Serotonin Binding Proteins: An *In Vitro* Model System for Monoamine-related Neurotoxicity[a]

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INTRODUCTION

Serotonin Binding Proteins and the Housekeeping of Serotonin

Catecholamine- and serotonin- (5-HT) secreting cells have developed specific mechanisms to reduce the free concentration of monoamine within their storage vesicles and, hence, the osmotic pressure. For catecholamines, it is accepted that they are stored as macromolecular complexes with ATP.[1] On the other hand, 5-HT has long been thought to form complexes with soluble proteins: the "serotonin binding proteins" (SBP). This contention was based on the finding by Tamir and Huang[2] that 5-HT binds with high affinity to soluble proteins from brain. Later on, SBP were also found to be present in 5-HT-secreting cells from neuroectodermal origin in the periphery.[3–5]

In different species, including rat, sheep, and man, SBP have actually been shown to comprise two forms with different molecular weights: that is, 45 and 56 kDa.[5–7] Both proteins have been purified from rat brain,[6,8] and comparative studies, involving a variety of experimental approaches, all argue in favor of their close structural similarity.[6,9,10] Much research has also been devoted to determining the exact subcellular location of SBP. Cell fractionation studies suggested that at least part of the SBP could be inside 5-HT storage vesicles and, hence, be involved in the storage of 5-HT. However, this model did not comply with the observation that 5-HT binds preferentially to the 45 kDa protein when the amine is taken up by intact neurons (reflecting the presence of the 45 kDa protein in terminal synapses), whereas the binding to the 56 kDa protein predominates when the amine is added to extracted SBP *in vitro*.[3,11] To integrate these findings, it was admitted that part of the SBP, and more particularly the 56 kDa form, might be located at the cytosolic side of the vesicles or even be freely present in the cytosol. As possible functional roles of the 56 kDa protein, it was proposed that it could protect 5-HT from degradation by monoamine oxidase and/or be involved in the transport of 5-HT.[8]

[a]This work was supported by grants from the Koningin Elizabeth Foundation Belgium.

[b]This author is Onderzocksdirecteur at the National Funds voor Wetenschappelijk Onderzoek, Belgium.

SBP Bind Catecholamines as Well as 5-HT

We initially investigated SBP in soluble extracts from bovine frontal cortex.[12] These extracts were found to contain soluble proteins whose [^{3}H]5-HT binding properties and molecular weights are very close to those previously reported for SBP in rat brain. Similar to SBP from rat brain, the binding of [^{3}H]5-HT to bovine brain SBP was dependent on the presence of Fe^{2+}, whereas Fe^{3+} was ineffective.[12] Saturation binding experiments indicated that, in the presence of 0.1 mM Fe^{2+}, [^{3}H]5-HT binds with high affinity to a single class of sites (TABLE 1). SBP are also present in different regions of the human CNS (TABLE 1).

Competition binding studies with 5-HT analogs indicated that binding of these compounds to SBP is dependent on the presence of a hydroxyl group on the aromatic ring, while the structure of the aliphatic side chain is irrelevant.[12] Dopamine and related catecholamines were also able to compete with [^{3}H]5-HT for binding to bovine SBP.[12] To our surprise, catecholamines and analogs competed with about the same potency as 5-HT (IC_{50}-values between 0.2 and 0.3 μM), at least if they possessed an intact catechol moiety.[12] Since catecholamines possessed high affinity

TABLE 1. Binding of [^{3}H]5-HT and [^{3}H]dopamine to SBP from Bovine

	[^{3}H]serotonin Bound		[^{3}H]dopamine Bound	
Bovine Tissue	B_{max} (pmol/mg)	K_D (μM)	B_{max} (pmol/mg)	K_D (μM)
Frontal cortex	120 ± 12	0.12 ± 0.04	279 ± 64	0.19 ± 0.02
Retina	242 ± 10	0.22 ± 0.04	505 ± 30	0.34 ± 0.04
Adrenal medulla	124 ± 28	0.50 ± 0.01	685 ± 118	0.46 ± 0.06
Chromaffin cells	64 ± 13	0.12 ± 0.01	243 ± 5	0.44 ± 0.04
Granules	12 ± 4	0.10 ± 0.01	39 ± 4	0.49 ± 0.01

