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Functional Properties of *Drosophila* Dopamine D1-Receptors Are Not Altered by the Size of the N-Terminus

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Molecular cloning revealed the existance of at least five pharmacologically different dopamine receptors in vertebrates. Functionally, dopamine receptors either activate (D1-type), inhibit or do not interact with adenylate cyclase (D2-type). A recently cloned dopamine receptor from *Drosophila melanogaster* shares many structural and functional properties with vertebrate D1-type receptors but the pharmacological properties are very different. In contrast to most aminergic receptors, DmDop1 contains a long N-terminal extension. Here we describe a deletion-mutagenesis approach to study whether the N-terminus of DmDop1 participates in receptor–ligand interactions. All mutants gave rise to functional receptors after heterologous expression in HEK 293 cells. The pharmacological properties, however, remained unchanged. A comparison of DNA and deduced amino acid sequences revealed that some *Drosophila* strains express a truncated version of the DmDop1 receptor.

Several neurological disorders have been attributed to a malfunction of the dopaminergic system in mammals, including Parkinsonism, schizophrenia, and epilepsia (1–4). Therefore, the dopaminergic system and especially the analysis of structural and functional properties of dopamine receptors is of general interest for the treatment of such diseases.

Only little is known about the role of the dopaminergic system in invertebrates. A contribution in behaviour and neuronal differentiation was assumed (5, 6). The cloning of a dopamine D1-receptor homologue from *Drosophila melanogaster* (7) revealed that the functional properties of the D1-receptor subfamily are well conserved throughout evolution. Receptor activation occurs after binding of dopamine to specific amino acid residues located within the transmembrane segments of the receptor polypeptide. Most notably, amino acid residues which interact with ligands in vertebrate dopamine receptors (8) are strictly conserved in DmDop1. When heterologously expressed, the *Drosophila* receptor activates adenylate cyclase after application of dopamine and dopamine receptor agonists. In contrast to these pronounced similarities with mammalian D1-type receptors, it was surprising that DmDop1 did not bind with high affinity to vertebrate D1-receptor ligands, i.e. SCH 23390 and SKF 38393 (7).

In comparison to vertebrate dopamine receptors, DmDop1 contains an unusual long N-terminal segment of 142 amino acid residues. Long amino-termini are a signature of peptidergic receptors and have only been described for two serotonin receptors so far (9, 10). We suspected that this segment might interfere with the binding properties of DmDop1. Thus, a mutagenesis approach was undertaken to systematically delete portions of the N-terminus of DmDop1. After transient expression in HEK 293 cells, all mutants gave rise to functional receptors. The properties of the constructs, as monitored by the ability to activate AC, remained largely unchanged in comparison to the original receptor.

A recent publication also described the molecular cloning of a *Drosophila* dopamine receptor

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Abbreviations: AC, adenylate cyclase; DmDop1 (=D-Dop1, accession no.:X77234), *Drosophila* dopamine receptor; EDTA, ethylenediaminetetraacetic acid; GPCR, G-protein coupled receptor(s); hD1, human dopamine D1-receptor; IBMX, 3-isobutyl-1-methylxantine; MEM, minimal essential medium; PBS, phosphate buffered saline; PCR, polymerase chain reaction(s); TM, transmembrane segment(s).

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(*d*DA1, 11). In comparison to DmDop1 the deduced amino acid sequence of *d*DA1 contains three substitutions in the intracellular loop between TM1 and TM2. Most notably, *d*DA1 lacks the N-terminal extension, though only 16 amino acid residues preceed TM1. This difference might be due to several nucleotide substitutions and insertions in the exonic DNA sequence which encodes the N-terminus of the dopamine receptor in wildtype strain *Canton S.* Such variations do neither exist in wildtype strain *Berlin,* from which DmDop1 was isolated, nor in cDNA molecules amplified by PCR from wildtype strain *Oregon R.* These results indicate that certain domains of receptor molecules might vary significantly in different strains of *Drosophila* but the structural changes do not alter the functional properties of the expressed polypeptides.

