1,88) = 0.041, p = .84 \)).

Paroxetine-treated animals neither differ from controls in the resident intruder paradigm. For number of attacks, the comparison yielded an \( F(1,67) = 1.24; p = .27 \), while latency to the first attack resulted in a chi square of 0.1, \( p = .75 \). There was no effect of pharmacological challenge on number of attacks [by group \( F(1,22) = 3.62; p = .07 \) or dose \( F(1,22) = .07; p = .798 \)] or on latency to first attack [by group, \( F(1,22) = 0.28; p = .60 \) or by dose \( F(1,22) = .015; p = .90 \)].
DISCUSSION

In this study we have shown that inhibition of MAO during murine development may produce behavioural phenotypes that differ as a function of the specific form of MAO (A vs. B) that has been inhibited. There was no effect of these treatments upon either gross morphology or neurological function. This suggests that our findings are the direct or indirect consequence of inhibiting MAO activity and are not secondary to more general adverse effects (blindness, motor hyper- or hypoactivity, etc.).

The most important findings are:

- Combined inhibition of MAO A and B had the highest inhibitory effect in mice brain and strongest effects on experimental offspring. These mice were the least successful in learning to delay a response; they had the highest number of attacks and the shortest latency to the first attack. In this group, behaviour was not significantly worsened by postnatal challenge with MAO inhibitors, probably because of a ceiling effect.

- Prenatal inhibition of MAO A produced offspring with normal behavioural performance under baseline conditions and indistinguishable effects upon MAO A or B activities. Subjects in this group displayed low number of attacks, long latency to the first attack and a good capacity to withhold a response. However, when MAO A prenatally inhibited mice were challenged by inhibiting MAO activity during adulthood, a remarkable deterioration appeared, as judged by a potentially substantial (albeit not statistically significant) increase in the number of attacks when compared to baseline. Latency to the first attack was also decreased after challenge. Doses of inhibitor did not play a role in the effects of challenge as proven by the use of high and low dose trials. This pre-natal sensitization model mimics the
pathological responses seen in Brunner's syndrome in the presence of environmental challenges.

• Prenatal MAO B inhibition produced mice whose behaviour was intermediate between the MAO A group and the group with combined prenatal inhibition. Percentage of inhibition as expected, was more pronounced for MAO B than for MAO A. As compared to controls, there was a non-statistically significant increase in number of attacks. In the DRL, this group demonstrated a significantly lower capability to delay a response as compared to the MAO A inhibited group. Postnatal pharmacological challenges reduced the latency to first attack but did not increase the number of attacks significantly.

• The behavioural profile of offspring treated prenatally with the serotonin selective re-uptake inhibitor paroxetine did not differ from that of the control group. Thus, the behavioural changes seen in mice with prenatal MAO-inhibition cannot solely be attributed to an increase in serotonin levels.

Effects of the pharmacological inhibition of MAO during development

This is the first study in which an attempt to selectively inhibit MAO activity is used to discern the behavioural effects of blocking specific subtypes of MAO activity during development. Our findings with combined MAO A and -B inhibition are similar to those of Whitaker-Azmitia et al. (1994), who treated rats with the same MAO inhibitors during pregnancy and was able to identify an aggressive pattern of behaviour in the treated animals.

These behavioural similarities are remarkable, taking into account how different these models are. Besides the species difference, Whitaker-Azmitia et al. (1994) used higher doses of inhibitors
[clorgyline (3 mg/kg vs. 1 mg/kg/day), deprenyl (3 vs. 0.25 mg/kg/day)] making inhibition non-selective. Their behavioural evaluations included tests for catalepsy, righting reflex, olfaction, percent of time spent in light/dark, passive avoidance and open field tests, compared to clinical neurological evaluations, DRL, and resident intruder battery in the current experiment. Although in Whitaker-Azmitia's report it is mentioned that rats were aggressive towards handlers and cage-mates, no systematic tests for aggressive behaviour were performed. According to these two studies, it appears that MAO deprivation during development is a reliable and replicable biological model for the study of aggressive behaviour. Moreover, our study shows that the behavioural effects seen by Whitaker-Azmitia et al. (1994) are not linked to a specific MAO subtype. Predominantly MAO B inhibition elicits weak baseline effects, including a tendency towards increased attack behaviour and an impaired ability to withhold response; while the attempt to selectively inhibit MAO A did not cause impairments at baseline but show significant sensitization to post-natal challenge. The combined inhibition is clearly greater than the sum of the parts. Since different functional roles have been proposed for each form of the enzyme (Berry et al., 1994; Lenders et al, 1996; Shih and Chen, 1999), it is no surprise to discover that the most severe behavioural manifestations appear in completely inhibited mice, which resemble the closest Whitaker-Azmitia et al.'s non-selectively inhibited rats. Finally, our study suggested that despite overlapping substrate specificities for MAO A and B, the presence of one isoform does not entirely compensate for the lack of the other.
Genetically mediated loss of MAO

Comparative information on the specificity of MAO A vs. MAO B enzymatic activity is provided by natural or engineered genetic experiments (mutations and deletions or transgenic manipulations respectively). Selective (A or B) or combined deficiencies have been documented in animals and human males hemizygous for these genes.

A point mutation in exon 8 of the MAO A gene leads to the complete abrogation of enzyme activity manifested by borderline intelligence, stereotyped hand movements and a marked increase in impulsive aggressive responses disproportionate to the provocation (Brunner et al., 1993 a and b). Another disorder, Norrie Disease, results from a variable size microdeletion in the Xp11.3 band of the X chromosome where the MAO genes and the Norrie disease gene reside. Subjects with deletions including the Norrie disease gene and both MAO genes exhibit congenital blindness due to pseudo-gliomas, progressive mental retardation, growth retardation, and impaired sexual maturation. Severe cases also have tonic seizures, myoclonic contractions, sleep disturbances, autistic-like behaviour, peripheral autonomic dysfunction and self-mutilatory conducts such as biting and punching (Murphy et al., 1990; Collins et al., 1992). By contrast, restricted deletions in the Norrie disease region may include MAO B, but not MAO A. These patients are blind and deaf but show no cognitive or behavioural impairment (Murphy et al., 1990; Collins, 1992; Lenders, 1996).

Both MAO A and MAO B knockout mice have been transgenically developed (Cases et al., 1996; Grimsby et al., 1997). MAO B knockouts do not differ from controls in motor activity or open field performance (Grimsby et al., 1997). However, they are incapable of habituating to the forced swim test, they show hyperactivity in
response to stress (Grimsby et al., 1997), but they were not impulsive or aggressive (Scremin et al., 1999; Shih and Chen, 1999a).

