1

The Dopaminergic System

1.1 Introduction

The dopaminergic system can be defined as a group of nerve cells (dopaminergic neurons) mainly located in the midbrain that extend their axons to different sections of the forebrain. Five pathways of dopaminergic system are identified [1] (Figure 1.1): (i) *Nigrostriatal*, going from the substantia nigra to dorsal striatum, involved in facilitating movements, (ii) *Mesocortical*, going to the frontal lobes, particularly the prefrontal cortex, involved in modulating cognitive function, (iii) *Mesolimbic,* innervating the ventral striatum (nucleus accumbens), involved in emotions and reward, (iv) *Tuberoinfundibular*, located in the arcuate nucleus of the hypothalamus and controlling prolactin secretion from the anterior pituitary gland, and (v) *Thalamic*, described in primates. It innervates the thalamus, and its function is not well known although it is involved in sleep and arousal mechanisms.

The central dopaminergic neurons can be divided into ascending, descending, and local neuron systems [2] and are directly implicated in Parkinson's disease (PD) [3] and related complications such as depression, [4] schizophrenia [5], and several neuroendocrine disorders. Depending on the affected pathway, a different neurological disorder can arise. The most known, affecting 2% of population, is PD resulting from the death of neurons in the substantia nigra (nigrostriatal pathway) [6, 7] and, consequently, causing movement disorders such as shaking, rigidity, slowness, and difficulty with walking. Chorea and addiction are also related to nigrostriatal pathway. Schizophrenia is related to mesocorticolimbic projection; in particular, mesocortical and mesolimbic pathways are related to positive and negative symptoms of the pathology, respectively. The mesocorticolimbic pathways are also linked to addiction and attention-deficit hyperactivity disorder (ADHD). Finally, the tuberoinfundibular pathway is affected in hypoprolactinemia.

At a molecular level, the main actors of the dopaminergic system are the neurotransmitter dopamine (4-(2-aminoethyl)benzene-1,2-diol; DA) and dopamine receptors (DRs). DA is biosynthesized from the amino acid tyrosine by the action of tyrosine hydroxylase, which forms dihydroxyphenylalanine (DOPA) that is decarboxylated by DOPA decarboxylase to yield DA (Figure 1.1).

The DRs play a crucial role in DA signaling since they are responsible for the signal transduction. In fact, their drug-mediated stimulation can compensate the

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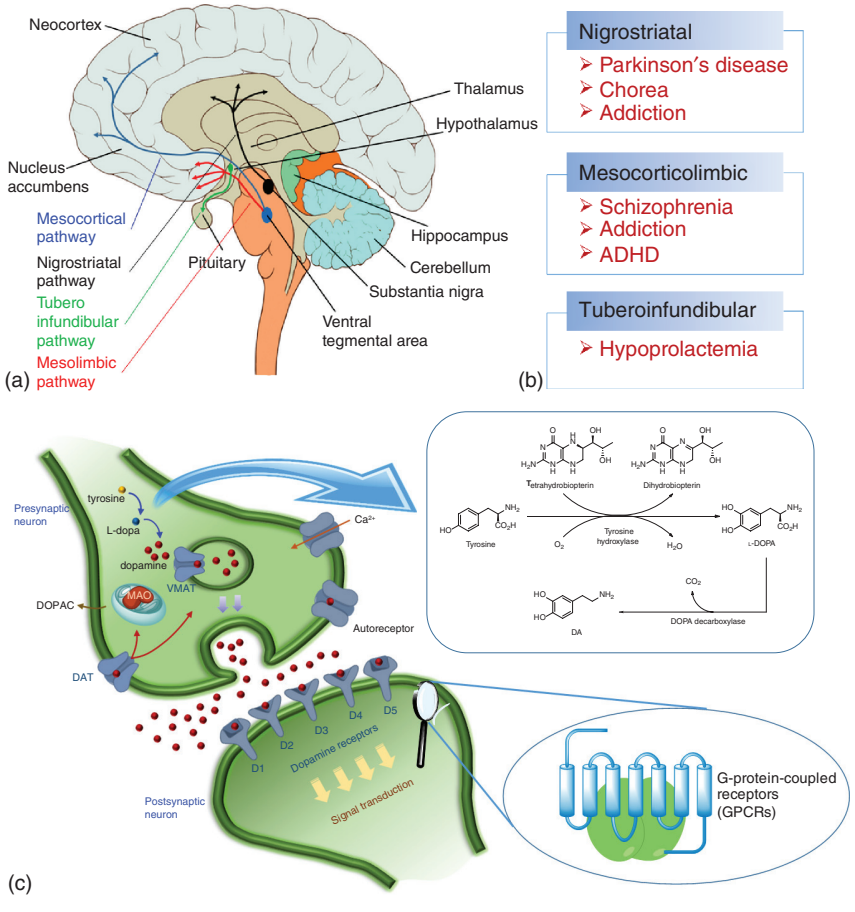


Figure 1.1 The dopaminergic system. (a) Pathways of dopaminergic system (only the four identified in humans are shown). (b) Neurological disorders associated with the pathways. (c) Dopaminergic synapse. Dopamine (DA) is synthesized from tyrosine inside the dopaminergic neuron. After packaging in vesicles by VMAT, DA is released into the synapse. DA is recognized by dopamine receptors that produce the signal. The excess of DA is removed from the synaptic area by DAT and reintroduced in the neuron for being reused or catabolized by mitochondrial MAO. VMAT: vesicular monoamine transporter; DAT: dopamine transporter; D_1–D_5: dopamine receptors; MAO: monoamine oxidase; DOPAC: 3,4-dihydroxyphenylacetic acid.

lack of DA in the first stages of neurodisorders such as PD [8, 9]. DRs are part of the catecholamine-binding family of G-protein-coupled receptors [10]. The superfamily of receptors are membrane proteins for which several computational predictions of their structures have been reported [11]. Recently, some X-ray structures have been resolved, but none are related to dopamine receptors [12, 13]. Biological aspects of DRs related to selective drugs, modulation of signaling, and trafficking have been collected in a volume edited by Neve in 2010 [14]. The treatise also includes links between DRs and neurodisorders such as schizophrenia, PD, and other neuropsychiatric disorders.

Once DA is synthesized inside dopaminergic neurons, it is packed into synaptic vesicles by a vesicular monoamine transporter (VMAT) and then released at nerve terminals into the synapse (Figure 1.1). The released DA binds to DRs to produce a signal in the postsynaptic neuron. However, DRs are widely expressed away from dopamine synapses; hence it is not evident how dopamine synapses are involved in dopaminergic transmission. Recent studies have demonstrated that dopamine synapse is a contact formed between dopaminergic presynaptic and GABAergic postsynaptic structures [15]. After the signal transduction, to stop signaling, dopamine is removed from the synaptic cleft by the dopamine transporter (DAT) that reenters DA to the presynaptic neuron for repackaging into vesicles. DA accumulated in the cytosol is recycled to form 3,4-dihydroxyphenylacetic acid, DOPAC, by the action of monoamine oxidase, MAO.

1.2 Dopamine Receptors

1.2.1 Classification

Dopamine receptors are classified into two groups according to initial pharmacological and biochemical studies [16]. These studies suggested two types of dopamine receptors: (D_1) activating adenylyl cyclase (AC) (the enzyme that converts adenosine triphosphate, ATP, into cyclic adenosine monophosphate, cAMP) and the other inhibiting the enzyme (D_2) [17]. Further analysis of cloned full-length cDNAs by several groups [18–21] clearly identified the D_1 receptor. Further cloning revealed two additional receptors named D_3 [22] and D_4 [23]. According to their pharmacology and structure, the two new receptors were included in the initially assigned D_2-type. At the same time, a novel D_1-like receptor (based on its sequence and pharmacological profile) was cloned and identified as D_5 [24, 25]. So, two groups are defined on the basis of their ability to activate/inhibit adenylyl cyclase: D_1-like receptors and D_2-like receptors [26]. The D_1-like group includes D_1 and D_5 receptors, which in some texts are referred to as D_{1A} and D_{1B}, respectively. These receptors present very high homology in their transmembrane domains and ligand-binding features. The D_2-like group comprising D_2, D_3, and D_4 receptors also shares a similar homology between them. Genetics also serves to identify the two groups of dopamine receptors. While D_1-like receptor genes are intronless [27], the genes encoding D_2-like receptors are interrupted by introns [28]. The presence of introns leads to the generation of splice variants and thus there are two D_2 receptor isoforms: D_{2L} and D_{2S} [29], different nonfunctional isoforms of D_3 [30] and polymorphic forms within the coding sequence of D_4 receptor [31].

1.2.2 Location

Dopamine receptors are localized in different parts of the brain. D_1 receptors are the most widespread and expressed than any other receptor in the central nervous system (CNS) [32]. Associated with chromosome 5, they are highly expressed in the caudate putamen, the nucleus accumbens, the zona reticulata of substantia

nigra, and the olfactory tubercle. Lower expression of D_1 receptors has been found in the dorsolateral prefrontal cortex, cingulate cortex, habenular, and hippocampus [33]. D_5 receptors are associated with chromosome 4 and expressed in the hippocampus, cerebral cortex, and hypothalamus, so they are related to pain stimuli. Actually, D_1 and D_5 receptors cannot be distinguished by radioligand binding, the usual methodology for locating dopamine receptors; hence, their distribution has been determined using receptor-selective antibodies [34]. D_5 receptors are predominant in pyramidal cells of the hippocampus and frontal cortex, and they are also found in the cerebellum [35]. D_2 receptors, associated with chromosomes 6 and 7, are preferentially located in the zona compacta of the substantia nigra. Other locations include glomerular layer of the olfactory bulb, bed nucleus of the stria terminalis, hypothalamus, habenula, hippocampus, and the intermediate lobe of pituitary [36]. D_3 receptors, associated with chromosome 3, are mainly expressed in two regions of the limbic system called the islands of Calleja and nucleus accumbens as well as the olfactory tubercle [37, 38]. D_4 receptors are associated with chromosome 11, and they are mainly located in the hippocampus and caudate putamen [39]; less predominant but in similar amounts they are also located at olfactory tubercle and substantia nigra. So, D_4 receptors are associated with both limbic structures and motor areas [40].

