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The pharmacology of a dopamine receptor in the locust nervous tissue

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Abstract

A dopamine receptor in the nervous tissue of the desert locust (*Schistocerca gregaria* Forskål) was studied using [^{3}H]lysergic acid diethylamide (LSD) as the radioligand. Its expression is almost entirely restricted to the mushroom bodies, centres for learning and memory in the insect brain. This G-protein coupled receptor is present in relatively low concentrations in the locust brain (35 fmol/mg protein). The pharmacological characterisation reveals high affinity for the putative natural agonist dopamine (K_i = 28 nM). Substances with high subtype specificity for vertebrate dopamine receptors such as SCH 23390 (K_i = 639 nM) and sulpiride (K_i = 21,200 nM) have low affinity for the locust neuronal dopamine receptor. In opposite, substances with a broad pharmacological profile such as LSD, spiperone (K_i = 7.26 nM), and chlorpromazine (K_i = 9.52 nM) have high affinity properties. Comparison of the pharmacological data reveals no significant homology to any vertebrate dopamine receptor class characterised so far. This uncertainty about the pharmacological relatedness of insect dopamine receptors mirrors the available molecular data. It is almost impossible to classify cloned insect dopamine receptors into vertebrate dopamine receptor schemes. This lack of pharmacological relatedness opens the opportunity to develop highly specific insecticides against insect dopamine receptors.

Keywords: Dopamine receptor; [^{3}H]Lysergic acid diethylamide (LSD); (Locust); (Insect); Mushroom body

1. Introduction

Dopamine, one of the classical catecholamine neurotransmitters, is long known to be a neuroactive substance in insects. It is present in relatively high concentrations in the entire insect nervous tissue (Klemm and Axelsson, 1973; Brown and Nestler, 1985). The widespread distribution of dopamine-like immunoreactivity, as well as the extensive arborizations of the respective neurones into all major neuropils, is indicative for an important role as a neurotransmitter or neuromodulator in the insect nervous system. More than 300 dopamine containing neurones are present in the bee's brain. In other insects such as locusts, dopamine-containing neurones show a different pattern of distribution. Among the 3100 dopaminergic neurones of each locust hemisphere, 3000 are restricted to the optic lobes (Wendt and Homberg, 1992), which is devoid of dopaminergic cells in the honey bee (Schäfer and Rehder, 1989). Dopamine is synthesised from tyrosine via dihydroxyphenylalanine (L-DOPA). The rate limiting enzyme is the tyrosine hydroxylase, which could easily be inhibited, resulting in dopamine depletion (Neckameyer, 1998).

1.1. Physiological role of dopamine in insects

Depletion of dopamine from insects through inhibition of the tyrosine hydroxylase leads to numerous behavioural defects. The best studied peripheral organ, which is modulated by dopamine, is the salivary gland (Brown and Nestler, 1985). Electrical stimulation as well as dopamine application leads to hyperpolarisation and the initiation of the secretory response of the salivary gland (Evans and Green, 1990a). In the nervous system, various effects of dopamine are documented. It appears to play an important role in the neuronal escape circuit (Casagrand and Ritzman, 1992) located in the abdominal ganglia of the cockroach. Together with other biogenic amines, dopamine has pronounced effects on the flight motor pattern of the hawkmoth *Manduca* (Claasen and Kammer, 1986). During development, dopamine seems to be essential as inactivation of tyrosine hydroxylase activity results in akinesia,

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developmental retardation, and decreased fertility (Neckameyer, 1996).

1.2. Dopamine in learning an memory

Dopamine appears to play an important role, together with other amines, in the process of learning and memory. The mushroom bodies, known to be essential for olfactory learning in insects have a central role in this context. As mentioned above, numerous studies dealing with the localisation of dopamine-immunoreactivity were performed. It ruled out that dopaminergic neurons supply the Kenyon cells, the cellular constituent of the mushroom bodies, with dopamine (Wendt and Homberg, 1992). Global injection of dopamine into the bee brain inhibits the retrieval of memory (Bicker and Menzel, 1989). Additionally, dopamine-releasing neurons might be involved in the motor expression of learned behaviours (Menzel et al., 1989). Kenyon cells held in primary culture respond to dopamine application by a brief increase in intracellular calcium (Cayre et al., 1999). Recently, Müller was able to show that dopamine leads to an activation of the protein kinase PKA II (Müller, 1997). Taken together, these results show that dopamine has significant effects on learning and memory in insects, transmitted through G-protein coupled dopamine receptors.