Comparatively, **MAO A knockouts** (Cases et al., 1996) not only showed aggressive behaviour as measured by resident-intruder tests, but also displayed reduced frequency of social exploratory responses and an increased frequency of offensive aggressive incidents toward cage-mates. The presence of nonspecific abnormal behaviours in these animals (i.e.: trembling, sustained reactions to pinching, prolonged righting, head nodding, etc.), suggested generalized developmental problems. These phenotypical anomalies disappeared in adulthood but relapsed with acute doses of MAO B inhibitors. Furthermore, a serotonin synthesis inhibitor (para-chlorophenylalanine) administrated during development effectively antagonized the effects of the genetic manipulation (Cases et al., 1996).

In summary, it is clear that genetic obliteration of the activity of MAO (A, or B, or both) produces distinct behavioural effects. Total lack of MAO activity is compatible with life but results in serious neurological, biochemical and behavioural consequences as seen in humans with severe forms of Norrie disease. Accordingly, in the present experiments mice with prenatal inhibition of both MAO subtypes had the most pronounced behavioural impairments.

Mice with developmental MAO B inhibition showed some baseline behavioural changes, but like MAO B knockouts, had little response to post-natal pharmacological challenge (Lenders et al., 1996). Heritable lack of MAO A activity, by contrast, seems to elicit a behavioural syndrome in humans and rodents in which aggressive behaviour occurs only in response to pharmacological challenge or psychosocial stress (Brunner et al., 1993). A similar effect was discovered in our experiment in that aggressive behaviour was
obvious in mice prenatally treated with MAO A inhibitors only after pharmacological challenge, despite the lack of a selective inhibition during the prenatal period.

**Developmental expression of MAO subtypes**

As noted above, developmental expression of MAO genes is quite distinct, and it may play a role in the observed behavioural differences amongst treatment groups through its ontogenetic evolution. MAO A appears shortly after the development of the primary neuraxis and reaches adult levels almost immediately. MAO B expression rises in parallel with astrocyte proliferation promptly after birth in rodents and during the third trimester of gestation in primates (Koide and Kobayashi, 1984; Weyler et al., 1990; Rao et al., 1995; Cases et al., 1995; Berry et al., 1996; Shih and Chen, 1999b).

The finding that the combined MAO A/B inhibited group had the greatest behavioural alterations at baseline is consistent with the idea that absolute inhibition of MAO A spanning brain development emulates behavioural changes seen in severe Norrie disease. Contrast, mice show developmental sensitization of the brain to later challenges, as do patients with Brunner's syndrome. Unfortunately, MAO B-inhibited mice exhibit baseline behavioural alterations that MAO B knockout mice did not, making it difficult to reconcile the two models. Perhaps, the permanent absence of MAO B activity in the knock-out mouse in comparison to the transiently inhibited model, encourages the appearance of compensating mechanisms that do not develop in the pharmacological model as MAO B activity goes back to normal.
**Anatomical expression of MAO subtypes.** A third dimension to consider in understanding the effects of absent MAO activity is that both forms of the enzyme coexist not only in the same tissue but also in the same cell in different proportions. However, MAO A is predominantly expressed in noradrenergic, adrenergic and Dopaminergic neurons, while MAO B is located primarily in serotoninergic neurons and glia (Berry et al., 1994). Even when both forms of the enzyme are present, intra-neuronal catabolism (predominantly MAO A) accounts for >90% of total degradation of most neurotransmitters. The usual 10% or less of glial inactivation (mainly MAO B), becomes much more relevant when the intra-cellular mechanisms are overwhelmed (Lenders et al., 1996; Berry et al., 1999).

Extrapolating these facts to interpret the current results, we could postulate that the AB inhibited mice did not have any form of compensation because both enzymes were inhibited. In MAO A inhibited mice, intracellular MAO activity was blocked during development, forcing glial activity to exert any possible counteracting event. However, glial proliferation peaks only after birth, leaving only a narrow three-week window of development within which counterbalancing enzymatic effects would have to occur. Furthermore looking at the current experience, MAO B activity appeared as inhibited as MAO A was, making it more difficult for that counterbalancing period to offset the deleterious effects of earlier disruption, and making the close-to-normal baseline behaviour in the clorgyline treated mice, the more puzzling.

Comparatively, loss of MAO B would have eliminated any possible compensation should intracellular mechanisms fail. To understand the behavioural effects of MAO B inhibition let us consider not only the anatomical localization, but also the quantitative relationship
between the different forms of the enzyme in the whole brain. It was mentioned above that MAO A is responsible for about 90% of intraneuronal MAO activity, presumably sufficient to metabolize the majority of neurotransmitters present before birth in human and mouse brain. However, during growth and adulthood this level of enzymatic activity probably accounts for only 20% of the total activity, such that MAO B is responsible for most amine metabolism in the post-natal period (Weyler et al., 1990; Berry et al., 1994). Accordingly, during periods of high catabolic demand, the brains of MAO B-inhibited mice would be exposed to potentially damaging conditions as a consequence of the great amount of non-catabolized neurotransmitter. This may explain why this group had greater baseline impairment than controls or A-inhibited mice, while performing better than A/B-mice. These findings also highlight how important the first weeks of postnatal life may be for the still incomplete murine brain development, even when MAO activity returned to baseline levels within a month after weaning.

**Possible mechanisms of developmental disregulation in MAO-inhibited mice.**

Serotonin has been acknowledged to have a guiding role in neural development (Whitaker-Azmitia et al., 1996). It is one of the first neurotransmitters to appear both phylogenetically and ontogenetically. Serotonergic neurons develop in the raphé nuclei and project to almost every part of the future cortex. They are thought to coordinate consecutive steps in development by releasing molecules such as βS100, a growth factor produced by astrocytes (Whitaker-Azmitia et al., 1996). When the effects of serotonin are blocked with antagonistic agents, aberrant growth of axons occurs, as demonstrated by both *in-vivo* and *in-vitro* studies. High levels of
serotonin on the other hand, have also been related to developmental disorders (Upton et al., 1999; Bou-Flores et al., 2000.)

The mechanisms involved in the neurotrophic effects of serotonin are possibly related to the integrated effects on many different receptors. It is well known that the receptors for most neurotransmitters are over-expressed during prenatal development (Larsson et al., 1985; Quirion and Dam, 1986; Seeman et al., 1987; Slesinger et al., 1988; Kinney et al., 1990; Whitaker-Azmitia, 1992; Whitacker-Azmitia et al. 1996). Some receptors require a certain level of stimulation to establish a stable set-point. In the absence of stimulation they may suffer atrophy. Other receptors seem to be transiently expressed. They produce diverse neurotrophic factors and predominate in glial cells even in regions in which they become obsolete in adult life. Finally, it is important to consider that receptor affinity may be different in-utero compared to that in the adult brain (Whitaker-Azmitia, 1992).