Note: Extracts were incubated with Fe^{2+} (0.1 mM) for 15 min with increasing concentrations of radioligand (10 to 500 nM). Specific binding refers to Fe^{2+}-dependent binding, and saturation curves were analyzed to yield B_{max} (pmol/mg of protein) and K_D (in μM). Values are means and SD of three experiments. (Data from Jimenez Del Rio *et al.*[12,13] and Pinxteren *et al.*[14])

for bovine SBP in competition experiments, we tried to find out whether these monoamines were specific enough to label these proteins directly. Experiments with [^{3}H]dopamine were affirmative. Indeed, binding of [^{3}H]dopamine was increased up to 5-fold when Fe^{2+} was included in the medium, but not when Fe^{3+} was included.[12] These data suggested that [^{3}H]5-HT and [^{3}H]dopamine label bovine SBP via a similar Fe^{2+}-dependent mechanism.

The putative functional roles that have been attributed to SBP were limited to the housekeeping of 5-HT (storage, protection from MAO, transport) in 5-HT-secreting cells which are derived from the neuroectoderm.[3,4,8] Since very little attention had been paid to the possibility that SBP might also interact with neurotransmitters other than 5-HT, the possibility arose that SBP might also possess some catecholamine-housekeeping function.[12] This hypothesis was strongly supported by the demonstration that SBP is present in tissues (bovine retina and adrenal medulla) and cells (bovine chromaffin cells) which are known for their ability to secrete catecholamines.[13,14] The concentration of SBP in these extracts was equivalent to or even higher than in the extracts of the frontal cortex (TABLE 1). These findings revealed that SBP may occur in neurons wherein the concentration of 5-HT is either

nil or at least too low to be detected[15,16] and, hence, that there is no positive correlation between the SBP concentration and the 5-HT content of a tissue.

MODELS FOR MONOAMINE AND RELATED NEUROTOXIN–SBP ASSOCIATIONS: INVOLVEMENT OF OXYGEN FREE RADICALS

The observation that the binding of 5-HT to SBP is strongly enhanced by Fe^{2+} but not by Fe^{3+} ions[17] constituted initial, important information concerning the molecular mechanism of the 5-HT–SBP interaction. Subsequently, it was demonstrated that SBP contain essential sulfhydryl groups at or near the binding site of 5-HT.[18] Based on this information, Tamir and Liu[18] advanced an elegant model to describe the 5-HT–SBP interaction, wherein it was postulated that Fe^{2+} first binds to sulfhydryl groups of SBP and that then, up to four 5-HT molecules should be linked to the trapped iron by coordination bonds. The ability of 5-HT analogs to bind SBP with high affinity when at least one hydroxyl group is present on the indole ring[19] was in full agreement with this model. Because of the important role of the catechol ring, this model could also be extended to describe catecholamine–SBP interactions.[12] Accordingly, catecholamines and 5-HT were proposed to bind to SBP by a similar mechanism, involving the formation of coordination bonds between the trapped iron and aromatic hydroxyl groups, that is,

$$\text{SH–SBP–SH} + Fe^{2+} \rightarrow \text{SBP–(S)}_2\text{–Fe}$$

$$\text{SBP–(S)}_2\text{–Fe} + 4\ \text{5-HT} \rightarrow \text{SBP–(S)}_2\text{–Fe–(5-HT)}_4$$

$$\text{SBP–(S)}_2\text{–Fe} + 2\ \text{catecholamine} \rightarrow \text{SBP–(S)}_2\text{–Fe–(catecholamine)}_2$$