1.2.3 General Structural Considerations

Dopamine receptors such as other G-protein-coupled receptors (GPCRs) are characterized by seven transmembrane domains with three intracellular loops (il_{1-3}) and three extracellular loops (ol_{1-3}), the amino and carbon termini being extracellular and cytoplasmic, respectively [41]. Despite the similarity in the transmembrane regions, there are several structural differences between D_1-like and D_2-like receptors. In D_1-like receptors, the C-terminal tail is about seven times longer and the third intracellular loop is shorter, compared to D_2-like receptors (Figure 1.2) [42, 43]. In both D_1-like and D_2-like receptors, the C-terminal tail is rich in threonine and serine residues, containing a cysteine residue. Figure 1.2 also collects the amino acid sequence for the five dopamine receptors. Dopamine receptors are subject to posttranslational modifications including glycosylation in Asn residues, palmitoylation in Cys residues, and phosphorylation in Ser or Thr residues (Figure 1.2).

The presence of isoforms for dopamine receptors furnishes a wider range of choices to couple to G-proteins. Due to this diversity along with the heterotrimeric nature of the G-proteins themselves (Figure 1.2, a,b), Dopamine receptors can couple different families of G-proteins [44]. Indeed, both types of dopamine receptors form dimers and oligomers. Examples of homo- and hetero-oligomerization are reported in several studies. Homo-oligomerization of D_1 [45] and D_3 [46] dopamine receptors has been demonstrated. The formation of homo-oligomers for D_2 receptors has also been evidenced by ligand binding studies [47], cysteine cross-linking [48], and visualization of oligomeric complexes by Western blotting [49]. Hetero-oligomers of D_2 receptors with D_1 [50] and D_3 [51] have been demonstrated *in vitro*. Heteromers D_1–D_3 have been studied in detail as a target for the treatment of schizophrenia and motor dysfunctions

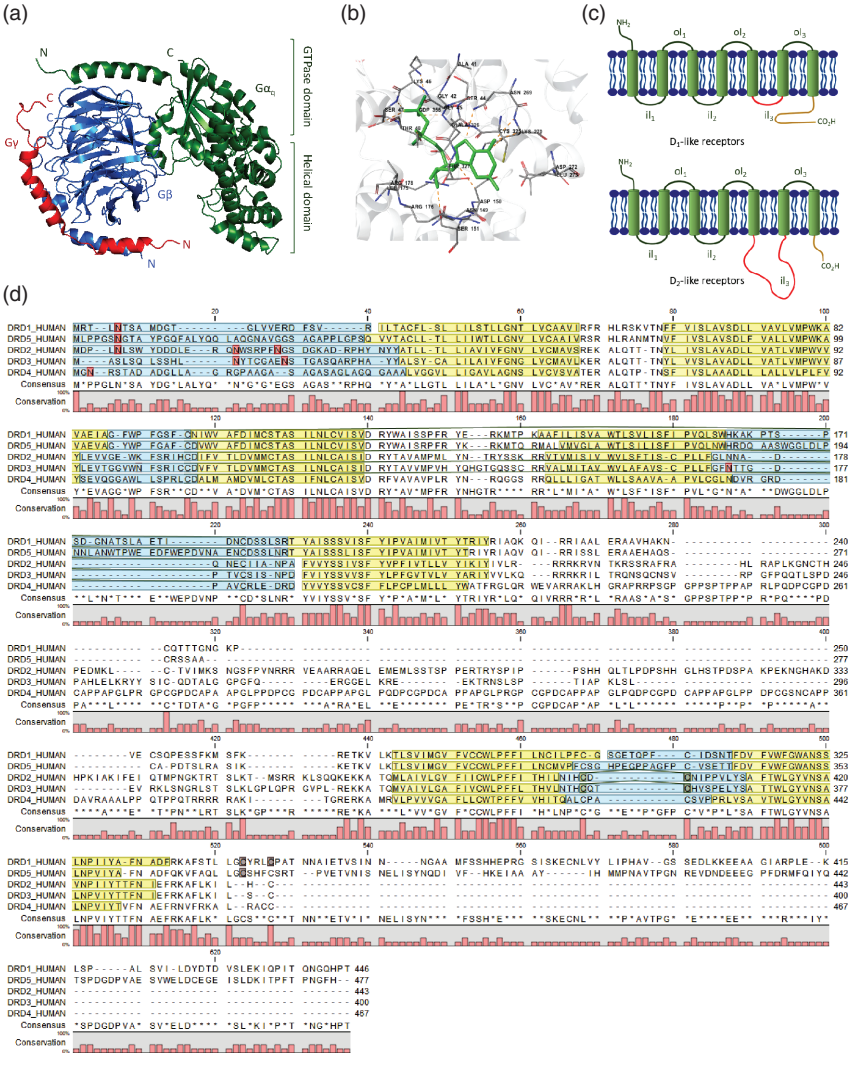


Figure 1.2 (a) Overall structure of the $G\alpha_{i/q}\beta\gamma$ heterotrimer (PDB ID: 3AH8). The heterotrimer is viewed with the expected orientation at the plasma membrane. $G\alpha_{i/q}$ (green) consists of the GTPase and the helical domains. Gβ and Gγ are blue and red, respectively. (b) Details on the interactions between GDP (green) and residues of $G\alpha_{i/q}$ (PDB ID: 1GG2). (c) General schematic structures of D_1- and D_2-like dopamine receptors. Differences are highlighted for the third intracellular loop (red) and the C-terminal tail (brown). (d) Sequence alignment of the five dopamine receptors. Conservation graphic and consensus sequence are shown. Disulfide bonds (green), N-glycosylation sites (red), palmitoylation sites (brown), extracellular domains (light blue), intracellular domains (white), and transmembrane domains (yellow) are indicated on sequences.

[52]. D_2 receptors also form hetero-oligomers with other receptors such as CB_1 cannabinoid receptor [53], A_{2A} adenosine receptor [54], and somatostatin receptor subtype SSTR5 [55]. D_1 receptors also form hetero-oligomers with A_1 adenosine [56] and glutamate *N*-methyl-D-aspartate (NMDA) receptors [57].

1.2.4 Effector Mechanisms

Dopamine receptors activate multiple and diverse signaling pathways through selective coupling to different G-proteins. Indeed, both D_1-like and D_2-like dopamine receptors have a "typical" GPCR structure, but differ in that the D_1 receptor has a smaller third intracellular loop (il_3) but a longer intracellular C-terminal tail compared to the D_2 receptor. GPCRs are a superfamily of membrane proteins, which sense diverse molecules outside of the cell and transfer the information inside the cell where they regulate a number of signaling pathways [58]. GPCRs have been extensively studied, and several comprehensive dedicated treatises have been published elsewhere [59–61]. A number of various volumes published are dedicated to particular aspects such as ligand design [62], pharmaceutical targets [63] and drug discovery [64, 65], pharmacology [66, 67], modeling [68], methods [69], structure and function [70], and their relationship with neurobiology [71], and related diseases [72]. The reader is directed to these volumes for detailed information on GPCRs.

Regarding effector mechanisms, D_1-type receptors couple to G_s (and G_{olf} in the striatum), which causes activation of adenylyl cyclase and consequently, increasing cAMP formation, which activates protein kinase A (PKA) (Figure 1.3). On the contrary, D_2-type receptors couple to $G_{i/o}$ guanosine triphosphate (GTP)-binding proteins inhibiting cAMP formation and dissociating βγ subunits from $G_{i/o}$. The dissociated βγ subunits inhibit voltage-dependent calcium channels (VDCCs) and activate voltage-sensitive potassium channels G-protein-coupled inwardly rectifying potassium (GIRKs) as well as a phospholipase C isozyme, resulting in an increase in intracellular calcium, which leads to activation of kinases and phosphatases including mitogen-activated protein kinase (MAPK), protein kinase C (PKC), and calmodulin-stimulated enzymes such as calcium/

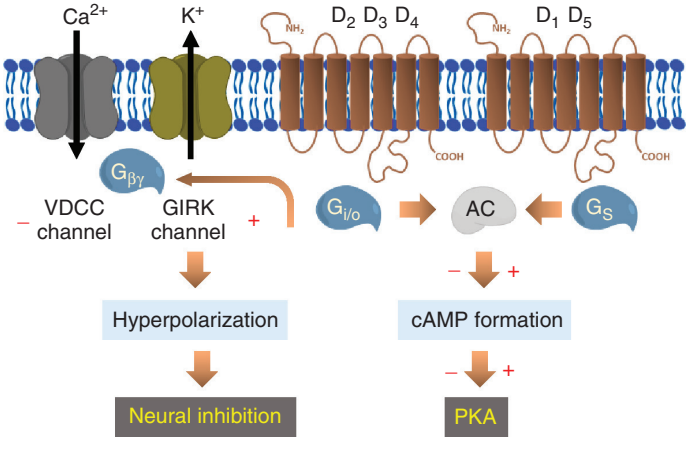


Figure 1.3 Signal transduction of dopaminergic receptors via coupling to individual G-proteins (e.g. $G_{i/o}$, G_s, and G_q). AC: adenylyl cyclase; GIRK: G-protein-coupled inwardly rectifying potassium; VDCC: voltage-dependent calcium channel; PLC: phospholipase C; PKA: protein kinase A.

calmodulin-stimulated protein kinases (CaMK), as well as protein phosphatase-2B (PP-2B).