**Clinical implications**

Recently it has been reported that antidepressants may increase the expression of neurotrophic factors (Russo-Neustadt et al., 2000). Similarly, the neuroprotective effects of deprenyl might be significantly correlated with increased output of neuronal growth factors (Semkova et al., 1996). The extent to which these effects depend upon elevated levels of serotonin (Liu and Lauder, 1992), similar to those thought to result from MAO inhibition, remains to be determined. Whatever the mechanism, the fact remains that adult brains apparently respond differently to MAO inhibition than does the developing brain. As we show in the present study, MAO inhibition during gestation has both
general and specific behavioral effects unrelated to those antidepressant effects sought in post-natal pharmacotherapy.

These findings also have implications for the management of pregnant women. On the one hand, it may sometimes be desirable to continue a maintenance dose of psychopharmacological agent. There are unfortunately few data available to suggest whether some MAOI's might be safe for such use. Certainly, the types of behavioural effects described here would not be noticed for many years given the apparently normal attainment of neurobehavioral milestones. A sobering example is provided by the recent study of Brennan et al. (1999) suggesting that maternal prenatal smoking is significantly associated with criminal outcome in male offspring; even when parental characteristics and other perinatal problems are controlled. Although not the only possible mechanism, inhibition of MAO activity is a well-documented effect of cigarette smoking (Weyler et al., 1990; Fowler et al., 1996).

In future research, the analysis of physiopathological changes caused by MAO inhibition using molecular biology and histological tools should provide information about receptor density, schedule of appearance, distribution, affinity and expression. In order to understand the mechanisms through which variation of enzymatic activity levels result in the behavioural phenotypes documented by the vast number of articles associating low levels of MAO activity with psychopathology, it will be also crucial to understand the regulation of MAO expression. (Shih and Chen, 1999b).

In summary, this communication demonstrates that pharmacological inhibition of MAO activity during development has behavioural effects that strongly parallel those observed in animals with genetic manipulations that obliterate MAO functions. This adds to the evidence already accumulated in favour of the postulated
physiological differences between the two forms of the enzyme. In clinical context, this also sheds light towards a better understanding of the effects of MAO inhibitors on the developing brain that are qualitatively different from MAO inhibition in the adult.
REFERENCES


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Scremin OU, Holschneider DP, Chen K, Li MG, Shih, JC (1999): Cerebral cortical blood flow maps are reorganized in MAO B deficient mice. *Brain Research* 824:36-44.


Appendix 1

Pivoting [appears at 2 days, disappears at 15 days of age] (one minute observation period) Circular locomotion caused by side motion of front legs with hind legs essentially inactive.
   0 = response not present
   1 = weak attempts to pivot, typically of short duration
   2 = moderate pivoting movements
   3 = pivoting movements pronounced; pivoting occurs throughout most of the observation period

Straight walking [appears at 5-10 days] (one minute observation period) Locomotion in an approximate straight line with all four legs involved in adult fashion.
   0 = response not present
   1 = straight walking for a distance at least equal to body length
   2 = more than half the observation period spent in straight walking

Righting reflex [appears at 5 days] (best of two trials) Turning to rest in normal position on all four legs after being placed on side by experimenter.
   0 = response not present
   1 = more than one second required to attain upright position
   2 = upright position attained almost immediately

Back righting (average of two trials) Turning to rest in a normal position on all four legs after being placed on back by experimenter.
   0 = response not present
   1 = righting within 5 seconds
   2 = righting within 1 second

Rooting reflex [appears at 2 days, disappears at 15 days of age] (20 second test period) Pushing forward after bilateral stimulation by experimenter's finger on face area.
   0 = response not present
   1 = 1 or 2 brief responses
   2 = 1 sustained response
**Cliff drop aversion** [appears at 5 days] Withdrawing from the edge of surface when forepaws are placed over the edge.

- 0 = response not present
- 1 = backing within 10 seconds
- 2 = backing within 3 seconds
- 3 = backing almost immediately, within one second

**Grasp reflex** [appears at 2 days of age] Foot flexion with grasping of metal rod when plantar surface is stroked by the rod.

**Front placing** [appears at 2 days, disappears at 15 days of age] Raising and placing foot on table top when dorsum of foot is placed in contact on edge of table top (animal suspended by loose skin at the back of the neck during test).

**Crossed extensor** [disappears at 8 days of age] Flexion of hind limb when pinched and extension of opposite hind limb.

**Bar holding** [appears around ten days] (best of three trials) Grasping a wooden pencil with front legs and paws and supporting own weight.

- 0 = response not present
- 1 = momentary support of body weight
- 2 = support of body weight for 5 seconds or longer

**Auditory startle** [appears at 12 days of age] Immediate flight or “freezing reaction” following hand clap of experimenter.

**Eyes open.** [appears at 12 days of age] Time of opening of eyes.

- 0 = both eyes closed
- 1 = at least one eye partially open
- 2 = both eyes fully open

**Hyperreactivity.** Overreaction or exaggerated responsiveness to novel stimuli (the “popcorn stage”) with exaggerated freezing or jumping being elicited by ordinary noises and movements.

**Mass reaction.** Exaggerated squirming and rolling with occasional whole body convulsions following tail pinch.

- 0 = response not present
1 = reaction restricted to tail region
2 = reaction involving tail and hind quarters
3 = reaction involving entire body with writhing, squirming and rolling.