1.3. Insect dopamine receptors

Receptors for dopamine have been characterised in the nervous tissue of various insect species using different methods. Dopamine-sensitive adenylate cyclases were studied in moth (Bodnaryk, 1979a,b), mosquitoes (Pratt and Pryor, 1986), and cockroaches (Orr et al., 1987). The pharmacological investigations performed with the dopamine sensitive adenylate cyclase of the cockroach nervous tissue revealed that it may represent a peculiar receptor type that is not homologous to other invertebrate dopamine receptors (Orr et al., 1987). Using electrophysiological methods, the pharmacology of a dopamine receptor in cockroach somata was characterised (Pitman and Davis, 1988). Recently, two types of dopamine receptors were cloned from the fruitfly *Drosophila*. Whereas the first of them shows a widespread expression within the nervous system (Gotzes et al., 1994; Sugamori et al., 1995), the second one, called DAMB is concentrated in the mushroom bodies (Han et al., 1996; Feng et al., 1996). Unfortunately, these receptors are pharmacologically not well studied.

The main goal of the current investigation is to study the locust dopamine receptor in terms of tissue localisation and pharmacology. It should be evaluated if these receptors have pharmacological features peculiar for insect dopamine receptors, or of they fit into the vertebrate type of dopamine receptor classification. If the pharmacology of insect dopamine receptors is different from that of vertebrates, the corresponding receptors could be interesting targets for highly specific insecticides.

2. Material and methods

Desert locusts (*Schistocerca gregaria* Forskål) of both sexes were reared at a light/dark cycle of 12:12 h (35°C) under crowded conditions. They were fed with a diet of fresh wheat and bran.Two to three weeks after imaginal moult, nervous tissue (brain and thoracic ganglia) was dissected and immediately stored frozen on ice. The nervous tissue was homogenised in ice-cold incubation buffer (10 mM HEPES/NaOH, 5 mM $MgSO_4$, pH 7.4) supplemented with 100 μM phenyl methyl sulfonyl fluoride. It was homogenized using 10 strokes of a glass/teflon homogenizer followed by 10 strokes of a glass/glass homogenizer. The homogenate was centrifuged ($20{,}000 \times g$, 30 min), the supernatant discarded, and the pellet was resuspended in the original volume. This was repeated twice to obtain washed pellet, which were stored frozen until use (−70°C). For the study of the tissue distribution, the nervous tissue was dissected into different parts and stored frozen until use.

Incubation was performed for 60 min at 21°C in a total volume of 0.5 ml. The incubation medium contained 1 nM [^{3}H]lysergic acid diethylamide (LSD) (65 Ci/mmol; NEN Dreieich, Germany), 10 μM 5-hydroxytryptamine (5-HT), and the appropriate concentrations of the tested substances. The incubation was started by addition of the homogenate (0.7–1.0 mg/ml protein) and stopped by filtration through glass fibre filters that were preincubated in 0.3% polyethylene-imine. Although the protein concentration appears to be relatively high, we experienced no problems with this. The filters were washed with ice-cold incubation buffer, and the radioactivity was measured using a scintillation-counter (40% efficiency). Experiments dealing with the G-protein coupling were performed in a buffer where $MgSO_4$ was replaced by ethylene diamine tetraacetic acid-Na_2(EDTA). The K_I-value for 5-HT was evaluated using saturation analysis and calculated accordingly.

The pharmacological and saturation data were evaluated using the ''LIGAND'' program (Munson and Rodbard, 1980). Each substance was tested in six to eight different concentrations at least three to four times in triplicate.

All substances tested were from Sigma Chemie (Deisenhofen, Germany) or from Research Biochem (Koeln, Germany), all other chemicals were of p.A. quality.

3. Results

The radioligand [^{3}H]LSD labels specifically two binding sites in the locust nervous tissue. If the binding is

performed in varying concentrations of either dopamine or 5-HT (serotonin) a biphasic decrease of the specific binding could be observed. The high affinity dopamine-sensitive as well as the high affinity 5-HT-sensitive binding sites are totally additive, which indicates that two different binding sites are labeled (Fig. 1). Whereas the 5-HT sensitive binding represents about 2/3 of the total binding, the remaining 1/3 represents the dopamine receptor.