MATERIALS AND METHODS

Construction of N-terminal deletions. PCR was performed to generate N-terminal truncated DmDop1 receptors. The pcDop1 construct (7) was used as a template in all reactions. DmDop1 contains four methionine residues in the N-terminal part at position 1, 16, 20, and 127, respectively. We used the internal methionine residues as novel translation initiation sites. Each 5′-oligonucleotide contained a unique Hind III recognition sequence in front of the Kozak consensus motif (12) to facilitate subcloning. To delete amino acid residues 1–15 (DmDop[−15]) were used primer I (# I): 5′-CCTAATCAAAGCTTCCACCATGACAAATGCAATGCGGGCG-3′. For the deletion of amino acid residues 1–19 (DmDop[−19]), we used # II: 5′-CGTTCCAAAAGCCTTCCACCATGCGGGCGATTGCTGC-3′. The third construct (DmDop[−126]) was generated with oligonucleotide # III: 5′-GGACAAAAGCTTCCACCATGGATACAATAGTTG-3′. The 3′-primer for all PCR reactions was: 5′-GACAGTAGATGCCAATC-3′ corresponding to nucleotide position 978–994 of pcDop1. PCR amplifications were performed in a total volume of 100 μl with 10 ng of template DNA, 0.3 nM of the 5′- and 3′-primers, 0.2 mM of each deoxynucleotide, and 2.5 U *Taq* DNA polymerase in 1 × *Taq*-buffer. PCR fragments and pcDop1 were digested with BamHI and HindIII, gel purified and ligated. All constructs were verified by sequencing.

Cell culture and transient transfection. HEK 293 cells were passaged in MEM supplemented with 1 × non-essential amino acids, 0.2 mM 1-glutamin, 1 × antibiotic/antimycotics (Gibco), and 10% fetal calf serum at 5% CO_2 and 37°C. For transient transfections exponentially growing cells were used ($\sim 4 \times 10^5$ cells/50 mm dish). The medium was exchanged 2h before transfection. 10 μg of the construct-DNA was introduced into the cells by a modified calcium phosphate method (13). The dishes were incubated at 3% CO_2 and 35°C over night. Thereafter cells were rinsed with PBS and PBS/EDTA to remove the DNA precipitate. Incubation was continued after addition of fresh medium at 5% CO_2 and 37°C for 24h.

Functional receptor analysis. Functional expression of the receptor constructs was monitored by cAMP assays. Transfected cells were rinsed twice with PBS containing 100 μM IBMX and incubated with various ligands for 15 min at 37°C in PBS/IBMX. After aspiration of the medium, cells were lysed with 1.5 ml of ethanol and incubated for 2h at 4°C. The ethanol was collected and lyophilized. cAMP-concentrations were determined using a cAMP assay kit (Amersham, TRK 432). Measurements were done in triplicate on at least two independent transfections.

Isolation and analysis of nucleotide sequences from different Drosophila strains. Genomic DNA fragments (*Canton S* strain) which harbor the exons (1–6) of the DmDop1 gene (7) were isolated from recombinant phages and subcloned into pBluescript SK>-vectors by standard cloning techniques (14). PCR was performed on 25 ng first strand cDNA preparations from *Drosophila* heads (*Oregon R* strain) with the following oligonucleotides: 5′-CCGCATTTAGTATCGAA-3′ and 5′-CCAACAACTACAATTGA-3′. The PCR product was gel purified and subcloned into the *Eco*RV site of pBluescript SK>. Sequencing was done on double stranded templates.

Chemicals. (+/−) Butaclamol, cis-flupentixol, dopamine, (+) SCH23390, and (+/−) SKF38393 were purchased from RBI.

RESULTS

Analysis of Heterologously Expressed Dopamine D1-Receptor Constructs

Hydropathy profile analysis (15) of the deduced amino acid sequences of DmDop1 (7) and hD1 (16) showed that the *Drosophila* polypeptide contains an elongated N-terminus (Figure 1). In order to examine whether this extracellular loop contributes to the ligand binding properties of DmDop1, we employed a systematical deletion approach. The deduced amino acid sequence of DmDop1 contains four methionine residues in the N-terminal segment (position 1, 16, 20, and 127). We used the internal methionine residues as novel translation initiation sites in successively truncated receptor constructs, missing 15, 19, and 126 amino acid residues from the N-terminus, respectively.