**Body weight.** Body weight measured to the nearest tenth of a gram.

**Appendix 1.** Neurological evaluation scale. The different items with the criteria for scoring are listed. Some of them include the expected time of appearance and disappearance as expected in developmentally mature populations.
Table 1. Weight of experimental subjects at 1 month of age per experimental group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10; 26.2; 0.57</td>
<td>5; 21.1; 1.34</td>
</tr>
<tr>
<td>B</td>
<td>10; 25.8; 2.25</td>
<td>5; 19.1; 1.81</td>
</tr>
<tr>
<td>AB</td>
<td>10; 27.1; 0.74</td>
<td>5; 23.2; 1.71</td>
</tr>
<tr>
<td>C</td>
<td>10; 25.4; 2.10</td>
<td>5; 20.2; 1.48</td>
</tr>
<tr>
<td>P</td>
<td>10; 25.4; 1.98</td>
<td>5; 19.3; 0.67</td>
</tr>
</tbody>
</table>

The numbers correspond to number of mice per group; mean per group; and standard deviation, respectively.
**Table 2.** Motor activity at one month of age per experimental group

<table>
<thead>
<tr>
<th>GROUP</th>
<th>MEAN</th>
<th>S.D.</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>83.4</td>
<td>36.5</td>
<td>12.2</td>
</tr>
<tr>
<td>B</td>
<td>109.1</td>
<td>43.5</td>
<td>13.8</td>
</tr>
<tr>
<td>AB</td>
<td>101.8</td>
<td>25.6</td>
<td>8.1</td>
</tr>
<tr>
<td>C</td>
<td>83.56</td>
<td>19.2</td>
<td>6.4</td>
</tr>
</tbody>
</table>

This table shows the mean values per experimental group with standard deviation and error. These values illustrate the absence of significant differences amongst groups.
Table 3: Monoamine oxidase activity in the brains of prenatally treated mice with clorgyline (MAO A inhibitor), deprenyl (MAO B inhibitor) and a combination of both.

<table>
<thead>
<tr>
<th>Prenatal inhibition</th>
<th>MAO A percentage of inhibition</th>
<th>MAO B percentage of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clorgyline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>24.11</td>
<td>32.09</td>
</tr>
<tr>
<td>A2</td>
<td>10.05</td>
<td>16.09</td>
</tr>
<tr>
<td>A3</td>
<td>13.86</td>
<td>21.19</td>
</tr>
<tr>
<td>A4</td>
<td>49.66</td>
<td>43.41</td>
</tr>
<tr>
<td>Mean</td>
<td>24.42</td>
<td>28.19</td>
</tr>
<tr>
<td>Deprenyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>-0.99</td>
<td>8.59</td>
</tr>
<tr>
<td>B2</td>
<td>1.06</td>
<td>24.55</td>
</tr>
<tr>
<td>B3</td>
<td>44.50</td>
<td>47.81</td>
</tr>
<tr>
<td>B4</td>
<td>18.08</td>
<td>33.43</td>
</tr>
<tr>
<td>Mean</td>
<td>15.66</td>
<td>28.59</td>
</tr>
<tr>
<td>Clorgyline+ deprenyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB1</td>
<td>49.22</td>
<td>48.94</td>
</tr>
<tr>
<td>AB2</td>
<td>17.75</td>
<td>36.13</td>
</tr>
<tr>
<td>AB3</td>
<td>29.91</td>
<td>35.69</td>
</tr>
<tr>
<td>AB4</td>
<td>56.60</td>
<td>49.71</td>
</tr>
<tr>
<td>Mean</td>
<td>38.37</td>
<td>42.62</td>
</tr>
</tbody>
</table>

Results are expressed as the proportion of inhibition. It was obtained by calculating what proportion of the mean enzymatic activity of controls, each experimental subject had. These values were in time subtracted from a 100% of expected activity giving the percentage of inhibition.
Figure 1. Successful attempts in getting a reward, per group of prenatal treatment. Each subject gets a food pellet if it pokes a poke-nose device only after a 15-second delay (DRL test). Bars represent mean and standard deviations. Significant pairwise comparisons are shown by the lines across the bars. ANOVA for these data resulted in F(3,186) = 3.58, p > .02.

Figure 2. Ratio of successful/total attempts in getting a reward, per group of prenatal treatment for DRL test with 15 second delay [F (3,186) = 3.5, p > .02]. Significant differences between pairs are shown with arrows above each pair with the corresponding p values.

Figure 3. Number of total attacks per group of prenatal treatment [F(3,110) = 5.89, p > .001]. Attacks were quantified during 3-minute periods of testing. Significant pairwise comparisons are shown with their corresponding p values.

Figure 4. Latency to the first attack by group as measured in the resident intruder test. The graphics show the cumulative survival and cumulative hazard per group of prenatal treatment. Latencies higher than 200 seconds were censored for this analysis. A significant difference related to prenatal exposure to different MAO inhibitors was demonstrated in this experiment (X^2= 19.43, p <.001).

Figure 5. Difference in number of attacks between baseline resident intruder tests and trials performed after acute single doses of combined inhibitors MAO A and MAO B. The light bar represents low doses (same as for prenatal treatment) and the dark bar represents high doses (three times the baseline dose of inhibitors). Groups are constituted by subjects allocated to the corresponding MAO inhibition treatments during development. The differences were obtained by subtracting baseline number of attacks from the number of attacks obtained after each acute challenge. The effect of prenatal treatment was significant [F(3,69) = 3.56, p > .02]; however, neither dose (F(1,71) = .02] nor dose and group combined [F(3,69) = .19] had an influence on the outcome.

Figure 6. Difference in latency to the first attack by group of prenatal treatment. Differences are calculated by subtracting the mean of the latency to first attack at baseline from that obtained after pharmacological challenge. This was done on two occasions with different doses of MAO inhibitor. In this analysis, the effect of prenatal treatment combined with the acute inhibition was significant [F (3,69) = 6.58, p> .001]. Dose [F (1,71) = .12] and the interaction between dose and group [(3,69) = .28] had no effect. Post-hoc analysis revealed that groups A and B accounted for most of the variance (A vs. AB, p>0.001; AB vs. B, p = 0.02; B vs. C, p = 0.04).
Figure 1

Group

Mean and SE

A B AB C

p<0.01

p< 0.01
Figure 2

Mean and SE

Group

A
B
AB
C

p<0.01

p<0.05

p<0.05
Figure 3

Mean and SE

Group

A
B
AB
C

p < 0.01

p < 0.05

p < 0.05

Mean
Figure 4
CHAPTER VIII

SUMMARY AND PERSPECTIVE
In this thesis I aimed to investigate the relationship between MAO activity and the occurrence of pathological impulsive aggressive behaviours as seen in genetic disorders such as Brunner's syndrome. In a more general perspective, I also hoped to increase knowledge about the etiopathological mechanisms underlying aggressive behaviours. In particular, the first study of this thesis sought to identify other cases with Brunner's syndrome mutation without success. Reviewing the literature, one realizes that no other group of researchers has been able to replicate Brunner's results (Murphy et al., 1998; Schuback et al., 1999). The general experience is that this mutation is extremely rare. Furthermore, this also suggests that even though aggressive behaviour in the general population may be influenced by MAO, they are not necessarily associated with absolute obliteration of enzymatic activity.

In the second study I looked for an association between MAO activity, behavioural manifestations of aggression and genetic markers with no success. We were not able to link the presence of four genetic markers in the MAO B and MAO A genes to platelet MAO activity. We were also unable to find an association between these specific markers and longitudinally defined aggressive behaviour. Finally, we were unable to link behaviour to MAO activity.