1.2.5 Implication in Neurodisorders

The complexity of dopamine signaling – largely conserved in both invertebrates and vertebrates – is increased as it can be affected by minor alterations in receptor/G-protein coupling. Such alterations may be responsible for imbalanced signaling detected in dopamine-related disorders such as schizophrenia, PD, and ADHD. Even though both D_1-like and D_2-like dopamine receptors are important for dopamine function in basal ganglia and frontal cortical areas, D_2-like receptors are the ones targeted therapeutically by antipsychotic and anti-Parkinson's disease drugs. In fact, D_2-like receptors are involved in several neurodisorders including PD, Tourette's syndrome, schizophrenia, and hyperprolactinemia. D_2-like receptors are blocked in varying degrees by antipsychotic drugs. D_1, D_2, and D_3 receptors are directly involved in motor activity. D_1 and D_2 receptors are also involved in reward and reinforcing properties of different drugs of abuse as well as in learning and memory [26]. D_2 receptor is directly involved in psychiatric disorders, especially schizophrenia [73], and D_3 receptor has been suggested as target for the treatment of depression [74]. In general, D_2, D_3, and D_4 receptors have similar pharmacology, although subtle differences are emerging with the development of more specific ligands; for instance, D_4 receptors have a particularly high affinity for the antipsychotic drug clozapine. All three subtypes have postsynaptic locations in dopamine terminal areas.

1.2.6 D_1-like Receptors

D_1-like receptors couple to G_S and α-units of G_{olf} in the striatum and stimulate adenylate cyclase to increase the concentration of intracellular cAMP [75]. This coupling has been demonstrated in neostriatum, where $G\alpha_{olf}$ is abundant and expression of D_1 is high, whereas expression of $G\alpha_s$ is low [76]. Similarly, in basal ganglia nuclei, the $G\alpha_{olf}$ is the primary G-protein coupled to D_1Rs [75]. Additional *in vitro* studies indicated that D_1 receptors can also couple to other proteins such as $G\alpha_z$, $G\alpha_o$, $G\alpha_{i1}$, and $G\alpha_{i2}$ [77]. Details of the interactions between D_1 receptors and G-proteins have been obtained using several fluorescence techniques. In the absence of receptor activation, no preassociated complexes were detected, which suggests that D_1 receptors alter the distribution of $G\alpha_s$ and $G\alpha_{i3}$ subunits inside the membrane [78]. In PD patients, $G\alpha_{olf}$ is highly expressed in striatum, where dopamine is deficient; on the contrary, $G\alpha_s$ is less abundant. D_1 receptors are also involved in the regulation of calcium, sodium, and potassium channels in striatal and prefrontal cortical neurons. The intracellular Ca^{2+} concentration is a key parameter for neurotransmitter release and synaptic plasticity [79]. However, the mechanism through which D_1-mediated Ca^{2+} current regulates neuronal activity is still unknown.

The structure of D_1 receptors is common for all the mammals having 446 residues. The general structure for D_1-like receptors is shown in Figure 1.3. Potential sites for N-glycosylation (Asn5 at the amino terminus and Asn175 in the third

extracellular domain) have been identified, but no effect of glycosylation on the function (or expression) has been revealed [80]. Phosphorylation by cyclic-adenosine monophosphate (c-AMP)-dependent kinase (PKA) takes place at (i) Thr136 in the second cytoplasmic loop, within the sequence R-K-X-T, (ii) Thr268, at the C-terminal end of the third cytoplasmic loop, within the less commonly phosphorylated sequence R-X-T, and (iii) Ser380 (R-X-S) [81]. Most isoforms, but not all, of PKC can phosphorylate D_1 receptors, regulating signaling [82]. Phosphorylation by the GPCR kinase family is also possible at multiple Ser and Thr residues present in the long cytoplasmic tail in proximity to acidic residues. At Cys347 and Cys351, the polypeptide is palmitoylated [83]. Notably, when palmitoylation is blocked by mutation of Cys347 and Cys351, D_1 receptor activation of G-proteins is not affected [84]. On the other hand, replacement of Leu344 and Leu345 at C-terminus by alanines decreased glycosylation, suggesting an important role in plasma membrane trafficking for such di-leucine motif [85]. The N-terminus plays a crucial role in defining the specific ligand (D_1 or D_5) affinity and ligand-induced adenylate cyclase activation [86].

The difficulty in obtaining suitable crystals for GPCRs, for which the only available high-resolution crystal structure is that of bovine rhodopsin [87], has limited the availability of three-dimensional structures of D_1-like receptors. To gain insights into dopamine receptors function and structure, homology-modeling techniques based on the crystal structure of bovine rhodopsin have been used [88–91]. However, further studies demonstrated that due to the low sequence identity of GPCRs with rhodopsin (less than 20%), homology-based approaches provide unreliable three-dimensional structures to be used for making predictions [92]. Actually, homology models based on bovine rhodopsin provide good correlation for antagonists but not for agonists [93]. Other authors have used MembStruk [94, 95] and HierDock [96] methods for prediction of structure and ligand-binding sites of several dopamine receptors [97–99]. In particular, the structure of D_1 dopamine receptors has been predicted based on the modeled D_2 structure using the MembStruk procedure. Seven transmembrane helices of 19–29 residues per helix were predicted (Figure 1.4) [101].

The binding site of dopamine was determined using the HierDock procedure [97], and selective pharmacophore models have been developed [102]. Several interactions between dopamine and D_1 receptors have been reported. At physiological pH condition, the amine functionality is protonated, and it is known it interacts with receptors [103]. In particular, dopamine forms a salt bridge with the highly conserved Asp103 located in transmembrane segment 3 (TM3) [104]. The catechol moiety of dopamine forms H-bonds with conserved Ser198, Ser199, and Ser202 located in TM5 [105, 106]. In fact, any mutation from serine to alanine in that position reduces activity of dopamine and catechol-agonists of D_1 receptors [107]. The conserved aromatic residues Trp285, Phe289, Ph296, and Phe297 have been suggested to participate in π-interactions playing an important role in D_1 activation [103]; however, it has not been tested with D_1 receptors yet. The interaction of D_1 receptors with non-catechol derivatives has been studied with ergoline-based ligands [108], which are known to have antipsychotic properties [109]. Conserved aromatic residues at TM6 are crucial for binding affinity of ergolines [110].

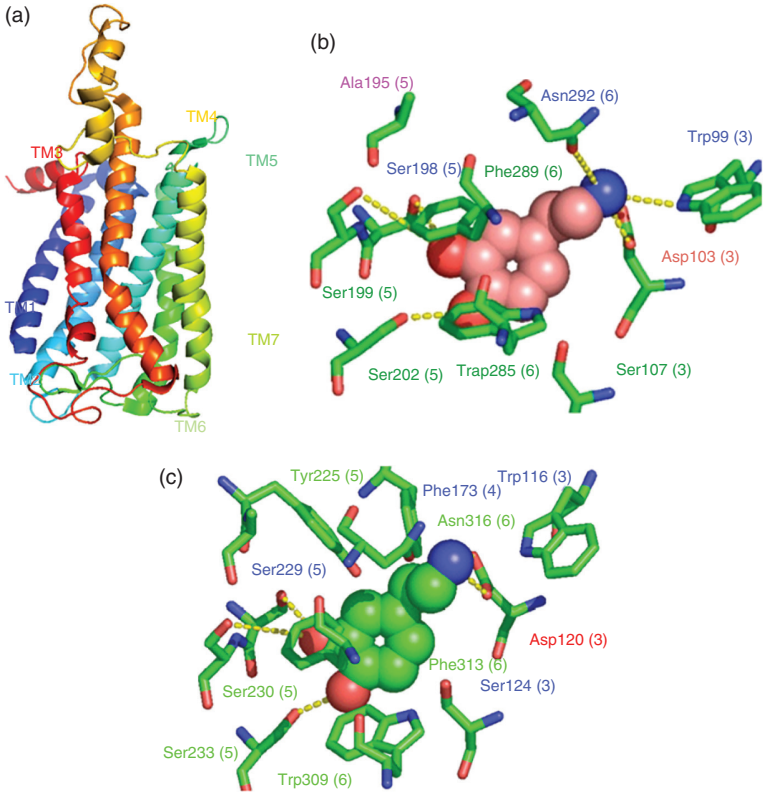


Figure 1.4 (a) Predicted structure for D_1 dopamine receptor. The seven transmembrane helices are given in different colors. (b, c) Predicted binding sites and interactions for dopamine in D_1 (b) and D_5 (c) receptors (numbers in the brackets indicate the TM helices to which the residues belong to). Source: (b, c) Reproduced with permission from Kalani 2004 [100]. Copyright 2004, California Institute of Technology.

D_5 receptors have 477 residues, and although they are genetically different from D_1 receptors, both receptors share c. 60% amino acid identity overall and 82% identity in the transmembrane-spanning regions [111]. D_5 receptors couple to $G\alpha_s$ [112] and in a minor extent to $G\alpha_{olf}$ [113, 114]. Binding of dopamine or an agonist induces a conformational change that promotes interaction and, thus, activation of $G\alpha_s$. Through this interaction, exchange of guanosine diphosphate (GDP) for GTP is facilitated, leading to dissociation of the α and β-γ subunits of G-proteins and stimulation of adenylate cyclase with subsequent production of c-AMP and activation of PKA. There is also further evidence for coupling of D_5 with $G\alpha_z$ [114], suggesting various signaling responses upon simultaneous stimulation of both D_1 and D_5 receptors [113].