If the incubation is performed in the presence of either 10 μM 5-HT or 10 μM dopamine, the 5-HT as well as the dopamine-sensitive binding are accessible to a detailed pharmacological study. All further experiments were performed in the presence of 10 μM 5-HT.

This high-affinity dopamine sensitive [^{3}H]LSD binding was sensitive to heat treatment which indicates the proteinaceaous nature of the binding site. Incubation at 40°C (15 min) reduces the specific binding by approximately 25%. If the temperature is increased to 60°C (15 min), less than 5% of the maximal specific binding was detectable. If the concentration of the radioligand is increased, saturation of the dopamine sensitive specific [^{3}H]LSD binding could be observed at LSD concentrations of about 1.5–2 nM (Fig. 2). We used two higher [^{3}H]LSD concentrations (7.5 and 15 nM) that showed specific binding in the same range (data not shown). Non-specific binding, determined in the presence of saturating concentrations of dopamine (10 μM), usually represented 30–40% of the total binding. A closer evaluation of these saturation data was performed using the Scatchard-plot. It shows a linear decrease, which indicates that LSD binds to a single class of non-interacting binding sites (Fig. 2, inset). This is further supported by Hill-plot analysis revealing a Hill-coefficient close to 1 (0.992) which is also indicative for the presence of a single class of non-interacting binding site. The maximal concentration of binding sites obtained from the Scatchard-plot was approximately 35 fmol/mg of protein and the K_D-value of the binding was 0.97 nM (Fig. 2, inset).

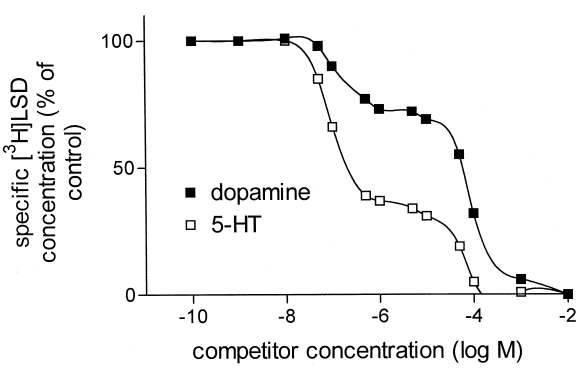

Fig. 1. Displacement of specific [^{3}H]LSD binding to locust nervous tissue membranes by increasing concentrations of dopamine or 5-HT, respectively. Each point represents the mean of at least three different experiments performed in triplicate.

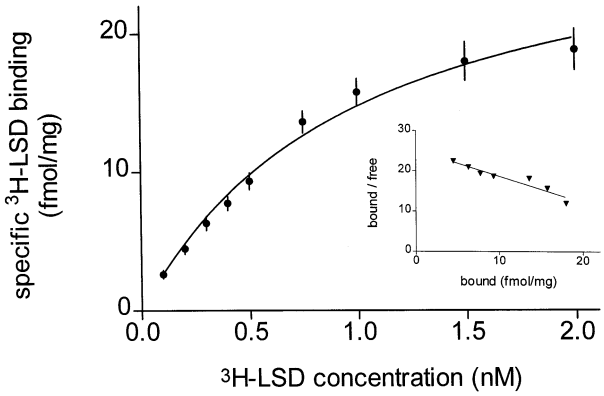

Fig. 2. Saturation isotherm of [^{3}H]LSD binding to locust nervous tissue membranes in the presence of 10 μM 5-HT. The binding saturates at [^{3}H]LSD concentrations of about 1.5–2 nM. Scatchard-plot analysis of these data (inset) revealed a linear decrease of the function, indicating the existence of a class of non-interaction binding sites. A K_D-value of 0.97 nM and a B_{max}-value of 35 fmol/mg protein were obtained. The vertical bars represent the S.E.M. Each data point is the mean of at least five experiments performed in triplicate.