Varying concentrations of dopamine (10^{-8}–10^{-6} M) were used to determine the dose-response curves for the receptor constructs and DmDop1. All deletion constructs gave rise to functional receptors in HEK 293 cells. In comparison to DmDop1, the apparent $K_{1/2}$ values for dopamine were

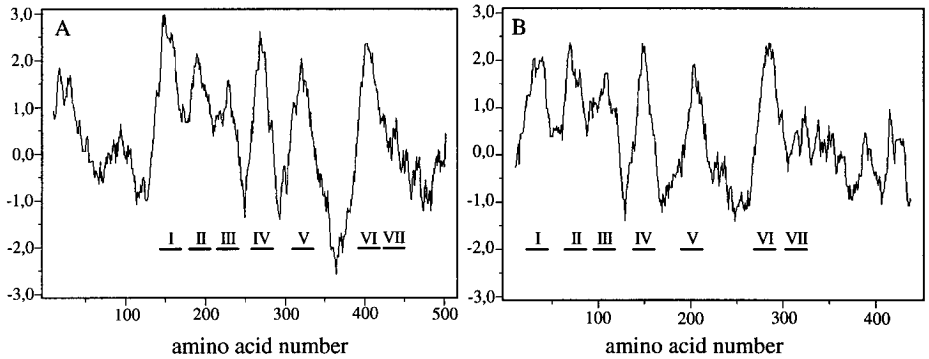

FIG. 1. Hydrophobicity profiles of deduced amino acid sequences of DmDop1 **(A)** and hD1 **(B).** Hydrophobicity index was calculated according to Kyte and Doolittle (15) with a window size of 19 amino acids and plotted against the amino acid number. Relative hydrophobicity values are given on the left hand side. Transmembrane regions (I–VII) are numbered.

similar in all deletion constructs (data not shown). Therefore, truncation of the N-terminus of DmDop1 does not alter the binding properties to its endogenous ligand.

To investigate the pharmacological properties of the deletion constructs in more detail, we examined the effects of dopamine receptor agonists and antagonists on the cAMP production. Either 1 μM dopamine or SKF 38393 were applied to transiently transfected HEK 293 cells. As for the original construct, DmDop1, SKF 38393 leads to $\sim$25% cAMP production in all deletion constructs compared to dopamine induced cAMP-levels (Figure 2). Similar to the dose-response curves measured with dopamine, the interaction between the mutants and the synthetic D1-receptor agonist SKF 38393 remained largely unchanged. In addition, receptor activation by dopamine or SKF 38393 was efficiently competed by 100 μM of the receptor antagonists butaclamol and flupentixol. For each construct, cAMP values were reduced to $\sim$5% by these ligands (Figure 2). Competition with SCH 23390 reduced the cAMP level to $\sim$15% in DmDop1, DmDop[−15], and DmDop[−19]. In construct DmDop[−126], however, competition of dopamine induced cAMP production by SCH 23390 was less efficient. Here, $\sim$28% of the cAMP level remained (Figure 2D). SCH 23390 is a high affinity antagonist to vertebrate dopamine D1-receptors (17). We have previously described that this compound does not bind with high affinity to DmDop1 (7). A deletion of 126 amino acid residues from the N-terminus of the *Drosophila* receptor, further decreases the affinity to their compound. Although DmDop[−126] and hD1 receptors possess similar short N-terminal segments and many of the residues involved in ligand binding are highly conserved (7), the pharmacological properties are still different. Therefore, specific receptor-ligand implications in these receptor polypeptides most likely are governed by structural determinants which are evolutionary less conserved.

Different Drosophila Strains Express Alternate Dopamine Receptors

A recent publication described the cloning and expression of a dopamine receptor (11) which is almost identical to the DmDop[−126] construct. Whether the structural differences between DmDop1 and *d*DA1 are due to strain variations was presently unknown. Thus, we sequenced cDNA and genomic DNA from different wildtype *Drosophila* strains. DmDop1 cDNA was originally cloned from wildtype *Berlin* (7). A cDNA fragment which spans most parts of exon 1 and 2 of the DmDop1 gene was amplified by PCR from *Oregon R* flies. Sequence comparison of the PCR product and DmDop1 cDNA showed that both were identical (data not shown). Thus *Oregon R* and *Berlin* flies should express dopamine receptors with long amino termini.

In contrast, genomic DNA-fragments from wildtype strain *Canton S* contained several nucleotide substitutions and insertions. Restriction fragments harboring the exons of the DmDop1 gene were