Despite the structural and pharmacological similarity between D_1 and D_5 receptors, there are functional differences in the cellular expression and distribution, indicating that they cannot reconstitute each other when a pathology is present [115]. However, until now there are no highly selective D_1 or D_5 receptor ligands, dopamine being the most discriminating (D_5 receptors have a higher

affinity for DA than D_1 receptors); consequently, their design constitutes a current challenge [116]. Recent studies have demonstrated that D_5 stimulation may enable cells to better support adjacent neurons [117]. The structure of D_5 dopamine receptors has been modeled based on the template of high-resolution crystal structure of human β_2 adrenergic receptor (PDB ID: 2RH1A) [118]. The predicted binding sites included all the seven transmembrane helices corresponding to the general structure outlined in Figure 1.4. Sites for N-glycosylation are found in first and third extracellular (ol_3) domains. While glycosylation at Asn7 and Asn198 is required for receptor trafficking [119], it is not necessary for ligand binding [80]. The palmitoylation sites of D_5 are conserved from D_1 receptors. Phosphorylation by PKA and G-protein receptor kinases takes place at the third cytoplasmic loop (il_3) and carboxy-terminal tail, respectively. The D_5 dopamine receptors form a complex with the GABA-A ligand-gated ion channel, leading to reciprocal inhibition of both inhibitors [120]. Deficiencies in D_5 receptors are associated with learning problems directly connected to ADHD [121]. Polymorphism of D_5 receptors is associated with schizophrenia [122] but not with PD [123].

As in the case of D_1 receptors, the structure of D_5 receptors has been predicted using the MembStruk procedure. The binding site of dopamine has been determined using the HierDock procedure and a library of 11 ligands has been studied [124]. Seven helical motifs were identified and very similar to those of D_1 receptors. From the predicted structure, docked conformation of dopamine showed a stabilizing salt bridge with Asp120 located at TM3. In addition, a network of H-bonds formed by Ser229, Ser230, and Ser233 located at TM5 was found to interact with catechol moiety. Up to eight residues (Trp116, Ser124, Phe173, Tyr225, Ala226, Trp309, Phe313, and Asn316) were identified in the binding site as candidates to constitute a hydrophobic pocket. A model based on the X-ray structure of human β_2 adrenergic receptor led to very similar results in predicting binding sites [118].

1.2.7 D_2-like Receptors

D_2-like receptors couple to heterotrimeric $G\alpha_i$ or $G\alpha_o$ GTP-binding proteins to inhibit adenylate cyclase activity, reducing the concentration of cAMP, and modulate potassium and calcium ion channel activities. The large third intracellular loop il_3 – essential for G-coupling [125–127] – is a characteristic of all D_2-like receptors. Depending on the receptor subtype, different subunits are coupled. Several studies on coupling between D_{2S} and D_{2L} receptors and G-proteins have been carried out. Depending on the studies, evidences on preferential coupling with $G\alpha_{i2}$, $G\alpha_{i2}$, $G\alpha_{i3}$, or $G\alpha_{o1}$ subunits have been observed [128–130], the latter being the most strongly activated subtype [131]. Initially, D_3 receptor was reported not to couple G-proteins [132]; however, it has been demonstrated in some cell lines that it can inhibit adenylate cyclase [133], suggesting coupling with $G\alpha_q$ or $G\alpha_{1l}$ subunits [134]. The human D_4 receptor activates $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{o1}$ subunits in a similar way to D_2 [135]. The rat D_4 receptor preferentially couples to $G\alpha_z$ and the transducin subtype $G\alpha_{t2}$ [136].

As in D_1-like receptors, in D_2-like receptors there are potential sites for N-glycosylation and phosphorylation although the regulation of the latter is not as well understood as that of the former. Nevertheless, it is known that phosphorylation is exerted by second-messenger activated and GPCR-specific protein kinases, and it has been demonstrated for D_2 and D_3 subtypes. In D_2-like receptors, the short C-terminus ends with a region containing a cysteine residue, conserved in many GPCRs, and identified as the palmitoylation site.

The two isoforms of D_2 receptor, D_{2S} and D_{2L}, differ in the insertion of a stretch of 29 amino acids in the third intracellular loop il_3. In general, the D_{2L} isoform is predominant over the D_{2S} isoform although the latter is the sole autoreceptor on dopaminergic neurons [137]. A third isoform (longer) has been identified in which Val270 is replaced by the sequence Val-Val-Gln.

Initial studies using predicted structures suggested that highly conserved residues within the hydrophobic TM domains define a binding pocket corresponding to the agonist-binding site [97]. In particular, the carboxyl group of Asp114 (a residue located in TM3) forms a salt bridge with the amino group of dopamine, as demonstrated by mutagenesis [138]. Hydroxyl groups at meta- and para-positions in dopamine form hydrogen bonds with Ser193 and Ser197 (located at TM5), respectively. It has been hypothesized that Ser194 might be an alternative to Ser193 for a slightly different conformation resulting from activation. Residues Asp114, Ser193, Ser194, and Ser197 are conserved in all five human dopamine receptors. A hydrophobic pocket for dopamine is formed in D_{2L} receptor by residues Phe110, Met117, Cys118, Phe164, Phe189, Val190, Trp386, Phe390, and His394 [97]. Transmembrane proline residues are highly conserved in all GPCRs, particularly Pro201, Pro388, and Pro423, the most conserved residues in class A GPCRs. Those three prolines as well as Pro89 and Pro169 have been located in the transmembrane region of the D_2 receptor, suggesting an important role in its function by eliminating a backbone hydrogen donor when necessary [139].

The structure of D_2 receptors has been predicted from primary sequence using the MembStruk procedure [97]. The predicted structure has been validated against experimental evidences on affinities of dopamine, three agonists (antiparkinsonian) and seven antagonists (antipsychotic), and correctly predicts the critical residues for binding dopamine (Figure 1.5a).

Differences between the binding sites of agonists and antagonists have also been identified. The different interactions for class I and II antagonists, represented by clozapine and haloperidol, respectively, are illustrated in Figure 1.5b,c.

Binding interactions of dopamine and the agonist apomorphine with high and low states of human D_2 receptor have been determined using computational and experimental techniques. Monomeric and dimeric forms of D_2 were considered. The observed results showed that dimerization of D_2 receptors have negative cooperative effect on agonist ligand binding [140].

The only X-ray structure resolved in which D_2 receptor is involved corresponds to the neuronal calcium sensor-1 (NCS-1) in complex with two peptides derived from the receptor [141]. Although structural information on the receptor cannot be obtained, the structure provides very important information about

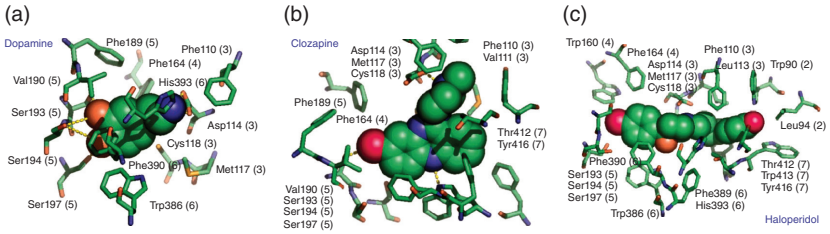


Figure 1.5 Residues within 5.5 Å of dopamine (a), clozapine (b), and haloperidol (c) bound to human D_2 receptor. Source: Reproduced with permission from Kalani et al. 2004 [97]. Copyright 2004, National Academy of Sciences, USA.

binding of D_2 with NCS-1. Two peptides (*a* and *b*) are bound as amphipathic helices with their C-termini pointing toward the center of NCS-1 in which a conformational change is induced (Figure 1.6a). The same residues are involved in both peptides in hydrophobic interactions with NCS-1. These residues are Ile431, Phe433, Ala436, Phe437, Ile440, and Leu441. In peptide *b* Leu438 is also involved (Figure 1.6b). The two copies of D2R peptides interact between them through hydrophobic interactions (*a*Leu438 and *b*Leu441) and hydrogen bonds (*a*Glu431 and *b*Ser433; *a*Lys435 and *b*His442; *a*His442 and *b*Arg434, and *a*Ser433 and *b*Arg434).

The murine D_3 receptor also presents two functional isoforms, short and long, similarly to the D_2 receptor [30]. Although such splice variation has not been found in humans, nonfunctional splice variants resulting in truncated chains have been elucidated. The variant $D3_{nf}$ has been suggested to be involved in schizophrenia [142]. Polymorphism has also been identified for D_3 receptor but no alterations in the function are detected. Some studies, however, suggested a relationship between schizophrenia and a polymorphism consisting of a Ser to Gly change in the ninth residue of the amino terminus [143].

The only full X-ray structure of a human dopamine receptor has been resolved for D_3 receptor in complex with the antagonist eticlopride [144]. The main fold of D_3 receptor consists of the seven-TM bundle of α-helices characteristic of GPCRs (Figure 1.6c). The extracellular region has high sequence diversity among the GPCR family, and two different conformations of intracellular loop il_2 are observed. Starting from the crystal structure of D_3 receptor, a homology model of D_2 receptor was created. A comparison of both models revealed subtle differences that might be the origin of the different selectivity between the receptors.