Considering the numerous studies associating MAO activity and psychopathological traits, and genetic markers with behavioural traits a relationship amongst them might well be expected. The absence of any association between these three factors - MAO activity, behaviour and genetic markers - may suggest a type II error that a bigger sample could solve.

Our final study demonstrated that pharmacologically induced MAO inhibition during development is sufficient to cause behavioural disturbances in the resident-intruder paradigm, a standard test of
increased aggressive behaviour in pharmacologically manipulated animals. We also demonstrated that selective inhibition of different forms of MAO during development results in different patterns of abnormal behaviour, with the MAO A inhibited mice being comparable in certain particulars to men affected by Brunner's syndrome. Only combined inhibition of MAO A and B produced pervasive behavioural changes. This model proved also to be a very useful way of studying MAO deprivation in a fashion closer to that which occurs in the preponderance of cases that are not caused by genetic disorders.

The ultimate goal of this project was to conduct both clinical and basic studies that would expand our understanding of the overall involvement of MAO -mutations, polymorphisms, enzymatic activity and developmental expression- as a contributing factor to aggressive behaviour. In retrospect, we can conclude that genetic disorders such as Brunner's mutation are rare. It would also be desirable to look at factors such as tobacco smoking (Fowler et al., 1996 a and b) or psychotropic substances that more frequently affect MAO activity. The possibility of physiopathogenic interaction between markers, biochemical processes and behaviour, not detected in our study, remains relevant as one of the potential mechanisms to be involved in the influential effect of genes upon behaviour. The concept of associating traits and not diagnostic categories should prevail in search of the said associations.

Finally, we demonstrated that the lack of MAO during critical periods of neural development is directly involved in the causation of behavioural disturbances as previously reported (Whitaker et al., 1994; Cases et al, 1995 and 1996; Lenders et al, 1996). Interestingly, we also obtained different patterns of behavioural disturbances by inhibiting MAO activity in a selective or combined fashion, which
strengthens the concept that different forms of MAO play different roles in metabolism.

Simply put, we now understand that aggressive and impulsive behaviour could be induced by impaired MAO activity during development, resulting from multiple common environmental factors, as well as by genetic disorders which are much rarer and more dramatic in consequences.

**Effects of MAO inhibition**

To appreciate the effects of deprivation of MAO activity requires understanding of the normal functional effects of these enzymes. As discussed previously throughout this thesis, Berry et al. (1994a) reviewed the literature and concluded that, given the similar distribution of MAOs in the brain of rodents and primates, their functions ought to be similar which allows extrapolating results between the two species.

For the specific effects upon the dopaminergic and noradrenergic metabolism, there are several points to consider. *In vitro* metabolism of dopamine is largely due to MAO A, particularly because this process occurs in the intrasynaptosomal space where this form of the enzyme is more abundant. Meanwhile, the metabolism of dopamine in the extrasynaptosomal space is largely performed by MAO B and becomes relevant only in the presence of MAO A inhibition or dopaminergic recapture blockade. The latest issue is supported by the effects observed after the administration of low doses of the MAO B inhibitor deprenyl. Normally, MAO B has negligible effects upon dopaminergic metabolism, but such effects become significant after high doses of the inhibitor are administered causing the drug to loose specificity and to extend its inhibitory effects to MAO A. *In vivo* studies
in rats rendered complementary results when MAO A blockade with the MAO A inhibitor clorgyline decreased the concentration of dopamine metabolites DOPAC and HVA. Comparatively this was not achieved by the MAO B inhibitor deprenyl (Berry et al., 1994 a and b). Chronic administration of clorgyline also induced an increase in norepinephrine release in response to potassium chloride stimulation and reduced the number of α2 and β receptors in cortex and brainstem (Cohen et al., 1982; Finberg et al, 1993). This contributes to the notion of norepinephrine being mainly degraded by MAO A activity (Eisenhofer & Finberg, 1994).

Paradoxically, since 1977, Glover et al., (reviewed by Berry et al., 1994a) suggested that up to 75% of the metabolism of dopamine in the human brain occurred because of MAO B. The explanation to that phenomenon resides in the abundance of MAO B in human brain and the presence of high concentrations of dopamine. When the said catabolic activity is assessed in brain regions in which MAO A is present in a 1:1 ratio with MAO B, the metabolic ratios appear more congruent with the predominance of MAO A metabolism. From a different perspective, dopamine metabolism seems to happen mainly in the glial space, where MAO B is much more abundant in comparison to MAO A. In conclusion, low doses of dopamine elicit twice as much activity from the A form of MAO as from the B form.

However, only the absolute and chronic inhibition of both forms of the enzyme is capable of achieving an increase in the length and intensity of dopaminergic activity (Mercuri, 1997). Furthermore, other enzymes such as cathechol-O-methyl-transferase participate in dopaminergic catabolism at the same proportion that MAO does, illustrating how complex dopaminergic metabolism is.

Probably more relevant to dopaminergic and noradrenergic metabolism is the degradation of phenylethylamine by MAO B, as this
transmitter seems to be a regulator of dopamine, norepinephrine and serotonin release. Phenylethylamine is synthesized in very low amounts by catecholaminergic neurons, and is metabolized in glial cells almost exclusively by MAO B to produce phenylacetic acid. It is not stored in vesicles. It diffuses out of the cell when production increases and goes into the synaptic cleft, maintaining a constant concentration between the intra- and extraneuronal spaces. Administration of high doses of phenylethylamine, or transgenic induction of MAO B knockout, results in changes in release, recapture and receptor numbers of serotonin, dopamine and norepinephrine as explained in the text below (Boulton, 1991).

At physiological levels, phenylethylamine seems to potentiate responses to dopamine and norepinephrine without affecting other neurotransmitters. Comparatively, high levels of dopamine tend to produce a decrease in synthesis of phenylethylamine, while dopamine antagonists stimulate the production of phenylethylamine. Finally, MAO B inhibition, resulting in an increase of phenylethylamine concentration, also potentiated the effects of dopamine agonists without having any effect upon dopamine or its metabolites (Boulton, 1991; Berry et al., 1994 a and b).