The kinetic behaviour of the binding site was studied at room temperature at a concentration of 1 nM [^{3}H]LSD. Under these experimental conditions, association of the binding reached a constant value after approximately 20 min and was stable up to 90 min (Fig. 3). At equilibrium (45 min association), dissociation was initiated by the addition of 10 μM dopamine which inhibits reassociation. The specific binding decreased exponentially within 15–20 min to a stable value representing approximately 20% of maximal specific binding (Fig. 3). This decrease of the specific binding demonstrates its reversibility. Quantitative evaluation of these kinetic experiments revealed a dissociation constant K_{-1} of 0.142 min^{-1}, and an association constant K_{+1} of 8.3×10^7 min^{-1} mol^{-1}. Using these two

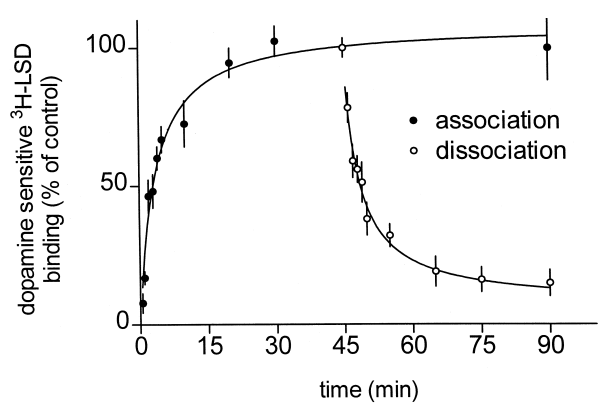

Fig. 3. Kinetic analysis of the dopamine sensitive [^{3}H]LSD binding to locust nervous tissue membranes. Incubation was performed at room temperature with 1 nM [^{3}H]LSD for the times indicated. Dissociation was studied by addition of 10 μM dopamine after 45 min association to prevent reassociation. Quantitative evaluation of these data revealed a K_{-1}-value of 0.142 min^{-1}, and a K_{+1}-value of 8.3×10^7 min^{-1} mol^{-1}.

constants, it is possible to determine the dissociation constant K_D as the quotient K_{-1}/K_{+1}. The resulting value of 1.71 nM is in good agreement with the K_D-value obtained from the saturation experiments ($K_D = 0.97$ nM).

The G-protein coupling of the receptor was studied by determination of the affinity of dopamine in both the G-protein-coupled and the uncoupled state. The G-protein was uncoupled from the receptor molecule by depletion of Mg^{2+}-ions from the incubation medium (K_I of dopamine in the uncoupled state = 30.3 μM). If Mg^{2+}-ions are present in the incubation medium, the affinity rises up to 28 nM (Fig. 4). This approximately 1000-fold higher affinity in the G-protein coupled state is characteristic for G-protein coupled receptors. The affinity of LSD itself is not changed under these conditions, which is characterisitic for antagonists.

3.1. Pharmacology

Using a variety of different compounds, it was possible to obtain a pharmacological profile of the dopamine-sensitive [^{3}H]LSD binding site. Dopamine itself was the agonist with highest affinity for this binding site ($K_I = 28$ nM; Table 1). The biological precursors of dopamine, L-DOPA ($K_I = 886$ nM) as well as tyramine ($K_I = 2900$ nM) had much lower affinities which is also true for other neurotransmitter candidates (Table 1).

For a closer evaluation of this binding site, the affinity of different dopaminergic antagonists was determined. Among them, the radioligand LSD itself had highest affinity ($K_I = 0.97$ nM) followed by the dopamine D_2 receptor antagonist spiperone ($K_I = 7.26$ nM), and chlorpromazine ($K_I = 9.52$ nM). All other antagonists tested had only moderate or even low affinities (Table 1). Especially those substances that are characterised by very high subtype specificity for vertebrate dopaminergic receptors failed to have high affinity properties. Among them are the D_1 antagonist 2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol HCl (SCH 23390) ($K_I = 0.64$ μM), and the dopamine D_2 receptor antagonist sulpiride ($K_I = 2.12$ μM). The higher affinity of the (+) isomer of butaclamol ($K_I = 242$ nM) compared with its (−) isomers ($K_I = 4590$ nM) demonstrates the stereospecificity of the dopamine-sensitive [^{3}H]LSD binding. The Hill slopes, calculated for the displacement curves, were all between 0.92 and 1.08 indicating that only single classes of non-interacting binding sites were labeled.