Catecholamines are easily oxidized, and in an attempt to prevent this process, we assessed the protection action of sodium ascorbate. To our surprise, this antioxidant abolished the binding of both [^{3}H]dopamine and [^{3}H]5-HT to SBP. This finding suggested that binding of monoamines to SBP could involve an oxidative step. A number of subsequent findings confirmed this hypothesis.[20] First, the binding of [^{3}H]dopamine and [^{3}H]5-HT to SBP was also inhibited by alternative reducing reagents such as vitamin E (TABLE 2). On the other hand, powerful oxidants such as sodium periodate[21] and superoxide radicals were able to promote the binding of both monoamines in the same way as Fe^{2+} (FIG. 1). The superoxide radicals could be added either directly as potassium superoxide or be generated by an indirect mechanism such as the xanthine oxidase–catalyzed oxidation of xanthine.[22] Finally, adrenochrome, an oxidation product of adrenaline, potently competed with the binding of [^{3}H]5-HT (TABLE 3) and [^{3}H]dopamine to SBP.

Since Fe^{2+} ions are also known to promote the oxidation of catecholamines,[23] the above findings led us to formulate an alternative model to describe the [^{3}H]dopamine- and [^{3}H]5-HT–SBP interactions.[20] In this new model, it is proposed that Fe^{2+} ions do not participate in the binding process by itself, but rather that they initiate the oxidation of 5-HT and dopamine into electron-deficient species (quinoneimines, *o*-quinones) which are capable of forming covalent bonds with external nucleophiles such as the sulfhydryl groups of SBP; that is,

$$\text{Dopamine, 5-HT} \rightarrow (Fe^{2+}) \rightarrow \text{oxidation products}$$

$$\text{Oxidation products} + \text{SBP–SH} \rightarrow \text{SBP–S-oxidation products}$$

Fe^{2+} is not an oxidant by itself, but it can react with dissolved molecular oxygen to produce superoxide radicals (i.e., $Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot-}$). These radicals are known to oxidize catecholamines,[23–25] and they have recently also been found to

oxidize 5-HT.[26–28] The involvement of superoxide radicals in the Fe^{2+}-mediated binding of [3H]dopamine and [3H]5-HT to SBP was evidenced by the ability of superoxide dismutase, an enzyme which catalyzes the dismutation of superoxide radicals into molecular oxygen and hydrogen peroxide,[22] to inhibit the binding (TABLE 2).[20] The superoxide radical does not necessarily represent the only oxidant species, especially since autoxidation reactions of catecholamines are complex and may involve the generation and utilization of various reactive oxygen species such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals.[29,30] Moreover, iron is capable of catalyzing the Haber-Weiss reaction, wherein the superoxide radicals and hydrogen peroxide generate hydroxyl radicals.[30] Although these latter radicals constitute even more reactive oxygen species than the superoxide radicals,[31] we were unable to prove their paticipation in the Fe^{2+}-mediated monoamine–SBP binding (i.e., hydroxyl radical scavengers such as thiourea and ethanol did not affect the binding, TABLE 2).[20]

TABLE 2. Comparison on the Binding of [^{3}H]5-HT to Bovine Brain SBP and Actin: Effect of Reagents and Superoxide Dismutase

	[^{3}H]5-HT Bound (% Control) to			
	SBP		Actin	
Reagent Added	Total	Nonspecific	Total	Nonspecific
None (control)	100	8 ± 1	100	9 ± 1
Glutathion	9 ± 3	5 ± 1	21 ± 4	7 ± 1
b-Mercaptoethanol	8 ± 1	5 ± 0	28 ± 2	7 ± 1
Dithiothreitol	8 ± 1	6 ± 1	24 ± 3	7 ± 1
Sodium ascorbate	5 ± 1	6 ± 1	13 ± 3	9 ± 3
Vitamine E (1 mM)	7 ± 1	7 ± 1	18 ± 1	9 ± 1
EGTA	6 ± 1	8 ± 1	21 ± 2	8 ± 1
Desferal	8 ± 1	6 ± 1	23 ± 1	6 ± 1
SOD (1000U)	33 ± 2	11 ± 2	29 ± 2	12 ± 1
Ethanol (2.5 mM)	84 ± 3	9 ± 2	65 ± 8	10 ± 1
Thiourea (5 mM)	76 ± 4	7 ± 1	35 ± 2	9 ± 1