Up regulation of D2 receptors and supersensitization of D1 receptors seem also to be due to the excess of phenylethylamine. Similar effects have been reported for norepinephrine (Boulton, 1991; Berry et al., 1994 a and b; Chen et al., 1999). Taking into account the effects reported here, it is easy to appreciate that MAO activity is an integral part of catecholaminergic regulatory mechanisms. Yet, MAO inhibition has to be cautiously interpreted when trying to understand how it would influence brain development. Directly related to the gestational age, the action of MAO A increases prenatally while MAO B remains absent until after birth (Berry et al., 1994a). Also, placental MAO A
activity may decrease the transfer of biogenic amines to the fetus (Abell & Kwan, 2001). Furthermore, some of the most important structures targeted by MAO activity may be present in a very primitive state depending on the age of the developing brain. Hence, let us not forget that the understanding of this particular point of neurotransmitter function is greatly not understood and largely extrapolated from adult models of neural function.

**Effects of MAO deprivation on central nervous system during development**

In order to understand how MAO A activity relates to aggressive behaviour, let us start by revisiting the effects of its absence. Complete deficiency of MAO A activity arising either through gene deletions (Warburg, 1966), or null mutations (Brunner et al., 1993 a and b), results in borderline to severe mental retardation, stereotypical movements, impulsive aggressive behaviours and sexual deviance. Biochemically these patients display an excess of normetanephrine, 3-methoxytyramine and tyramine, and very low levels of monoamine metabolites (Brunner et al., 1993 a and b; Lenders et al. 1996 and 1998;).

Monoamine oxidase A deficiency induced in mice by transgenic manipulations also produced distinct effects that resemble those seen in Brunner's syndrome. Newborn MAO A knockout mice showed stereotyped movements, impulsive and aggressive behaviour and altered mating conduct. Histochemically, serotonin was abnormally accumulated in noradrenergic neurons of the locus coeruleus and dopaminergic neurons of the substantia nigra and ventral tegmental area. Biochemically, knockout mice had excessive amounts of serotonin and norepinephrine with normal levels of dopamine and an
important decrease in the excretion of the serotonin metabolite 5 hydroxyindoleacetic acid (5HIAA) (Cases et al., 1995; Lajard et al., 1999). Interestingly, these alterations disappeared during adulthood or with the administration of the serotonin synthesis inhibitor para-chlorophenylalanine (Cases et al., 1995).

In recent years, this model has been very useful in exploring the neurodevelopmental effects of obliterating MAO activity. An early finding was that transgenic mice (Tg8) were devoid of the barrel-like configuration normally displayed in layer IV of the somatosensory cortex, and that this effect was critically related to excessive levels of serotonin, as it could be reversed administering para-chlorophenylalanine during development (Cases et al., 1996). Interestingly, normal mice that received MAO A inhibitors during development also had an abnormal pattern of distribution of neurons (Cases et al., 1996). More recently, Cases et al. (1998), reported that serotonin is not only elevated in serotoninergic structures of MAO knockout mouse brain, but is also found in brain regions typically devoid of serotonin. Possibly, this is the consequence of a "promiscuous" uptake of excessive serotonin into non-serotoninergic neurons as pharmacological blockade of all aminergic transporters (serotonin, dopamine and norepinephrine) resulted in the abolition of abnormal neuronal localization of serotonin.

Equally relevant are the studies of Upton et al. (1999) which demonstrate aberrant patterns of neuronal migration in MAO knockout mice. In their studies, the usual patterns of retinogeniculate and geniculo-cortical projections were absent in these mice, but could be re-established by inhibiting serotonin synthesis with para-chlorophenylalanine.

Regional cortical blood flow, thought to be a measure of general metabolic activity in the brain, also shows differences in the Tg8 mice
in comparison to wild type animals. It is increased in the somatosensory and barrel field neocortex, while it is decreased in entorhinal and midline motor cortex of MAO A knockout mice. Acute administration of fenfluramine increased the regional cortical blood flow in the allocortex and amygdala, while decreasing it in the somatosensory, barrel field, midline motor and retrosplenial cortices (Holschneider, et al., 2000).

Serotonin is known to regulate the medullar respiratory rhythm generator at the cervical phrenic motoneurons. In a study of phrenic motoneuron activity and morphology, Bou-Flores et al. (2000) found that transgenic MAO A mice did not have an stable pattern of activity as the one seen in control mice. Furthermore, the morphology of the said neurons is altered in that the dendritic trees lost their bipolar aspect to adopt an elevated number of varicosities and spines. As in previous cases, para-chlorophenylalanine proved to be effective in preventing the expression of these effects in MAO A knockout mice. In normal mice, a similar pattern of abnormal features to the one described above was observed when mice received serotonin$_{2A}$ receptor antagonists (Bou-Flores et al., 2000). This indicates that normal and abnormal modulation of dendritic proliferation and activity are mediated by serotonin in mutant and wild mice (Bou-Flores et al., 2000). Taken together, all these studies converge on a central point: the neurodevelopmental abnormalities seen in MAO knockout mice are significantly related, first, to the excessive amounts of serotonin found in the brains of these animals and second, to the disruption in homeostasis of multiple receptor and transporter molecules produced by the excessive levels of this critical monoamine. Is this also the case for patients with Brunner's syndrome? Unfortunately, there is no direct evidence that can point clearly to this conclusion. Metabolic studies of patients with the Brunner mutation certainly suggest a
pattern of plasma and urinary metabolites consistent with this conceptualization (Brunner et al., 1993a), but to our knowledge, there are no post-mortem data available, and no physiological investigations have been reported yet.

**Neurotransmitters and development**

Traditionally neurotransmitters are molecules considered to be necessary for the communication of neurons amongst themselves. One might well ask, what role neurotransmitters might have during early development prior to the elaboration of mature neurons. Norepinephrine, serotonin, dopamine and acetylcholine have been detected in the embryos of diverse animal species, particularly at times when they might be related to cell migration and changes in cell shape. Neurotransmitters are also present during the embryological life of lower organisms lacking organized nervous systems, where they are temporally implicated in morphogenesis and regeneration. In invertebrates neurotransmitter actions are linked to such early functions as gastrulation and cleavage after fertilization (reviewed in Lauder 1985 and 1993).

Serotonin and norepinephrine are also located in key sites during the development of the neural tube in higher vertebrates (reviewed in Lauder 1985 and 1993). Neurons containing neurotransmitters develop early in brain formation, around the time of telencephalic vesicle formation in rodents and primates (Levitt et al., 1997), and seem to have a leading role in the development of other populations of neurons to which they will connect later in life (Lauder and Krebs, 1976 and 1978, reviewed in Lauder, 1985).

To encompass the developmental roles of neurotransmitters in both lower and higher species, Lauder (1988) introduced the idea that
neurotransmitters may function as "morphogens". In addition, the distribution of neurotransmitters in both the intra- and extra-cellular spaces suggests a "humoral" role. As ontogeny recapitulates phylogeny, embryos of different species show the same progression of functions from "humoral" transmission, which acts by diffusion across a gradient, to "neural" transmission, in which transmitter is delivered precisely from the terminal regions of one cell to the receptive fields of the adjacent cell. Hence, in very primitive organisms amine transmitters appear to be involved in the control of cell proliferation, ciliary activity, motility, cell shape and morphogenic cell movements.