Table 1
Affinity of agonists and antagonists for the dopamine-sensitive [^{3}H]-LSD binding site of the nervous tissue of the desert locust *Schistocerca gregaria*
K_I-values ± S.E.M. are given. Each experiment is performed at least four to five times in triplicate. In the last column, the specificity of the respective substance for vertebrate dopamine receptors is given.

Substance	K_I (nM)	Specificity
Agonists		
Dopamine	28 ± 20	
L-DOPA	890 ± 500	
Norepinephrine	1500 ± 1000	
Tyramine	2900 ± 1200	
5-HT	12,000 ± 2100	
Epinephrine	49,000 ± 16,000	
Antagonists		
LSD	0.97 ± 0.19	
Spiperone	7.3 ± 3.8	D_2
Chlorpromazine	9.5 ± 2.8	
Fluphenazine	110 ± 29	D_1/D_2
(+)Butaclamol	240 ± 140	D_1/D_2
(−)Butaclamol	4600 ± 1900	
Pimozide	370 ± 110	D_2
Thioridazine	430 ± 120	D_1/D_2
SCH 23390	640 ± 130	D_1
Metoclopramide	2500 ± 820	
Haloperidol	14,000 ± 7200	D_1/D_2
Sulpiride	21,000 ± 11,000	D_2

The comparison with other dopamine receptors from insects revealed higher degrees of homology (Table 2). The rankorder of affinities of selected antagonists for the locust dopamine receptor (spiperone > chlorpromazine > SCH 23390 > metoclopramide > haloperidol > sulpiride) differs substantially from those of the other three insect dopamine receptors. Whereas haloperidol and SCH 23390 are nearly equipotent for the dopamine receptor of the cockroach salivary gland (Evans and Green, 1990a), SCH 23390 is about 20 times more active than haloperidol for the locust dopamine receptor (Table 2; this difference in their affinities is statistically significantly different, $p < 0.05$). In addition, the locust receptor could easily be distinguished from dopamine receptors from the cockroach nervous tissue by approximately 15,000 times higher affinity of spiperone compared with haloperidol. For both cockroach nervous tissue receptors, haloperidol is at least as potent as spiperone (Table 2).

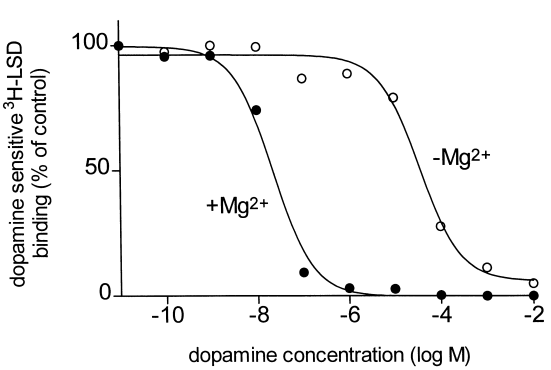

Fig. 4. Displacement of specific [^{3}H]LSD binding by dopamine in the presence and absence of Mg^{2+}-ions. Depletion of Mg^{2+}-ions was achieved by replacing Mg^{2+}-ions by EDTA. The obtained K_I-values are 28 nM in the presence of Mg^{2+}-ions and 30.3 μM in the absence of Mg^{2+}-ions.

Table 2
Pharmacology of dopamine receptors in insects
The K_I-values listed above are given in nM. Values are from the current study (locust nervous tissue), from Evans and Green (1990a) for the cockroach salivary gland (sg), from Orr et al. (1987) for the cAMP measurement in the cockroach nervous tissue, from Pitman and Davis (1988) for the electrophysiological study of cockroach interneurones, and from Blenau et al. (1998) for the honey bee receptor.

Substance	Locust nt	Cockroach sg	Cockroach cAMP	Cockroach elect	Honey bee	Specificity
Spiperone	0.97	–	44.5	> 10,000	64	D_2
Chlorpromazine	9.5	220	–	> 1 (potent)	15	D_2
SCH 23390	640	4060	–	1000	250	D_1
Metoclopramide	2500	246,700	–	< 100,000	–	D_2
Haloperidol	15,000	3770	47.9	1 (potent)	390	D_2
Sulpiride	21,000	–	309	> 1000	–	D_2

The only insect dopamine receptor with substantial homology is the honey bee dopamine receptor (Blenau et al., 1995, 1998; Fig. 5 top, Table 2). The affinities of most compounds tested are in the same range for both receptors.