Note: Proteins were incubated for 15 min with 0.2 μM [^{3}H]5-HT and the listed additives (0.1 mM, unless otherwise indicated) either in the absence (nonspecific binding) or presence (total binding) of 0.1 mM Fe^{2+}. Values are means and SDs of 2 to 5 experiments. (Data from Jimenez Del Rio *et al.*[20] and Velez Pardo *et al.*[39])

The oxidation of dopamine, and of catecholamines in general, has been investigated in great detail,[21,32] and several of the oxidation products are known to undergo covalent binding with external nucleophiles such as the sulfhydryl groups from glutathione and proteins.[33–36] The exact mechanisms for the oxidation of 5-HT have remained obscure for a long time, but a great number of oxidation products have now been identified without ambiguity, and several of them are capable of forming covalent bonds with external nucleophiles.[37,38]

Once bound to SBP, [^{3}H]dopamine and [^{3}H]5-HT can no longer dissociate in the presence of a 5000-fold excess of unlabeled monoamine or in the presence of potent iron chelators.[20] These observations are fully compatible with the revised model wherein the monoamines should be covalently bound to SBP, but they can hardly be explained by the initial model[12,18] wherein it was postulated that the monoamines should be linked to SBP-conjugated iron by coordination bonds. The covalent nature of the binding invalidates the interpretation of saturation and competition binding

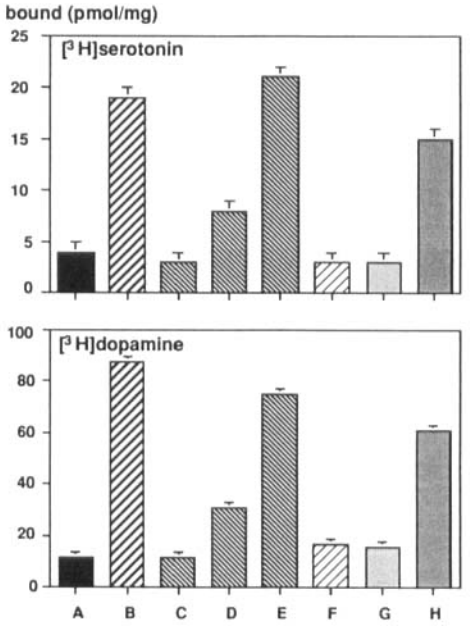


FIGURE 1. Binding of [^{3}H]5-HT and [^{3}H]dopamine to bovine brain SBP: Effect of oxidants and superoxide radical generation. Extracts were incubated with 0.2 μM of [^{3}H]5-HT or [^{3}H]dopamine for 15 min in the presence of **(A)** buffer only: **(B)** sodium periodate (100 μM); **(C to E)** potassium superoxide 10 μM, 100 μM, and 300 μM; **(F)** xanthine oxidase (0.02 U for [^{3}H]5-HT, 0.005 U for [^{3}H]dopamine); **(G)** xanthine (200 μM for [^{3}H]5-HT, and 50 μM for [^{3}H]dopamine); **(H)** xanthine oxidase + xanthine. Values are means SD of 2 to 6 experiments. (Data from Jimenez Del Rio *et al.*[20])

data in terms of reversible bimolecular interactions. Hence, the binding parameters shown in Table 1 and 3 are only apparent.