The utility and reliability of homology models has been further demonstrated through studies carried out with D_3 receptor in which the docking screen against the homology model was no less effective than that against the crystal structure [145]. Additional molecular dynamics refinement contributes to a better approach to the three-dimensional structure of the receptor [146] as it has been demonstrated for both D_1 and D_2 receptors [147] and GPCRs in general [148, 149]. These studies concluded that the homology models suit at least as well as the X-ray structure, thus being excellent surrogates to aid in drug discovery, [150] particularly for GPCRs [99, 151]. The resolution of the crystal structure of

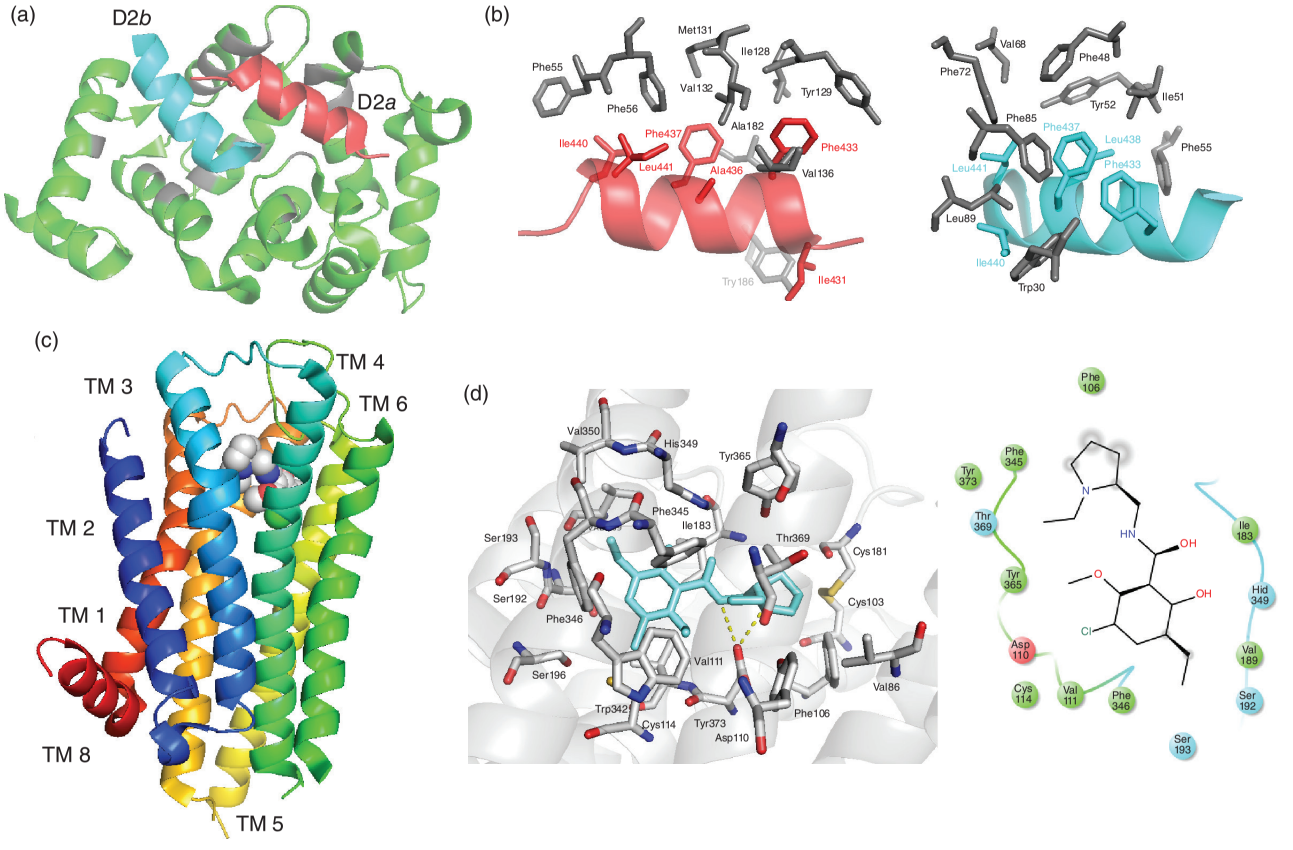


Figure 1.6 (a) Cartoon representation of NCS-1 in complex with two D_2 receptor chains bound independently. (b) Details on hydrophobic interactions between NCS-1 and D_2 receptor chains a (red) and b (cyan). (c) Crystal structure of D_3 receptor in complex with eticlopride (space-filling representation). (d) Details on interactions of eticlopride (cyan) at the binding site.

D_3 receptor has promoted further advances with D_2 receptor using reliable homology models [152]. These models served for determining key receptor–ligand interactions of several well-known dopaminergic compounds such as piperazines [153] and have been used as templates for antipsychotic virtual screening [154].

The functional role of various residues located at TM3 has been studied with homology models derived from D_3 X-ray structure. It was concluded that a microdomain packed by aromatic–aromatic interactions and connecting helices 3, 5, and 6 forms a barrier that prevents dopamine from binding further toward the intracellular surface [155]. At a molecular level, a sulfur–π interaction is responsible for those interactions. Homology models have been extended to D_2-Gα_i complex in the presence of dopamine and compared with aripiprazole-type partial agonists. It has been found that agonists induced different conformations of important structural motifs, including the extracellular loop regions, the binding pocket and, more importantly, intracellular G-protein-binding domains. Consequently, partial agonist efficacy is caused by an impaired coupling between the receptor and G-proteins [156]. Molecular dynamics of D_2 receptor interaction with the GIPC1-PDZ domain suggested a mechanistic role for helix 8 (located immediately after the end of TM7) in GPCRs in the course of signaling [157].

The selectivity of ligands for D_3 receptor over D_2 has also been studied by multiple fragment docking [158] and in combination with evaluations against chimeric and mutant receptors, suggesting that the selectivity of ligands is due to interactions at the extracellular end of TM7 [159]. The binding affinity of D_2 [160] and D_3 [161] receptors for a series of [4-(4-carboxamidobutyl)]-1-arylpiperazines has been evaluated at density functional theory (DFT) level. From these results, partial bidimensional pharmacophores have been proposed (Figure 1.7).

Key specific residues involved in activation of D_3 receptor signaling have been identified in TM6 and ol_3 [162]. The quaternary structure of the D_3 receptor has been investigated, and the formation of not only homodimers but also higher order oligomers has been confirmed. Those oligomers are formed by four protomers organized in a rhombic arrangement (Figure 1.8) [163].

D_4 receptor presents the structural features characteristic of D_2-like receptors including a carboxyl-terminal cysteine, which is believed to be a palmitoylation site. It has been demonstrated that glycosylation can take place on a single conserved Asn3 [164]. This glycosylation is not involved in ligand binding or receptor trafficking. D_4 receptor shares 53% identity with D_2 receptor in the transmembrane domains [43]. The human D_4 receptor is one of the most known polymorphic proteins [165]. Most of the diversity is found at the third intracellular loop il_3 – unusually proline-rich [166] for which 20 different variants of the receptor have been identified [167]. The $D_{4.4}$ variant is predominant followed by $D_{4.7}$ and $D_{4.2}$ [168]. Polymorphic variations encoding amino terminus are also known [165] although no major differences have been observed in G-protein coupling or signaling properties [169]. In this context, dimerization of D4 receptor is a key factor for the correct folding of the GPCR [170]. Several studies have investigated links between polymorphisms of D_4 receptors and disorders such as ADHD [171, 172] and schizophrenia [173, 174].

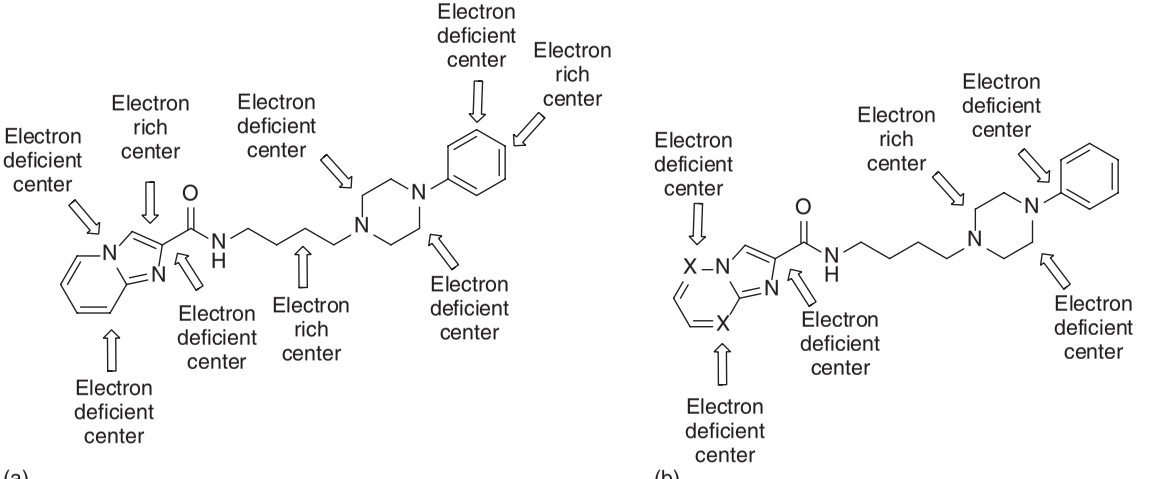


Figure 1.7 Examples of 2D pharmacophores calculated at DFT level for D_2 (a) and D_3 (b) receptors.

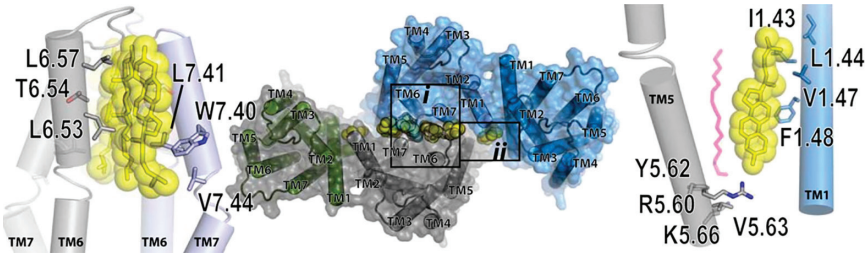


Figure 1.8 Molecular modeling of human D_3 receptor in tetrameric arrangements. Each dimer is shown as a semitransparent surface, whereas predicted cholesterols are shown as yellow spheres forming a buffer between the two dimers. Inset (a) shows details of the TM6 and TM7 interface, and the residues shown in sticks (gray and light blue) were found experimentally to affect hD3 quaternary structure. Yellow sticks and spheres depict predicted cholesterol molecules in positions as observed in adenosine A2A receptor, mu-opioid receptor, and the P2Y12 receptor structures. Inset (b) shows details of the predicted interaction between (transmembrane domain) TMD V Arg-5.60 and Tyr-5.63 (in gray sticks) of one dimer and the TMD I cholesterol (in yellow sticks and spheres). A predicted palmitoyl moiety, bound to Cys-8.60, is also shown in magenta semitransparent sticks. Source: Reproduced with permission from Marsango et al. 2015 [163]. Copyright 2015, JBC.