Receptors are the other half of the story about the morphogenic functions of neurotransmitters. Whitaker-Azmitia (1991) reviewed how receptors change in the developmental process and proposed some mechanisms of receptor-neurotransmitter interaction involved in shaping cell connections and networks. Considering what was said above about neurotransmitters as humoral factors that flood the intra- and extra-cellular environments, the specificity of development is then determined by the type of receptors that the developing cells express (reviewed in Whitaker-Azmitia, 1991 and Lauder 1985 and 1993). In other words, the function of a receptor depends on the time at which it is being considered. In this context, pharmacological manipulations occurring in immature brains may result in permanent changes manifested during adulthood.
Serotonin and development

Development of the serotoninergic system

Serotoninergic cells appear early in embryological development, on day 12 (E12), caudal to the mesencephalic flexure in the rhomboencephalon in a group of cells known as B7-9 and including the rostral raphe nuclei (Lauder, 1990). These cells appear to be functionally mature by E14 (Reisert et al., 1989). By E15 the cells in the dorsal portion of the B7-9 group, known today as B7, are subdivided in two subgroups. The dorsal subgroup remains separated in two subgroups to form the lateral portions of the dorsal raphe nucleus. The ventral subgroup migrates ventromedially and fuses along the midline in a rostro-caudal fashion. Group B8 forms the medial raphe nucleus with B5, and B9 remains separated in a rostral position in relationship to B8 and 7. Initially B9 migrates ventrally following the midline, to split later on in a bilateral fashion to its permanent location. Cells of groups B1-3 or caudal raphe nuclei, appear between E11-E12, but they are not immunoreactive until E14. They differentiate in the nuclei raphe magnus (B3), raphe obscurus (B2) and raphe pallidus (B1) (Lauder, 1990; Azmitia & Whitaker-Azmitia, 1991; Rubenstein, 1998).

Ascending projections of these nuclei are present as early as E12 and emanate from the B7-9 nuclei. They reach diencephalic structures (mammillary complex, stria terminalis, and amygdala) by E14-15, then branching out to structures such as lateral hypothalamus and supraoptic regions. By E17 they arrive at telencephalic structures in the frontal pole. In the hypothalamus these projections also connect with the ganglionic eminence crossing through the primitive globus pallidus, and medially they project to the
septal region. They also reach piriform cortex and the medial aspects of the frontal pole and hippocampus via subiculum and fornix (Lauder, 1990; Azmitia & Whitaker-Azmitia, 1991; Rubenstein, 1998). Descending projections from caudal nuclei appear by E14 and reach structures as far as the thorax through the anterior horn and the intermediolateral column (Rubenstein, 1998). Synapses start being established by E18 and adopt their adult pattern by postnatal day 21 (P21) (Lauder, 1990; Azmitia & Whitaker-Azmitia, 1991).

Serotonin receptors are also important elements in the orchestration of development. Serotonin1A, serotonin2C and serotonin2A receptors have been detected in brains of rat embryos (Lauder and Liu, 1994), as early as E17 (Roth et al., 1991). Serotonin2A receptors increase in number 8 times by P13, but decreases subsequently. Meanwhile, serotonin2C receptors increase only two fold. Comparatively, the mRNA's for these receptors augment after birth (Roth et al., 1991). Serotonin1 receptors were demonstrated to be functional in fetuses by observing the changes they undergo after administration of serotoninergic agonists or antagonists to pregnant rats. Binding assays were performed in the rat pups to measure the effects caused by the drugs, and it was discovered that these receptors adapted to the changes in neurotransmission just as adult receptors do (Whitaker-Azmitia, et al. 1987). The serotonin1A receptor seems to be particularly relevant as it is expressed in glial cells that produce growth factors when it is stimulated (Whitaker-Azmitia & Azmitia, 1989 and 1994; Azmitia et al., 1996). Immature neurons tend to express a great amount of such receptors, but as the cell matures, a decline in the receptor density is observed. The excessive amount of serotonin1A receptors is observed all over the central nervous system including regions where this receptor is not present during adulthood. The highest concentrations of serotonin1A
receptors in human brain occur in the cortex and hippocampus in the period that goes from 16 to 22 weeks of gestation, when serotonin\textsubscript{1A} receptors reside mainly in astroglial cells (Whitaker-Azmitia & Azmitia, 1994). In serotoninergic neurons, serotonin\textsubscript{1A} receptors are localized in somatodendritic areas or in the "axon-hillock". Stimulation of somatodendritic receptors produces hyperpolarization with a net inhibitory effect. Stimulation of the axon hillock receptors may have a localized effect to the axon without affecting the whole cell with hyperpolarization (Azmitia et al., 1996). In an analysis of human brains (Bar-Peled et al., 1991) it was demonstrated that serotonin\textsubscript{1A} receptors have a peak of expression in the early phases of pregnancy. Highest density occurs between 16 and 22 weeks of gestation in cortex and hippocampus. This correlates well with postnatal peaks previously reported in rodents as brain development in that period is roughly equivalent to the relatively more prolonged development in humans (Bar-Peled et al., 1991; Borella, et al., 1997).

**Serpotonin and neuronal differentiation**

Serotonin inhibits growth cone activity, induces retraction of neurites and produces abnormalities in synapse formation if applied to serotoninergic neurons in culture, which is mediated by neurite serotonin\textsubscript{1A} receptor (Haydon et al., 1987; Sikich, et al., 1990; Azmitia et al., 1996). The opposite effects are observed by the direct application of serotonin to neonatal rat brain. This suggests that perhaps *in vivo* serotonin also reacts with serotonin\textsubscript{1} astroglial receptors to release growth factors (Azmitia et al., 1990) which is manifested as neural differentiation and synaptogenesis, while *in vitro*, serotonin only stimulates autoreceptors that inhibit neurite outgrowth (Lauder, 1990; Azmitia et al., 1996; Borella, et al., 1997). More specifically, serotonin applied to serotoninergic neurons caused
complete inhibition of motility of serotoninergic growth cones. Consequently, structural changes occurred as neurite elongation was blocked. These inhibitory effects are restricted to the neuron that serotonin is acting upon. Furthermore, this action is present only if serotonin is administered adjacent to the growth cone to be affected. Other growth cones are not influenced by serotonin if it is not in close proximity. Serotonin also prevents growth cones from forming electrical synapses with other neurons by preventing them from being in close proximity in space and time (Haydon et al., 1987).