MONOAMINE–PROTEIN ASSOCIATIONS: POSSIBLE CONTRIBUTION TO NEURODEGENERATIVE DISEASES

SBP were initially proposed to store 5-HT[3] and, later on, their physiologic role was extended to include protection and transport functions for 5-HT as well as for catecholamines.[8,12] However, such potential functions of SBP can hardly be reconciliated with 1) the fact that they bind oxidation products rather than the neurotransmitters themselves, 2) the covalent nature of this binding, and 3) the fact that this binding only requires the presence of exposed nucleophiles such as sulfhydryl groups, a structural property which should be unrelated to the functional role of a protein. It is thus conceivable that the term "SBP" refers to proteins which are completely unrelated to the storage or housekeeping of monoamines. The identity and the actual physiologic roles of SBP therefore merit detailed examination. In this context, we have recently shown that Fe^{2+} is also capable of promoting the binding of [^{3}H]dopamine and [^{3}H]5-HT to actin, and this makes actin a likely candidate for the 45 kDa component of SBP.[39]

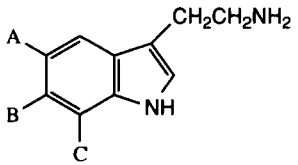
Several catecholamine- and 5-HT-related neurotoxins such as 6-hydroxydopamine, 6-aminodopamine, 5,6-dihydroxytryptamine, and 5,7-dihydroxy tryptamine undergo rapid oxidation,[35,36,40–42] and this property has traditionally been associated with the ability of these toxins to provoke nerve cell degeneration. These products only display major toxicity in a limited number of neurons, and it is generally accepted that this neuroselectivity is related to the presence of appropriate uptake sites on the target neurons. Up to now, three major molecular mechanisms have been advanced to explain the oxidation-related toxic effects of these molecules.

1. *Free-radical formation.* The auto-oxidation of, for example, 6-hydroxydopamine goes along with the production of reactive oxygen species such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals.[29,30] The radicals are particularly cytotoxic; they are prone to produce cell damage by the peroxidation of membrane lipids and breaking of DNA.[31,43]

2. *Alkylation of cell constituents.* Initial work by Saner and Thoenen[40] and subsequent studies by others revealed that the oxidation products of dopamine- and 5-HT-related toxins can be covalently attached to proteins. This could affect the activity of enzymes and structural proteins such as actin and so provoke the destruction of nerve terminals.

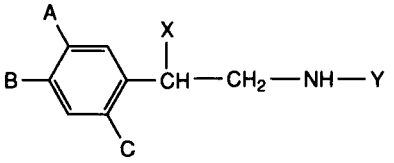
TABLE 3. [^{3}H]5-HT Binding to Bovine Brain SBP: Competition by 5-HT, Dopamine- and Related Neurotoxins

Competitor Structure



	Substitution at Position			
	A	*B*	*C*	IC_{50} (μM)
5-HT	OH	H	H	0.59 ± 0.14
5,6-DHT	OH	OH	H	0.17 ± 0.06
5,7-DHT	OH	H	OH	0.57 ± 0.16

Competitor Structure



	Substitution at Position					
	A	*B*	*X*	*C*	*Y*	IC_{50} (μM)
Dopamine	OH	OH	H	H	H	0.28 ± 0.04
6-OH-dopamine	OH	OH	H	OH	H	4.60 ± 1.20
TIQ	H	H	H	Closed ring		1200 ± 350
2-Methyl-6,7-diOH-TIQ	OH	OH	H	Closed ring		0.31 ± 0.01
Adrenochrome	=O	=O	OH	Closed ring + CH_3		0.78 ± 0.10

Note: Extracts were incubated with 0.1 mM Fe^{2+}, 0.2 μM [^{3}H]5-HT, and increasing concentrations (from 1 nM to 10 mM) of the competitors listed. IC_{50} values are means and SD's of three experiments. (Data form Jimenez Del Rio *et al.*[45])

3. *Redox cycling.* Sinhababu and Borchardt[44] proposed redox cycling as a possible mechanism for the neurodegenerative effects of 5,7-DHT. According to this mechanism, the monoamine should constantly cycle between its oxidized and its reduced form, thereby depleting the cell's oxygen as well as certain reducing agents.

The inhibition effect of the investigated catecholamine- and 5-HT-related neurotoxins on the [^{3}H]5-HT– and [^{3}H]dopamine–SBP association (TABLE 3)[45] adds support to the "alkylation theory." However, we do not exclude the possibility that all three mechanisms may act in concert to produce neurodegeneration and that their relative impact may vary from one toxin to another.