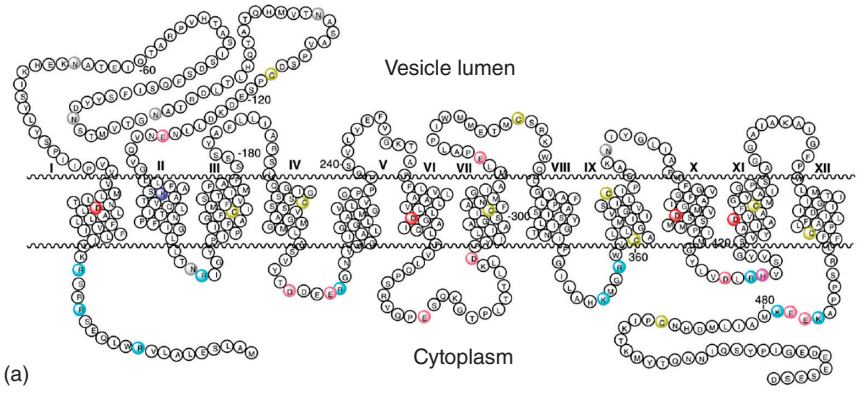
1.3 Vesicular Monoamine Transporter (VMAT)

The VMAT is a membrane protein that introduces monoamine neurotransmitters into storage vesicles to allow subsequent release into the synapse [175]. VMAT accumulates both newly synthesized and reuptaken from the synapse neurotransmitter molecules into the intraneuronal vesicles [176]. The VMAT exists in two distinct forms with different pharmacologies and tissues distribution: VMAT1 and VMAT2 [177]. These transporters are relatively nonspecific and transport the monoamine neurotransmitters serotonin, ephedrine, and norephedrine in addition to dopamine [178]. The neuronal selectivity for a given monoamine is determined by the particular biosynthetic enzymes expressed in the corresponding neuron. On the other hand, there are other plasma membrane neurotransmitter transporters that are selective for each monoamine, i.e. DAT for dopamine, SERT for serotonin, and NET for norepinephrine. In this section, an overview of VMAT2, present in the CNS, and DAT is presented, and NERT and SERT are discussed in Chapters 2 and 3, respectively.

VMAT1, previously known as the chromaffin granule amine transporter, is located in extraneural tissues although there is some evidence that it may also be present in the human brain, perhaps concentrated in the substantia nigra. Some studies suggest a relationship between VMAT1 gene and neuropsychiatric disorders [179] such as schizophrenia [180] and bipolar disorder [181]. VMAT2, previously known as the synaptic VMAT, is primarily located in neuronal cells of the central, peripheral, and enteric nervous systems [182]. VMAT2 selectively recognizes and transports all of the amine neurotransmitters across biomembranes, that is, dopamine, serotonin, histamine, norepinephrine, and epinephrine. VMAT2 is not selective, but the quantitative assessment of its density by

positron emission tomography (PET) scanning has been clinically useful for early diagnosis and monitoring of the progression of Parkinson's and Alzheimer's diseases and drug addiction [183, 184]. Genetic studies connected alterations of VMAT2 with depression [185]. The immunochemical analysis of VMAT2 has also been suggested to be used in the diagnosis of PD [186].

VMAT2 (as VMAT1) is a transmembrane glycoprotein with 12 transmembrane domains with both N- and C-termini in the cytoplasm and a large, hydrophobic, N-glycosylated loop between TM1 and TM2 facing the vesicle lumen. The structure has been predicted (Figure 1.9), but no crystal structures have been resolved. VMAT2 has 742 residues. It can be N-glycosylated by Glc-NAc at Asn84 and Asn91. A posttranslational phosphorylation takes place at Ser511 and Ser513.



(a)