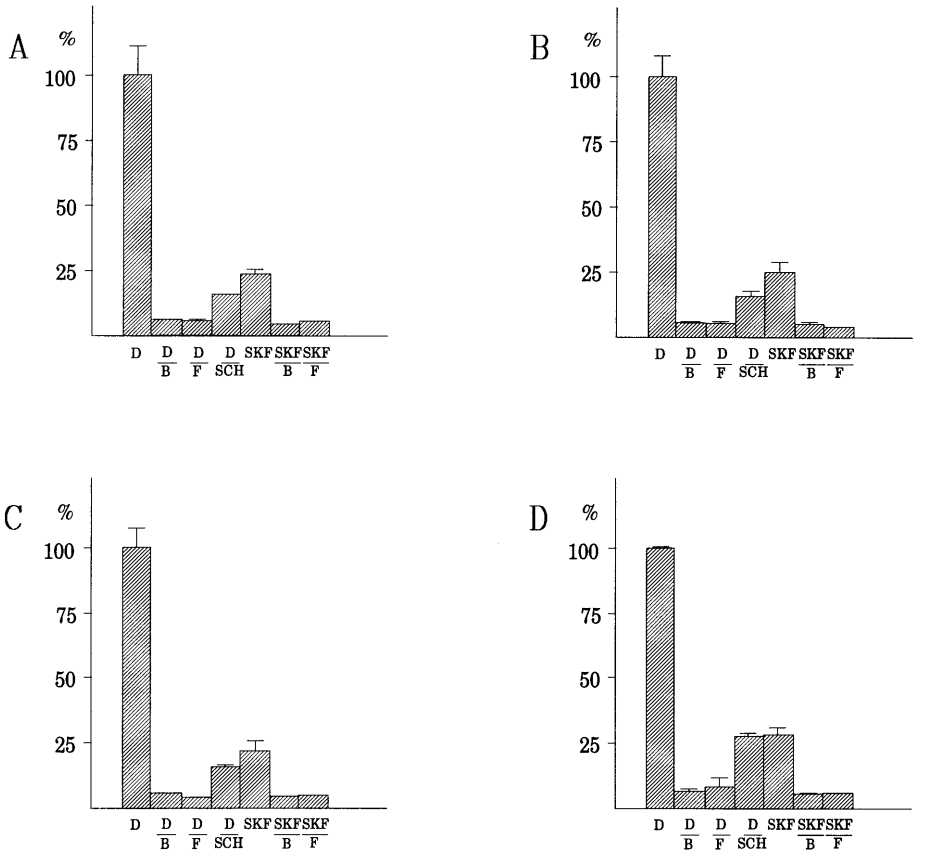

FIG. 2. Relative cAMP levels induced by various ligands in HEK 293 cells which were transiently transfected with DmDop1 **(A),** DmDop[-15] **(B),** DmDop[-19] **(C),** and DmDop[-126] **(D).** Concentrations of ligands used were: 1 μM dopamine (D) and (+/−) SKF 38393 (SKF), 100 μM (+/−) butaclamol (B), cis flupentixol (F), and (+) SCH 23390 (SCH).

purified from recombinant phages (7). All exons were completely sequenced on the genomic DNA. A partial alignment of genomic and cDNA sequences is shown in Figure 3. Insertions of one and seven nucleotides as well as five single nucleotide substitutions were found in the 5′ non-translated region (Figure 3A). Most notably, another single nucleotide substitution ($G_3 \rightarrow C$) leads to the disruption of the ATG ($\rightarrow$ ATC) codon of the open reading frame of DmDop1 (Figure 3B). Therefore, *Canton S* flies should contain dopamine receptors with altered N-terminal ends. In addition, four single nucleotide substitutions were detected in exon 1 (Figure 3C). All preceed the ATG codon of the shortest receptor construct, DmDop[−126], examined in this study. Only two of these substitutions, however, alter the deduced amino acid sequence: $A_{178} \rightarrow C$ would change $I_{60} \rightarrow L$ and $C_{206} \rightarrow T$ would change $T_{69} \rightarrow I$. We did not observe further differences between *Canton S* (genomic) and *Berlin* (cDNA) strain nucleotide sequences in any other exon of the DmDop1 gene.

DISCUSSION

Biogenic amine receptors share the common topology of GPCR and possess seven transmembrane segments. The N-terminus is located extracellularly (18). In contrast to peptidergic receptors which also belong to the GPCR superfamily and possess long N-terminal segments involved in ligand binding, aminergic receptors have fairly short N-termini (19, 20). Besides DmDop1, however, two serotonin receptors have been cloned which contain elongated N-terminal segments (9,