5-HT and dopamine oxidize much more slowly than their related neurotoxins,[35,36,40,41] and they also bind to an appreciably lesser extent to proteins.[40,46] However, Fe^{2+} is capable of enhancing monoamine oxidation and, consequently, their binding to proteins. In this context, we find it of particular interest that some of the oxidation products of 5-HT such as tryptamine-4,5-dione[28,47–49] and of catecholamines such as adrenochrome[50,51] are cytotoxic and are able to bind proteins. We therefore suspect that the test which was initially developed to evidence the 5-HT-storing role of SBP[2] might actually represent an *in vitro* model for neurodegeneration.

Based on these considerations, it is thus conceivable that 5-HT and dopamine may present cytotoxicity under conditions where the concentration of iron/oxygen free radicals is elevated and the concentration of reducing and sulfhydryl-group-protecting agents such as glutathione is low. Such conditions may occur in several pathophysiological conditions, including idiopathic parkinsonism. This disorder is characterized by a gradual degeneration of nigro-striatal dopaminergic neurons, and cell loss is especially pronounced in the pars compacta region of the substantia nigra.[52,53] The etiology of this degeneration is not known. However, neurochemical research has pointed out that there are marked differences in the substantia nigra between patients with idiopathic Parkinson's disease and age matched neurologically normal adults. In this context, recent evidence indicates that the substantia nigra of these patients contains increased iron (which enhances oxidation) and decreased glutathione (which protects against the formation of free radicals), and that these differences even accentuate as the disease progresses.[54–57] These and other changes are indicative of an oxidative stress syndrome, and this has led several authors to assume that free radicals generated from oxidation reactions may contribute to the pathogenesis of Parkinson's disease.[56,58–62]

Dopamine is generally believed to play a crucial role in the generation of oxygen free radicals in the substantia nigra. Such radicals may indeed be produced by dopamine autoxidation[35,36,61] and/or by its oxidative deamination by the monoamine oxidaze B enzyme.[63] Iron could therefore contribute to the generation of oxygen free radicals in different ways. It could convert hydrogen peroxide, which is produced along with the oxidative deamination of dopamine, into hydroxyl radicals.[64] In addition, since no tyrosinase activity can be detected in the substantia nigra,[65] iron could also promote the autoxidation of dopamine.

Dopamine autoxidation is likely to occur in various brain regions, even in normal adults. This statement is based on the presence in the brain of 5-*S*-cysteinyl-dopamine,[66] a metabolite whose formation requires the autoxidation of dopamine.[34,65,66] The highest levels of 5-*S*-cysteinyl-dopamine have been detected in the substantia nigra,[66] suggesting that the autoxidation of dopamine is most prominent in this brain region. Interestingly, 5-*S*-cysteinyl-dopamine also constitutes the main source of neuromelanin.[65] Like fossil remains, neuromelanin represents a "record" of the cell's past, and more precisely, of the conditions under which the autoxidation

of dopamine took place. Neuromelanin is mainly formed from 5-*S*-cysteinyl-dopamine,[65] and this indicates that the dopamine-derived quinones are rapidly inactivated by reduced glutathione in the healthy brain. However, part of the neuromelanin is also of the eumelanin type (i.e., no conjugated cysteine), and this has led Carstam *et al.*[65] to conclude that there may be occasional exhaustion of the glutathione reduction system at the site of neuromelanin formation. Taken together, there is solid biochemical evidence for the occurrence of dopamine autoxidation and for the occasional depletion of the small sulfhydryl group containing molecules in the substantia nigra. Such conditions are prone to promote covalent interactions between quinones and cellular proteins, and whereas such associations might be of limited importance in the healthy brain, it is conceivable that they could become more explicit when the cells are under increased oxidative stress such as in Parkinson's disease. We are therefore tempted to conclude that, besides the oxidative deamination pathway, the autoxidation of dopamine could also effectively contribute to the progression of Parkinson's disease both via the formation of free radicals and via the formation of reactive quinones.

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