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VMAT2_HUMAN  MALSELALVRWLQESRRSRKLILFIVFLALLLDNMLLTVVVPIIPSYLYSIKHEKNATEIQTARPVHTASISDSFQSIFSYYDNSTMVTGNATRDLTLHQ
VMAT2_HUMAN  TATQHMVTNASAVPSDCPSEDKDLLNENVQVGLLFASKATVQLITNPFIGLLTNRIGYPIPIFAGFCIMFVSTIMFAFSSSYAFLLIARSLQGIGSSCSS
VMAT2_HUMAN  VAGMGMLASVYTDDEERGNVMGIALGGLAMGVLVGPPFGSVLYEFVGKTAPFLVLAALVLLDGAIQLFVLQPSRVQPESQKGTPLTTLLKDPYILIAAGS
VMAT2_HUMAN  ICFANMGIAMLEPALPIWMMETMCSRKWQLGVAFLPASISYLIGTNIFGILAHKMGRWLCALLGMIIVGVSILCIPFAKNIYGLIAPNFGVGFAIGMVDS
VMAT2_HUMAN  SMMPIMGYLVDLRHVSVYGSVYAIADVAFCMGYAIGPSAGGAIAKAIGFPWLMTIIGIIDILFAPLCFFLRSPPAKEEKMAILMDHNCPIKTKMYTQNNI
VMAT2_HUMAN  QSYPIGEDEESESD
```

(b)

Figure 1.9 (a) Predicted secondary structure of the human vesicular monoamine transporter 2 (VMAT2) Conserved (in hVMAT1, hVMAT2, rVMAT1, rVMAT2, and bVMAT2) transmembrane Asp33, 262, 399, 426 (red), and Lys138 (blue); proposed vesicle lumen disulfide bridged Cys117, and 324 (yellow); and glycosylation sites Asn (gray) are shown. The conserved Cys (117, 302, 324, 360, 374, 430, 467, and 488) (yellow); cytoplasmic domain charged residues Lys (354, 476, and 479) (cyan), Arg (10, 16, 19, 155, 217, 357, and 413) (cyan), Glu (216, 278, 477, and 478) (pink), Asp (213, 291, and 411) (pink), His414 (red), and vesicle lumen Glu (127 and 312) (pink) are also shown (amino acid numbering is based on the sequence of hVMAT2). Source: Reproduced with permission from Van Liefferinge et al. 2013 [187]. Copyright subjected to CC-BY 4.0 License terms. http://journal.frontiersin.org/article/10.3389/fncel.2013.00139/full. (b) Amino acids sequence of human VMAT2. Disulfide bonds (green), N-glycosylation sites (red), extracellular domains (white), intracellular domains (light blue), and transmembrane domains (yellow) are indicated on sequences.

Studies with mammalian neurons suggested that VMAT2-mediated transport of monoamines is subject to regulation by two calcium-dependent activator proteins of secretion (CAPS1 and 2) linked to the heterotrimeric GTPase G_o [188]. The established proposed model for introduction of monoamines into vesicles is also based on efflux of two protons coupled with influx of the monoamine (Figure 1.10a) [175].

An intravesicular proton induces a conformational change, increasing amine-binding affinity outside the vesicle; the second proton induces a second and large conformational change that introduces the monoamine in the vesicle, reducing amine-binding affinity inside the vesicle. Finally, the monoamine is released inside the vesicle and two protons are liberated to cytosolic phase. The first proton-induced conformational change is assisted by His419. Mutagenesis experiments identified the residues involved in substrate recognition [190] and suggested that Lys139 and Asp427 interact to form an ion pair in the native protein, promoting high-affinity interaction with the substrate [191]. However, recent studies revealed that, during the transport cycle, VMAT2 is expected to occupy at least three different conformations: cytoplasm-facing, occluded, and lumen-facing (Figure 1.9b) [189]. The lumen to cytoplasm-facing transition, facilitated by protonation of at least one of the essential membrane-embedded carboxyls, generates a binding site for reserpine, a competitive inhibitor of both VMAT1 and VMAT2, that locks the

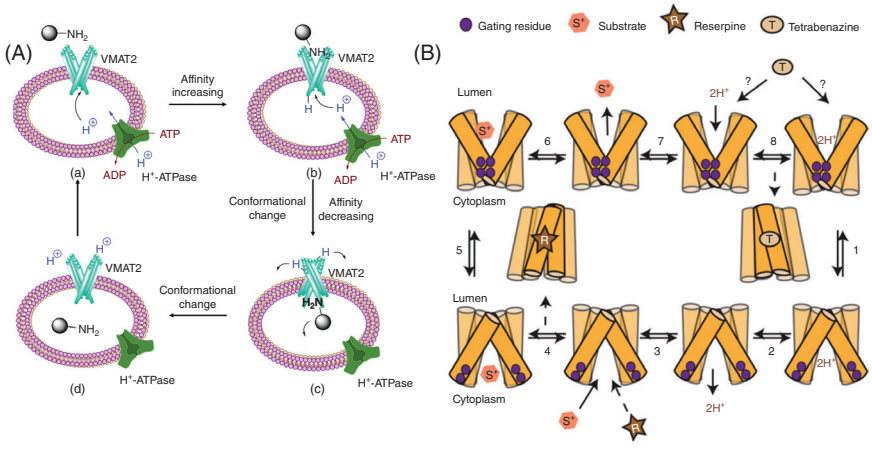

Figure 1.10 Models for introduction of monoamine neurotransmitters into intraneuronal vesicles mediated by VMAT2. (A) Simplified model. A conformational change induced by a proton (a) increases affinity by the amine (b), which is introduced into the vesicle through a second conformational change induced by a second proton (c). The amine is released and the two protons initially introduced by H^+-ATPase are liberated to cytosol (d). (B) Illustration of a third conformation. For simplicity, only six TMs are shown. In the absence of a proton gradient, the dominant population is of the lumen-facing conformation, as indicated by the transporter's ability to bind tetrabenazine but not reserpine. Binding of protons enables the conformational switch to the cytoplasm-facing conformation (step 1), whereas binding of substrate enables the change to the lumen-facing conformation (step 5). Source: Reproduced with permission from Yaffe et al. 2016 [189]. Copyright 2016.

transporter in a dead-end conformation. However, reserpine binding competes with substrate binding, suggesting that the reserpine-bound conformation is cytoplasm-facing. The VMAT2-selective inhibitor, tetrabenazine, locks the transporter in a conformation that appears incompatible with substrate binding and is therefore presumably not cytoplasm-facing.

1.4 The Dopamine Transporter (DAT)

The DAT is a transmembrane protein belonging to the family of sodium/chloride-dependent neurotransmitters, which removes the excess of dopamine from synaptic and presynaptic spaces and reuptake it into dopaminergic neurons through a process driven by the symport of sodium and chloride ions along their concentration gradients [192]. In the brain, DAT is expressed exclusively in dopaminergic neurons within the CNS, especially in midbrain neurons originating in the substantia nigra and ventral tegmental area. Dysfunction of DAT has been associated with several neurodisorders and psychiatric disorders including ADHD, bipolar disorder, clinical depression, drug addiction, and alcoholism [193]. Abnormal DAT densities are found in PD [194], Tourette's syndrome [195], dementia with Lewy bodies (DLB) [196], depression [197], and Lesch–Nyhan disease [198]. The quantitative assessment of DAT using PET and single photon emission computed tomography (SPECT) (DaTscan) imaging is found to be a powerful technique [199] for the diagnosis of PD [200] and other neurodisorders [201–203] as well as for the quantification of DAT in heroin- [204] and cocaine-dependent [205] subjects.

1.4.1 Structural Aspects

The human DAT has 620 residues with 12-transmembrane rings with a glycosylated large second extracellular loop and intracellular amino- and carboxy-termini (Figure 1.11a). N-glycosylation can take place at Asn181, Asn188, and Asn205. The transporter interacts with two sodium ions through residues Gly75, Val78, Leu418, Asp421, and Ser422 for one ion and Ala77, Asn82, Ser321, and Asn353 for the second one. Natural variants include replacement of Gly121 by Ser, Arg237 by Gln, Leu368 by Gln, Pro395 by Leu, Val471 by Ile, and Arg544 by Ser. Although the crystal structure of human dopamine transporter (hDAT) has not been determined, the X-ray structure of the *drosophila* dopamine transporter (dDAT) has been resolved (Figure 1.12). Figure 1.11b illustrates aligned sequences of both receptors showing a high degree of conservation. Hence, homology models of hDAT can be constructed from dDAT structural data.

The structure of a transport-inactive dDAT was resolved in complex with nortriptyline (PDB ID: 4M48) (Figure 1.12) and served for elucidating antidepressant mechanism [206] consisting of stabilizing the open conformation by sterically preventing closure of the extracellular gate and thus blocking the transporter from binding substrate and isomerizing to the inward-facing conformation. The same authors reported, two years later, X-ray structures of dDAT in complex with antidepressants nisoxetine (PDB ID: 4XNU) and reboxetine

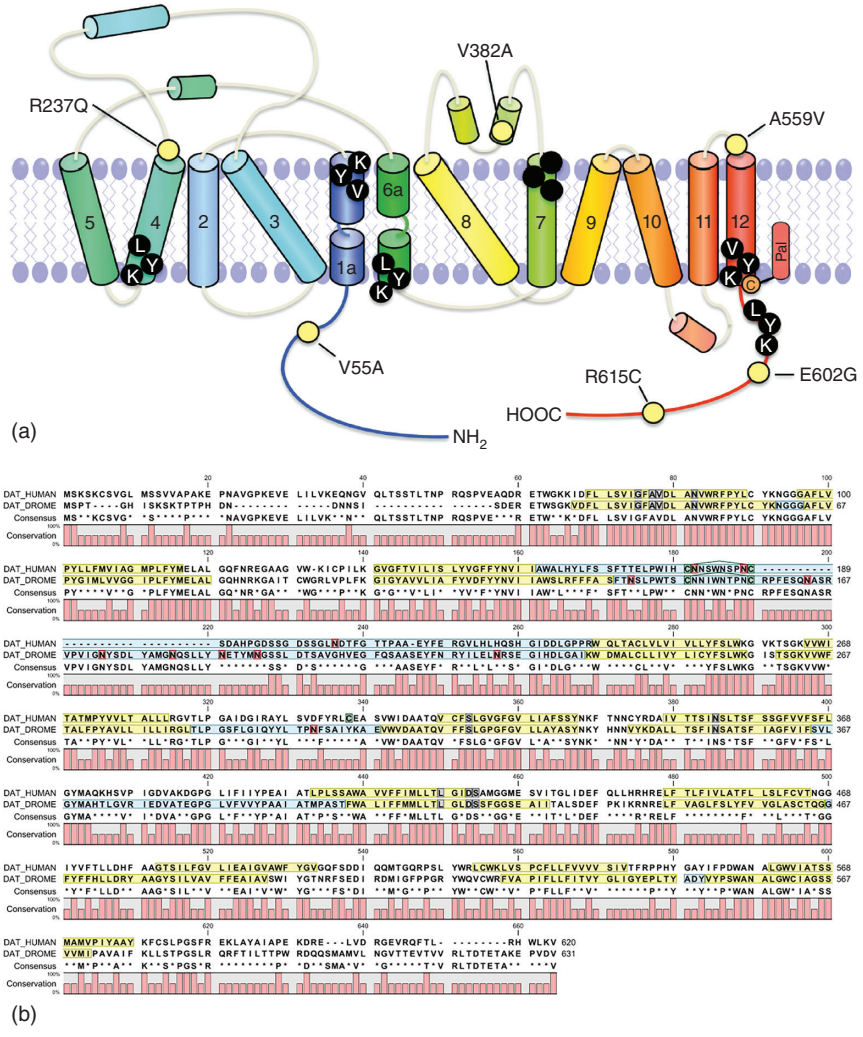

Figure 1.11 (a) Coding variants known to alter dopamine transporter (DAT) function (numbered yellow circles) and helical topological 2D architecture of DAT depicting essential cholesterol-interacting residues in putative Cholesterol Recognition Amino Acid Consensus (CRAC) motifs (black circles with white letters). Source: Reprinted with permission from Vaughan and Foster 2013 [193]. Copyright 2013, Elsevier. (b) Sequence alignment of hDAT and dDAT. Conservation graphic and consensus sequence are shown. Disulfide bonds (green), N-glycosylation sites (red), sodium interaction residues (gray), extracellular domains (light blue), intracellular domains (white), and transmembrane domains (yellow) are indicated on sequences.

(PDB ID: 4XNX) (Figure 1.12) [207]. The ligands occupy the substrate-binding site, contributing to the same stabilizing effect of the outward-open conformation observed with nortriptyline. These structures established that aromatic moieties and extended amine groups of antidepressants are important for generating high-affinity inhibition. The structural study further revealed that