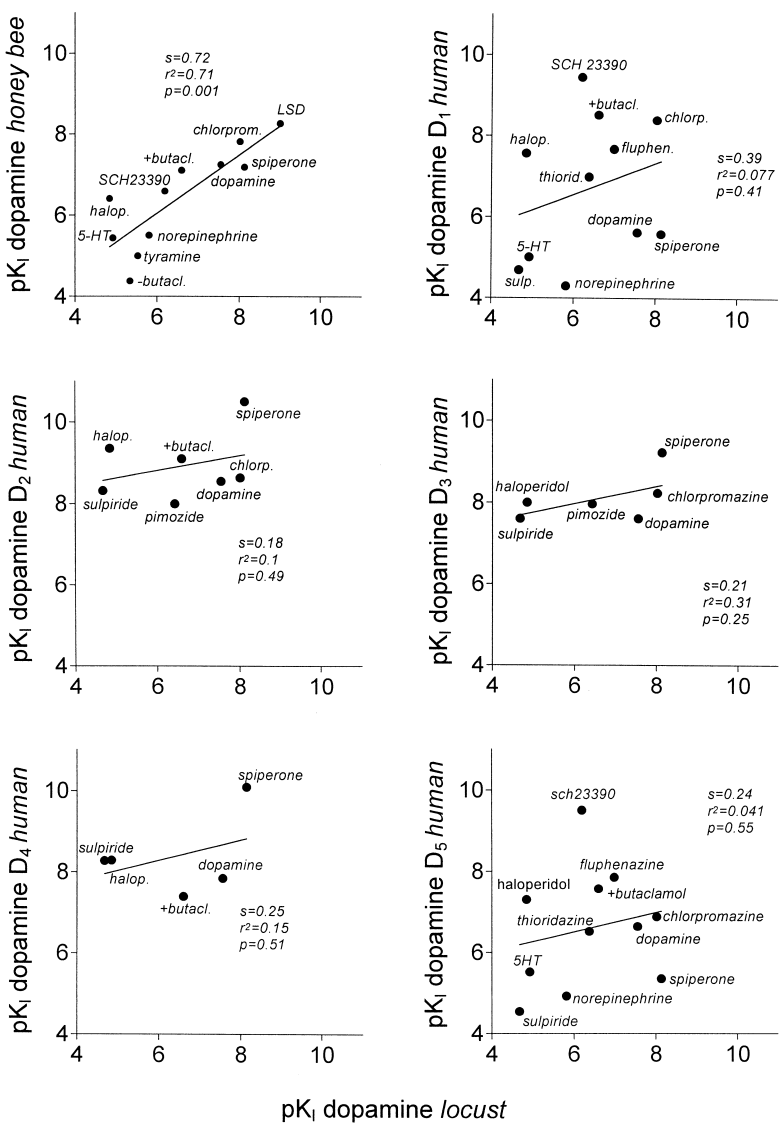

Fig. 5. Pharmacological relatedness of the locust dopamine receptor to dopamine receptors from honey bees and humans. The pK_I-values of selected compounds for the locust dopamine receptor, and the honey bee dopamine receptor, or the five human dopamine receptor are compared and aligned. Regression analysis of these data revealed the slope of the regression curve (s), the r^2, and the p-value. Halop. = haloperidol, butacl. = butaclamol, chlorprom. = chlorpromazine, fluphen. = fluphenazine, supl. = sulpiride, thiorid. = thioridazine.

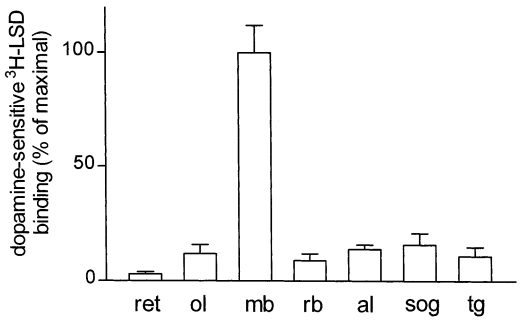

Fig. 6. Distribution of dopamine-sensitive [^{3}H]LSD-binding in different parts of the locust central nervous system. Non-specific binding is determined in the presence of 10 μM dopamine. Retina (ret), optic lobes (ol), mushroom bodies (mb), remainder of the brain (rb), antennal lobes (al), suboesophageal ganglion (sog), thoracic ganglion (tg). Each point represents the mean of at least.