Ivgy-May et al. (1994) studied the possibility that growth cones possess structures that are precursors of those elements present in synaptic structures later in life and eventually turn into them. In their analysis they found that growth cones share some characteristics present in the mature synapses such as plasma membrane serotonin transporter and vesicles to store serotonin and avoid catabolism by MAO. This suggests that while the growth cone is "travelling" to its target tissue, it keeps its primitive functions. Once it reaches the target, synaptic structures evolve from the existing structures to immediately initiate the synaptic neurotransmission. This may be crucial in differentiating those synapses to be pruned from those that remain functional during later phases of development and maturation (Ivgy-May et al., 1994).

Whitaker-Azmitia & Azmitia (1986) postulated that serotonin is an inhibitory developmental signal to its own system, which makes sense in the timeframe during which serotonin becomes the signal for growth and differentiation. In early periods, perhaps serotonin exists in moderate concentrations that stimulate autoreceptors to produce collateral connections. Once axons elongate to target tissues and serotonin levels increase, those target tissues mature and simulate maturation of serotoninergic cells (Whitaker-Azmitia & Azmitia, 1986).
Serotonin function and aggressive behaviour.

In a comprehensive review, Lee and Coccaro (2001) remind us of the apparent paradox about the correlation between low levels of serotonin in the brains of violent subjects, and low levels of monoamine oxidases, which must produce high concentration of serotonin, but are also associated with violent behaviour.

Asberg et al. reported in 1976 that cerebrospinal fluid contents of 5-hydroxyindoleacetic acid (serotonin metabolite) were low in those patients suffering from depression that attempted suicide. These findings were replicated by Brown et al. (1979), who included aggression, suicide and low cerebrospinal fluid 5-hydroxyindoleacetic acid in a causal triad. Linnoila et al. (1983), found that violent and recidivistic offenders, as well as those with suicide histories, had lower levels of cerebrospinal fluid 5-hydroxyindoleacetic acid in comparison to those that offended only once or had no history of suicide. Similar correlations had also been reported for arsonists and animal models of violence.

These reports have not existed without being contradicted. Five reports involving normal subjects, attention deficit hyperactivity disordered subjects and personality disordered subjects have not being able to replicate these associations. Furthermore, a meta-analysis by Balaban et al. (1996), did not found a relationship between serotoninergic function and violence after controlling for height, sex and age.

In search of an alternative model, other studies have used a pharmacological challenge with fenfluramine to elicit a serotonin release that will be manifested as an increase in prolactin. Coccaro et al. (1989), used this technique to differentiate those displaying aggressive behaviours amongst personality disordered individuals,
and affective disordered subjects with or without a history of suicide. Similar studies have been done in populations of individuals suffering antisocial violent behaviours, and depression with anger attacks. Primate studies have rendered similar results.

Coccaro et al. (1997) demonstrated in another study that subjects with personality disorder who have high scores in the Life History of Aggression showed low response to fenfluramine, but normal levels of cerebrospinal fluid 5-hydroxyindoleacetic acid, which suggests that the fenfluramine challenge may be more sensitive to serotonin variation than the measurement of cerebrospinal fluid 5-hydroxyindoleacetic acid. Similar challenges have been done using different drugs such as the serotonin 5HT₁a and 5HT₂ agonist m-chlorophenylpiperazine and ipsapirone resulting in prolactin and cortisol production respectively, showing an inverse relationship with regards of aggressive behaviours. Contrary to the former findings, a direct relationship between fenfluramine and aggression was reported in substance abusers and two out of four studies in children. However, more studies are needed to clarify these conflicting results.

Platelets have also been used to measure serotonin function in search of a correlation to aggressive behaviour, due to the similarities between 5HT₂ receptors in platelets and those in brain cells which suggests for them to be under an analogous genetic control. Similarly, violent subjects have shown low quantities of platelet serotonin receptors. Platelet ⁹H-imipramine binding sites, also reflective of serotoninergic function, appeared decreased in aggressive children in comparison to non-aggressive ones.

A widely used model for the study of the role of serotonin on behavioural states such as depression or aggressive responding is the tryptophan depletion model. This is performed with some variations, by administering a beverage containing all amino acids save
tryptophan. This amino acid load stimulates protein synthesis, which in turn draws tryptophan from cellular stores resulting in a lowering of plasma tryptophan and brain serotonin. Conflicting evidence exists with regards to the extent to which this manipulation alters aggressive responding in the high trait aggressive individuals included in these studies (reviewed in Van der Does, 2001 and Bell et al., 2001). In normal monkeys, tryptophan depletion clearly increases aggressive responding only during period of elevated stress (Chamberlain et al., 1987).

Despite the presence of all this evidence, low levels of serotonin in these studies should not be taken out of the context of being measured in subjects whose brains have gone through complete development. Probably, the fact that low levels of MAO activity are related to aggressive behaviours as widely demonstrated in the literature, is much more influential during development when a delicate window of serotoninergic concentration is essential for the appropriate neural development. The high comorbidity of psychiatric disorders with aggressive phenotypes suggests biological predisposition that could be caused by circumstances present only during development but compensated for later in life.

In conclusion, we know that MAOs are one of the main elements exercising control of the concentration of neurotransmitters during central nervous system development. In this perspective, it is necessary to understand how MAO regulates the metabolism and synthesis of serotonin, in particular in placenta and in the developing organs. It is important to know whether serotonin outside the central nervous system can contribute to the concentrations of such neurotransmitter within the central nervous system, whether placental metabolism can help decrease excessive levels of serotonin.
in the product, and whether therapeutic maneuvers could be implemented in the case of disorders such as Brunner's syndrome and probably Norrie disease.

We also know that total deficiency of MAO A causes Brunner's syndrome possibly through excessive levels of serotonin during central nervous system development. However, it is important to investigate the molecular mechanisms involved in the etiopathogenic events of such a disorder. That would provide an insight into the development of more common forms of deviant aggressive and impulsive behaviour.

Even more relevant for public health is the aspect of investigating if common environmental factors such as tobacco, alcohol and drugs can affect MAO activity and developmental neurotransmitter homeostasis. Such knowledge, besides that of the genetic predisposition to low or high levels of MAO, may have predictive value in the detection of abnormal patterns of impulsive-aggressive behaviour. Such prophylactic elements should never, though, be aimed to the eugenic goal of suppressing behaviours that are essential for the survival of animal species. They should provide educational models that help to properly orient characteristics produced by the genetic-environment interactions into productive skills adaptive to life in society.
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