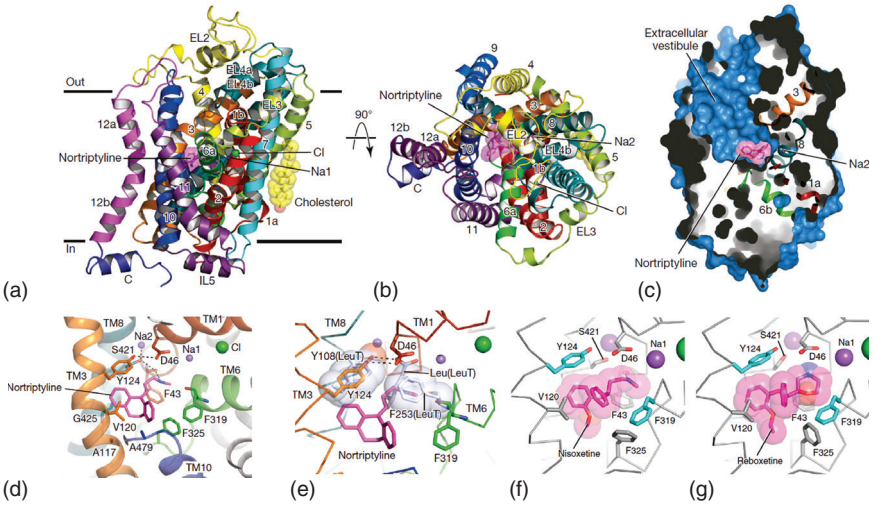

Figure 1.12 (a–c) Architecture of Drosophila DATcryst. (a) Structure of DATcryst viewed parallel to membrane. Nortriptyline, sodium ions, a chloride ion, and a cholesterol molecule are shown in sphere representation in magenta, purple, green, and yellow, respectively. (b) View of DATcryst from the extracellular face. (c) Surface representation showing that ligand- and ion-binding sites are accessible from the extracellular vestibule. Nortriptyline and TMs 1, 3, 6, and 8 are colored as in (a). (d) Close-up view of the drug-binding pocket. Sodium and chloride ions are shown as spheres. Nortriptyline is represented as sticks (magenta). The amino group of nortriptyline is 2.7 Å from the carbonyl oxygen of Phe43 (TM1a), and the *N*-methyl group of nortriptyline is 3.1 Å from the carbonyl oxygen of Phe319. Residues lining the drug-binding pocket with interfacial areas greater than 10 $Å^2$ are represented as sticks. (e) Comparison of the drug- or substrate-binding pocket of DATcryst with that of LeuT (PDB ID: 2A65). The distance between the carboxylate group of leucine and Tyr108 (spheres) is 2.7 Å in the occluded state (PDB ID: 2A65) and 5.1 Å in the inhibitor-bound state (PDB ID: 3F3A) of LeuT, whereas the equivalent interaction in DATcryst between Asp46 and Tyr124 is 3.1 Å. Source: (a–e) Reprinted with permission from Penmatsa et al. 2015 [206]. Copyright 2013, Macmillan Publishers Ltd. (f, g) Close-up views of the binding pocket for nisoxetine-bound (f) and reboxetine-bound (g) dDAT. Cyan, Tyr124 and Phe319; purple spheres, sodium ions; green spheres, chloride ions. Source: Reprinted with permission from Penmatsa et al. 2017 [207]. Copyright 2015, Macmillan Publishers Ltd.

chemical modifications on the aromatic rings of antidepressants encode selectivity between catecholamine transporters opening a door for the structure-directed design of more-selective biogenic amine transport inhibitors.

Several crystal structures of dDAT with cocaine (PDB ID: 4XP4), cocaine analogues (PDB ID: 4XP5), amphetamine (PDB ID: 4XP9), methamphetamine (PDB ID: 4XP6), 3,4-dichlorophenetylamine (PDB ID: 4XPA), and the natural substrate dopamine (PDB ID: 4XP1) were also reported [208]. The same outward-face orientation was found in all cases. Dopamine is located in the central binding site of the outward-face conformation defined by TMs 1, 3, 6, and 8. The primary amino group interacts with Asp46, and the catechol moiety is placed in a pocket formed by Ala117, Val120, Asp121, Tyr124, Ser422, and Phe325. All the resolved structures of dDAT have an outward-face orientation and similar interactions between the ligands and the residues forming the binding pocket. Amphetamines

lacking the catechol moiety present less affinity by the transporter although they are also placed in the binding site. However, amphetamines have a considerable weaker affinity by dDAT than by hDAT, probably due to differences in residues in the subpart of the binding pocket where the catechol unit is recognized. In fact, in mammalian DATs, amphetamines are closely as effective as cocaine at inhibition of dopamine uptake [209], suggesting that hDAT is more complementary to those drugs of abuse than DATs from invertebrates.

The resolution of dDAT served for the construction of reliable homology models of hDAT, which were the basis for unraveling the molecular mechanism of dopamine transport by hDAT [210]. By using homology models in combination with accelerated molecular dynamics calculations, it has been established that during dopamine transport, four conformational states are differentiated corresponding to global helical motions and local conformational switches. Initially, dopamine binding induces a spontaneous closure of extracellular gates (Arg85-Asp476 and Tyr156-Phe320) accompanied by 10–15° inward tilting of TM1 and TM6 that rapidly evolves to a more stable conformation. Disruption of Asn82-Asn353 hydrogen bond promoted by Na_2 dislocation induces permeation of intracellular water molecules while TM1 outward tilting breaks salt bridges Lys66-Asp345 and Arg60-Asp436. These transformations complete transition to inward-face conformation and release of chloride and one sodium cation. Finally, disruption of the H-bond between dopamine and Asp79 releases dopamine inside the neuron (Figure 1.9).

Recent studies revealed that the large N- and C-termini extended into the cytoplasm, synergistically contribute to dopamine affinity [211]. Molecular dynamics simulations combined with mutagenesis studies suggested that initial binding to DAT is favored by Val152 that leaves enough space for the substrate [212]. Regulation of domains responsible for binding is exerted through post-translational modifications such as phosphorylation [213] and palmitoylation [214] that control DAT kinetics [215]. The human DAT has a tetrahedral zinc(II) allosteric binding site that might be of interest for activating dopamine transport [216].

1.4.2 Molecular Mechanism

The DAT belongs to the SLC6 family of sodium- and chloride-dependent transporters. The cotransport of sodium and chloride ions provides the energy for the transporter to pump dopamine into the terminals against its concentration gradient. The sodium gradient in the membrane is maintained by the sodium–potassium pump Na^+, K^--ATPase.

The general reuptaking process of dopamine by DAT starts by binding one molecule of dopamine. Further binding of two sodium cations and one chloride induces a conformational change to an inward-facing conformation that releases both ions and dopamine into the cytoplasm. Actually, this process involves complex interactions between ions and DAT, initially foreseen by previous investigations [217] and ultimately identified by structural and computational studies (Figure 1.13). The use of homology models of human DAT, based on X-ray, determined dDAT and was complemented by full-atomic microseconds

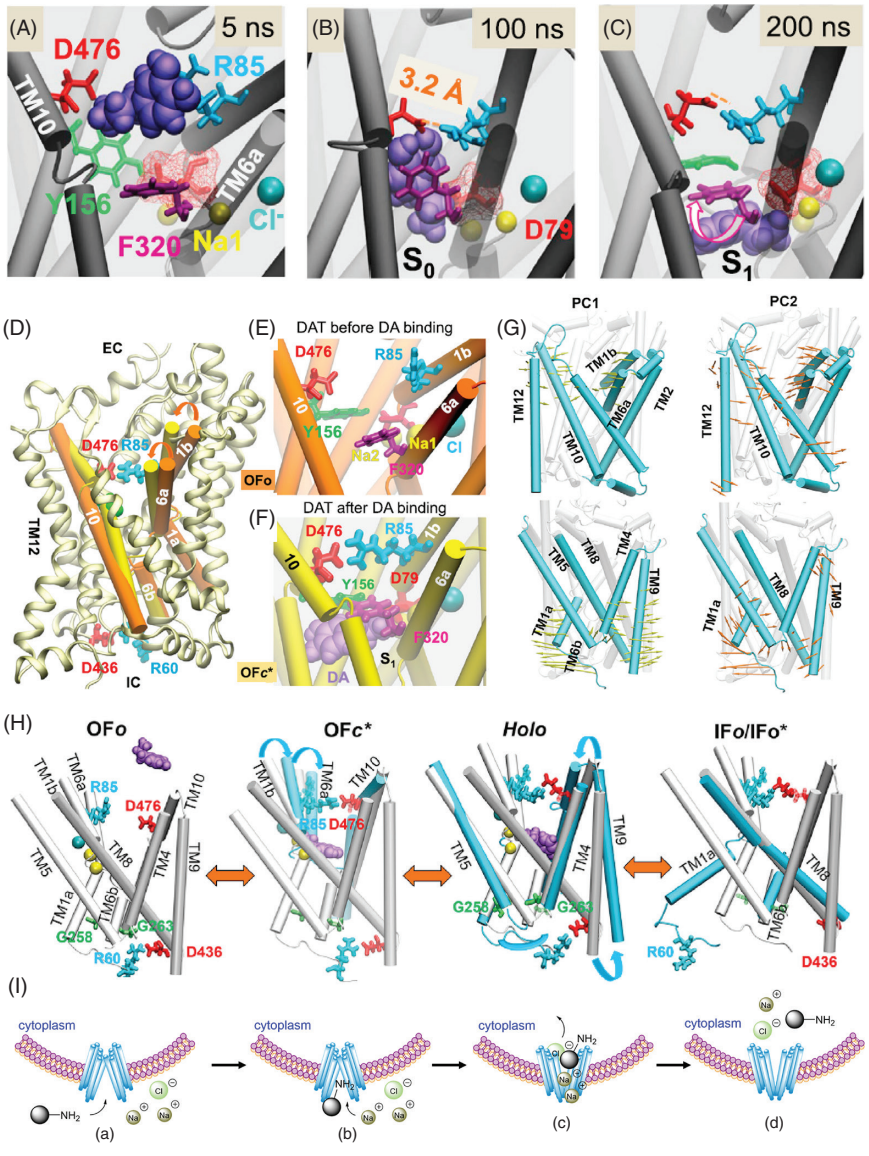

Figure 1.13 (A–C) Positions of DA (purple van der Waals spheres) with respect to Arg85, Asp476, Tyr156, Phe320, and Asp79 (red stick with mesh), at 5, 100, and 200 ns. (D) MD-resolved hDAT in the outward-facing closed (OFc*) state (cream ribbons). Alignment of a representative OFc* conformation (yellow) sampled in MD simulations with the initial hDAT in the OFo state (orange). (E) Substrate-binding pocket before DA binding, in the OFo state. (F) Same pocket after binding. Isomerization of F320 brings its aromatic side chain on top of DA. Tyr156-Phe320 and salt bridge by Arg85-Asp476 serve as EC gates. (G, H) hDAT Trajectory from outward-facing open to inward-facing open state, via holo-occluded state. (G) Movements along PC1 (yellow arrows) and PC2 (brown arrows). (H) Conformation states visited by hDAT along with substrate translocation. Four states are distinguished by global helical motions (cyan) together with local conformational switches. (I) Schematic mechanism of dopamine transport. Source: (A–H) Reprinted with permission from Cheng and Bahar 2015 [210]. Copyright 2015, Elsevier.

accelerated simulations, allowed determining the sequence of events during dopamine reuptake into dopaminergic neurons.

Dopamine binding to DAT induces closure of extracellular gates and a holo-occluded intermediate is stabilized. Then concerted motions of TM1 and TM7, accompanied by the TM1a–TM1b loop reordering, break the hydrogen bond between Asn82 and Asn353, causing exposure to intracellular water triggered by Na_2 dislocation. This process facilitates the release of sodium and chloride ions through a redistribution of a network of salt bridges at the intracellular surface in the inward-facing state and concerted tilting of intracellular helices. Finally, dopamine is released after protonation of Asp79.

The role of DAT in combination with VMAT2 is crucial for the subtle balance of neuronal dopamine exerted by dopaminergic neurons [218]. Increasing dopamine concentration in cytoplasm can lead to oxidative stress and neurotoxicity. On the other hand, enough but not excessive dopamine must be present in the synaptic and presynaptic environments. Thus, a correct compartmentalization of dopamine is critical for neuron function, and any misbalance results in severe neurodisorders. DAT and VMAT2 are responsible for a correct function and, consequently, therapeutic targets in diseases.

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