This is shown in Fig. 5 where most points, each representing a peculiar compound, are near the bisector of the angle (Fig. 5 top). If the corresponding values are compared with those of all five human dopamine receptors, it becomes obvious that no definite pharmacological homology is present. This holds true also if the dopamine receptor subfamilies, the D_1-like and the D_2-like, are used (Fig. 5).

3.2. Tissue distribution

For a quantitative analysis of the distribution within the central nervous system of the locust, the corresponding brain areas were dissected and probed separately for their actual dopamine-sensitive [^{3}H]LSD binding. It ruled out that most parts of the brain do not contain any detectable binding, indicating the absence of this dopamine receptor. This holds true for the retinae, the optic lobes, the antennal lobes, the suboesophageal and thoracic ganglia, and the midbrain minus antennal lobes and mushroom bodies. The only brain area with detectable radioligand binding are the mushroom bodies (Fig. 6). This restriction to only one brain area might be the major reason for the relatively low concentration of this dopamine receptor in the locust brain.

4. Discussion

The present study utilises a specific radioligand for the study of insect dopamine receptor molecules. A comparable approach for other invertebrates was described for the mollusc *Helix pomatia* (Drummond et al., 1978, 1980). [^{3}H]LSD labels in this system as well as in the locust nervous tissue, two different binding sites, a 5-HT-sensitive and a dopamine-sensitive binding site. In opposite to the 5-HT receptors of both species which share a relatively high degree of pharmacological similarities (Wedemeyer et al., 1992), the dopamine receptors have different pharmacological features. Although dopamine is present in high concentrations in the locust nervous tissue, the concentration of this dopamine receptor is relatively low (35 fmol/mg protein) if compared with the locust 5-HT receptor (79 fmol/mg; Wedemeyer et al., 1992), or the locust neuronal octopamine receptor (700 fmol/mg; Roeder and Gewecke, 1990). This receptor belongs, as all other dopamine receptors characterised so far, to the class of G-protein coupled receptors as it has been demonstrated by coupling experiments (Fig. 4).

Although dopamine is involved in a variety of different physiological circuits, studies which characterise dopamine receptors are very rare. Dopamine-sensitive adenylate-cyclases are present in the brain of different insect species, but quantitative pharmacological data are available only from a minority of these studies (Orr et al., 1987; Pitman and Davis, 1988). The only well characterised peripheral type dopamine receptor is from the acinar cells of the cockroach salivary gland (Evans and Green, 1990a,b). Although the affinity of only four antagonists was determined, the authors concluded that this receptor has a dopamine D_1-receptor like pharmacology. The rank order of affinities of these four antagonists (chlorpromazine > haloperidol > SCH 23390 > metoclopramide) showed on the other hand, that the cockroach receptor is clearly distinct from the locust neuronal dopamine receptor (chlorpromazine > SCH 23390 > metoclopramide > haloperidol).

If compared with the honey bee dopamine receptor, pharmacological similarities are obvious. This pharmacological similarity to the bees dopamine receptor, together with the very restricted expression pattern, indicates that the locust dopamine receptor is homologous to the fruitfly DAMB receptor, which shares also this type of distribution (Han et al., 1996). Unfortunately, the fruitflies dopamine receptors are not characterised pharmacologically in sufficient detail.

The expression in the mushroom bodies is indicative for a role in learning and memory, but to date almost no physiological correlate is available, that proves this assumption. Only two of the different dopamine receptors in insects are characterised at all (Gotzes et al., 1994; Sugamori et al., 1995; Kokay and Mercer, 1996, 1997). For both of them, a correlation with a functional significance of dopaminergic neurotransmission within the insect nervous system is not possible, because knock-out mutants are not available. Regarding the peculiar pharmacology of some peripheral dopamine effects (see above), it appears that additional dopamine receptors are present. Interestingly, if the pharmacological features of the locust dopamine receptor are compared with the ones of all five human dopamine receptors, no obvious pharmacological similarities could be observed. Therefore, it is not justified to classify the insect dopamine receptors according to the vertebrate pharmacology. At the moment, it is not obvious if the different dopamine receptors of insects are ancient or if they developed after vertebrates and invertebrates split.

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