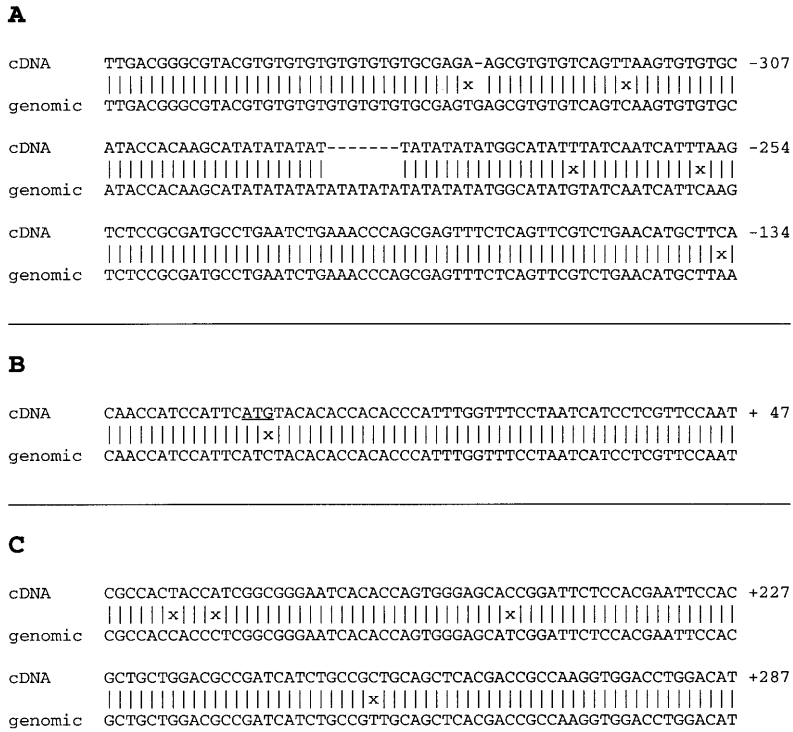

FIG. 3. Nucleotide sequence comparison between DmDop1 (cDNA) and *Canton S* (genomic) DNA. Nucleotide exchanges are labeled by 'x', dashes in cDNA sequence indicate insertions in the genomic DNA. **(A)** Sequence alignment of the 5′ non-coding region of the DmDop1 gene. In **(B)** the ATG codon of the open reading frame of DmDop1 is underlined. **(C)** Nucleotide exchanges in exon 1 preceding the ATG codon of construct DmDop[-126]. Numbering of cDNA is according to (7).

10). Interestingly, within the N-termini additional hydrophobic domains were identified. It was supposed that these hydrophobic segments could either serve as internal signal peptides or that they might form additional transmembrane segments (9, 10).

DmDop1 also contains two hydrophobic domains at similar positions in the N-terminus. The functional properties of deletion constructs of DmDop1 either lacking one (DmDop[−15]/[−19]) or both (DmDop[−126]) hydrophobic segments were indistinguishable from wildtype. Therefore, the N-terminus of DmDop1 and most likely those of cloned serotonin receptors (9, 10) are not necessary for proper integration of the polypeptides into the membrane.

In this study we also examined whether the N-terminus of DmDop1 participates in the ligand binding properties of the receptor. Analysis of truncated receptor constructs showed that activation by dopamine was not altered. However, substituted benzazepines, i.e. SCH 23390, which display high affinity binding to vertebrate dopamine receptors (17) show only low affinity to invertebrate dopamine receptors (7, 11, 21). Most notably, the affinity of SCH 23390 decreased even further in N-terminally deleted DmDop1 constructs, while interaction with other synthetic compounds remained unaltered. These results imply that additional residues in the extracellular loops and TMs which determine specific receptor-ligand interactions in vertebrate receptors either are substituted or missing in invertebrate receptors. Alternatively, DmDop1 and hD1 receptors might adopt a different quarternary structure which gives rise to variant pharmacological properties.

Interestingly, we found that *Drosophila* inbred strains do express dopamine receptors with alternative N-terminal ends. The N-terminus of DmDop1 contains five consensus motifs for N-linked glycosylation (7). In construct DmDop[−126] all of these glycosylation sites are deleted.

Glycosylation of membrane bound proteins is a common posttranslational modification and might be involved in receptor stability, turnover, and targeting to the membrane (22). Whether there do exist differences in receptor localization and turnover between different *Drosophila* strains requires immunohistochemical and biochemical examination. It is worth noting that the single nucleotide exchange $ATG_3 \rightarrow ATC$ in *Canton S* does not necessarily lead to the expression of the shortest, i.e. DmDop[−126] construct. Translation of the polypeptide might well be initiated at position 16 (= DmDop[−15]) which would preserve the glycosylation sites of the receptor.

In summary, we have found that the long N-terminal extension of DmDop1 could be deleted without altering the functional, i.e. AC stimulating properties of expressed receptors. *Drosophila* inbred strains, however, do express dopamine receptors which are structurally diverse but functionally indistinguishable.

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